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Research Article

Effect of Field Pea Replacement for Oats on Palatability, Feeding Safety, and Growth Performance in Yearling American Quarter Horses [1]

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[1] *Infrastructure for this study was provided by the Dickinson Research Extension Center (ND06253) and the North Dakota State University, Agricultural Experiment Station, Fargo, North Dakota, 58108, USA*

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Abstract

The objective of this study was to determine the palatability, feeding safety, growth performance, and replacement value of field pea (FP) for oats in yearling American Quarter Horses. Forty-eight yearling horses averaging 338.2±0.67 kg were used in a two-year replicated (84-d) study. The horses were randomly assigned based on sex and body weight (BW) to three pelleted experimental growing supplements: 1) 100% Oats and 0% FP (C), 2) 66.7% Oats and 33.3% FP (33.3PEA), and 3) 33.3% Oats and 66.7% FP (66.7PEA). The horses were fed a predetermined amount of alfalfa-bromegrass cubed (pelleted) hay. The supplement fed daily was divided into two meals and fed at 08.30 and 14.00 hours. Horse's BW, body length (BL), wither height (WH), hip height (HH), heart girth circumference (HG), cannon bone circumference (CB), forearm circumference (FA) and gaskin muscle circumference (GM) were taken at 28, 56, and 84 d. There was no statistical difference between supplement treatments for horse average daily gain (ADG) or final BW (P>0.10). There was a linear effect identified for 28, 56, and 84 d periods (P<0.01) for all treatment measurements. There was no statistical difference between supplement treatments for BL, WH, HH, HG, CB, FA, and GM (P>0.10). There was no evidence of discomfort, colic or hoof laminitis identified. When fed as two meals separated by 6 hours, FP was a suitable replacement for oats in yearling horse growing supplements up to a maximum of 66.7%.

Keywords: *Field pea, Feeding safety, Growing supplement, Laminitis, Oats, Yearling American Quarter Horse*

Yulafa İkame edilen Yemlik Bezelyenin Bir Yaşındaki American Quarter Atlarına Yedirilebilirliği, Beslenme Güvenliği ve Büyüme Performansı Üzerine Etkisi

Özet

Bu çalışmanın amacı yulaf yerine ikame edilen yemlik bezelye (FP)'nin bir yaşındaki Amerikan Quarter atlarına yedirilebilirliği, beslenme güvenliği, ikame oranı ve büyüme performansı üzerine etkisinin saptanmasıdır. İki yıl tekrarlanan (84-d) bu çalışmada, ortalama ağırlığı 338.2±0.67 kg olan bir yaşında, kırk sekiz adet at kullanılmıştır. Atlar cinsiyet ve vücut ağırlığı (BW) 'na göre rastgele tahsis edilmiştir. Peletlenmiş üç takviye yem grupları: 1) %100 Yulaf ve %0 FP (C), 2) %66.7 yulaf ve %33.3 FP (33.3PEA), ve 3) %33.3 yulaf ve %66.7 FP (67PEA). Atlar kaba yem olarak önceden belirlenmiş miktarlarda yonca-bromegrass küpleri (peletlenmiş) ile beslenmiştir. Günlük takviye yem ise iki öğüne bölünerek 08.30 ve 14.00 saatlerinde yedirilmiştir. Araştırmanın 28., 56., ve 84. gününde, atların BW, vücut uzunluğu (BL), yüksekliği (WH), kalça yüksekliği (HH), kalp çevresi (HG), incik çevresi (CB), önkol çevresi (FA) ve bacak kas çevresi (GM) ölçülmüştür. Takviye yem grupları arasında ortalama canlı ağırlık artışı (ADG) ve bitiş BW bakımından istatistiksel olarak bir fark saptanmamıştır (P>0.10). Tüm grupların 28., 56., ve 84. gün periyotlarındaki ölçümlerde doğrusal bir etki tespit edilmiştir (P<0.01). Takviye yem grupları arasında BL, WH, HH, HG, CB, FA, ve GM bakımından istatistiksel fark bulunmamıştır (P>0.10). Toynaklarda laminitis ile ilgili herhangi bir veri belirlenmemiştir. Bir yaşındaki Amerikan Quarter atlarının büyüme takviyesi yemlerinde FP 6 saat ara ile iki öğün olarak yediriliğinde yulaf yerine maksimum %66.7 oranında kullanılabilecek uygun bir alternatif yemdir.

Anahtar sözcükler: *Yemlik bezelye, Besleme güvenliği, Büyütme takviyesi, Laminitis, Yulaf, Bir yaşında Amerikan Quarter atı*

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INTRODUCTION

Horses are a simple stomach herbivore that can thrive well consuming a balance of grass and legume forage or pasture. However, when growing, or involved in light to heavy work, forages alone will not supply sufficient energy to meet the energy requirement for work, but heavy oats *(Avena sativa,* 14.5-17.2 kg/bushel), a common staple for horses, can supply the necessary energy for most working conditions.

Digestive integrity is vitally important for the horse. Oats are the grain concentrate of choice among horse owners, because after ingestion the grain forms a bulky loose mass in the stomach and supplies energy for maintenance and work. As a fiber, protein, and energy source, oats contain: crude protein (CP; 11-14%CP), lysine (LY; 0.40-0.55%), fiber (FIB; 12.0-15.9%), digestible energy (DE; 3.23-3.33 Mcal/kg), fat (4.9-5.2%), calcium (Ca^{++}0.05%) and phosphorus (P; 0.35%) [1].

Peas are considered to have originated from the region of southwest Asia encompassing present day north-western India, Pakistan, Afghanistan and Central Asia c. 7000 - c. 6000 BC. Cultivation of the crop spread east into present day China and north and west into Russia, the Mediterranean Basin, and Europe [2].

Field pea (FP) is grown in the northern Great Plains of the United States and the central provinces of Canada in crop rotations. The primary objective of farmers is to market the grain as human food or for export. In North Dakota, 2013 FP production was reported to be 43,338 MT [3]. When specifications for human food or export are not met, the grain becomes feed for all classes of livestock, with the exception of horses. FP is not commonly fed to horses in the USA.

FP is a protein and energy dense concentrate containing: CP (22-26% CP), LY (1.50-1.70%), FIB (6.0-7.0%), DE (3.40 Mcal/kg), starch (48-52%), fat (1.6%), Ca^{++} (0.05%) and P (0.48%), and is also a rich source of microminerals, water soluble and fat soluble vitamins [1,4,5]. The nutrient value of raw FP compared to soybean meal (SBM) has been evaluated in non-ruminant swine growing and finishing diets and shown to be a suitable substitute for SBM [6], and in ruminants, pea starch degradation rate has been reported to be slower than wheat, barley, and oats, but similar to the degradation rate of corn [7].

Colic is the expression of visceral abdominal pain in the horse, which can occur frequently, since the horse has a low threshold for pain and is most often associated with diet and dietary changes [8]. Improper feeding management, when feeding high starch non fibrous carbo-hydrate grains, can lead to grain overload associated with rapid fermentation of carbohydrates resulting in reduced pH (acidosis), intestinal mucosa damage and death of gram-negative bacteria, and the onset of endotoxemia (prodromal stage) and a cascade of events leading to laminitis of the hoof wall [9,10]. Feeding safety is of utmost importance when horses are fed non-fibrous carbo-hydrates, since overwhelming the digestive system leads to episodes of colic [11]. Because the young growing horse attains 80-90% of its mature size within the first two years of life [12], the young growing horse is an ideal growth model to evaluate the suitability of field peas as an ingredient in growing horse supplements.

Despite oat grain being the most common grain concentrate fed to horses worldwide, the objective of this study was to evaluate the value of FP as a non-fibrous carbohydrate replacement concentrate in yearling growing horse supplements using a reciprocal substitution protocol in which 33.3 and 66.7% of the oats in supplements was replaced with FP and compared to a control supplement without FP. Although, a paralleling digestibility study was not conducted, we hypothesized that due to the slower reported degradation rate of FP in ruminant diets [7] that FP would not be fully digested in the small intestine of the horse, but hindgut illness would not develop, because the supplements are fed in two meals separated by 6 hours. It was further hypothesized that there would be no difference identified for body linear and circumference measurements among horses fed the experimental FP supplements.

MATERIAL and METHODS

This research project was conducted at the Dickinson Research Extension Center, Ranch Headquarters, Manning, North Dakota, USA (47°11'34" N 102°50'17" W) in accordance with guidelines approved by The North Dakota State University Institutional Animal Care and use Committee (Protocol Approval Number: A0426).

Over a 2-year period, forty-eight yearling Quarter Horses (filly, n=30; stud colt, n=18) that averaged 338.2±0.67 kg were used in an 84 d feeding study to evaluate three oat growing supplements formulated with increasing levels of FP. Horses were randomly assigned to supplement treatment in the completely randomized design based on sex and yearling weight. Eight horses were assigned to each treatment each year and individual horse served as the experimental unit. Supplements were prepared to meet the nutritional requirements of the yearling growing horse [1]. The three reciprocal horse growing supplement treatments were: 1) 100% Oat and 0% Pea (C), 2) 66.7% Oat and 33.3% Pea (33.3PEA), and 3) 33.3% Oat and 66.7% Pea (66.7PEA).

The forage fed to the horses in the study was a cubed (3.81 x 3.81cm) alfalfa-bromegrass hay that *(Medicago sativa* and *Bromus inermis)* contained approximately one-third brome grass and two-thirds alfalfa. The nutrient composition of the alfalfa-grass hay was CP (15.5%), fat

(2.30%), FIB (33.70%), Acid Detergent Fiber (ADF) (35.2%), Neutral Detergent Fiber (NDF) (44.3%), starch (2.00%), LY (0.75%), Ca^{++} (1.04%), P (0.35%), and ash (7.1%). The nutrient composition of oat grain used to prepare the supplements was CP (13.5%), fat (5.60%), FIB (12.20%), ADF (14.2%), NDF (24.4%), starch (40.0%), LY (0.55%), Ca^{++} (0.01%), P (0.41%), and ash (3.1%), and the nutrient composition of FP used in the supplements was CP (23.5%), fat (1.60%), FIB (6.5%), ADF (8.0%), NDF (17.3%), starch (49.0%), LY (1.67%), Ca^{++} (0.05%), P (0.48%), and ash (2.8%). Soybean meal, which was used to balance protein across the supplement treatments, contained CP (44.5%), fat (1.0%), FIB (7.0%), ADF (10.0%), NDF (14.9%), starch (6.0%), LY (3.13%), Ca^{++} (0.40%), P (0.71%), and ash (4.4%).

The Supplement ingredient composition and nutrient analysis is shown in *Table 1*, and the calculated nutrient content of the daily diet is shown in *Table 2*. The alfalfa-bromegrass hay was sampled weekly, composited, and analyzed by a commercial laboratory for CP, ADF, NDF, fat, starch, LY, Ca^{++}, P, and ash (AgSource Soil and Forage Laboratory, Bonduel, WI, USA). Hay DE (Mcal/kg) for the horse was estimated according to: DE = 2118 +12.18* (%CP) -9.37* (%ADF) -3.83* (%hemicellulose) + 47.18* (%FAT) +20.35* (%NSC) -26.3* (%ASH); where hemicellulose equals ADF-NDF and Non-structural Carbohydrates (NSC) equal (100-%NDF-%FAT-%ASH-%CP) [1].

Before initiation of feeding, the experimental supplements were analyzed for CP, fat, ADF, NDF, Ca^{++}, P, and ash at the North Dakota State University Nutrition Laboratory. Samples were analyzed in duplicate [13] for DM by drying at 135°C (AOAC method 930.15), CP (AOAC method 2001.11), ether extract (AOAC method 920.39), Ca^{++} and P (AOAC methods 968.08 and 965.17), and NDF and ADF [14] and ash (AOAC method 942.05). Hay, supplement, and the total amounts fed to each horse daily, and the ration analysis is shown in *Table 2*.

Based on the daily feed fed and nutrient analysis shown in *Table 2*, starch from hay and supplement would supply 3.57, 3.78, and 3.99 g/kg of horse starting weight for the C, 33.3PEA, and 66.7PEA, respectively. Feeding this amount of starch in a single meal would overload the small intestine digestive capacity leading to acidosis, colic, founder, and laminitis [15]. Therefore, to avoid hindgut digestive disturbance, the amount of supplement fed was divided equally into two meals and after hay was fed one-half of the supplement was fed at 08.30 a.m. each morning and the remaining one-half was fed at 14.00 p.m. each afternoon.

Table 1. *Supplement composition and analysis (DM)*
Tablo 1. *Takviye yemin bileşimi ve analizi (Kuru Madde)*

Supplement Ingredients	C*		33.3PEA*		66.7PEA*	
	kg	%	kg	%	kg	%
Oats	2.72	80.24	1.81	57.10	0.91	30.95
Peas	0.00	0.00	0.91	28.71	1.81	61.57
Soya Bean Meal	0.45	13.27	0.23	7.25	0.00	0.00
Equine Vitamin/Mineral[1]	0.05	1.48	0.05	1.58	0.05	1.70
Molasses	0.17	5.01	0.17	5.36	0.17	5.78
Total	3.39	100.00	3.17	100.00	2.94	100.00
Analysis						
Crude Protein, %		17.61		17.97		19.00
Crude Fat, %		4.54		3.73		2.71
Crude Fiber, %		10.62		9.33		7.75
ADF, %		12.64		11.11		9.29
NDF, %		21.38		19.95		18.15
Starch, %		32.27		37.29		42.46
Lysine, %		0.91		1.02		1.20
Calcium, %		0.08		0.06		0.04
Phosphorus, %		0.43		0.42		0.42
Ash, %		3.09		1.31		1.21
Digestible Energy, Mcal/kg		3.34		3.46		3.60

C- 100% oats and 0% pea;* ***33.3PEA-* *67% oats and 33.3% pea;* ***66.7PEA-*** *33.3% oats and 66.7% pea;* [1] ***Equine Vitamin/Mineral;*** *Vit A 300.000 UI, Vit D 30.000 UI, Vit E 700 UI, Vit B_{12} 0.46 mg, Riboflavin 100 mg, Niacin 500 mg, d-Pantothenic Acid 300 mg, Choline 1.250 mg/Calcium 17.1-20.5%, Phosphorus 15.0%, Salt, 0.5-1.5% Sulfur 0.95%, Magnesium 0.75%, Potassium 0.45%, Iron 7.000 ppm, Zinc 3.000 ppm, Manganese 2.000 ppm, Copper 1.250 ppm, Iodine 147 ppm, Cobalt 50 ppm, Selenuim 30 ppm*

Table 2. *Daily feed intake and total ration analysis (DM)*
Tablo 2. *Günlük yem tüketimi ve toplam rasyon analizi (Kuru Madde)*

Parameter	C*	33.3PEA*	66.7PEA*
Daily Feed Intake, kg/Horse			
Alfalfa-Brome Hay	4.48	4.71	4.93
Supplement Pellet	3.47	3.18	2.95
Total Fed/Day	7.95	7.88	7.88
Total Ration Analysis			
Crude Protein, %	16.42	16.49	16.81
Crude Fat, %	3.28	2.88	2.45
Crude Fiber, %	23.63	23.88	23.99
Starch, %	15.21	16.21	17.14
ADF, %	25.36	25.50	25.51
NDF, %	34.30	34.49	34.52
Lysine, %	0.82	0.86	0.92
Calcium, %	0.62	0.65	0.67
Phosphorus, %	0.39	0.38	0.38
Ash, %	5.35	2.45	2.47
Digestible Energy, Mcal/kg	2.70	2.71	2.72

C- 100% oats and 0% pea; **33.3PEA- 66.7% oats and 33.3% pea; **66.7PEA**- 33.3% oats and 66.7% pea*

The supplements were not isocaloric (DE, Mcal/kg; *Table 1*); however, based on diet calculated DE, which has been previously described, the amount of supplement fed daily to each horse was adjusted so that all horses received the same amount of daily energy across treatments. Initially, 0.91 kg of supplement was offered at each morning and afternoon feeding. The amount of supplement fed each day was increased gradually during the first three weeks of the study until the desired amount of supplement was met. The caloric balance across treatments was met with 3.4, 3.12, and 2.95 kg/horse/d for the C, 33.3PEA, and 66.7 PEA treatments, respectively.

Each horse was housed and fed individually in rectangular 32' x 128' pens constructed with continuous steel fencing that provided face to face contact and social interaction. Initially, and at 28 d intervals, the horses were weighed using an alley way platform scale (Avery Weigh Tronic, 1000 Armstrong Drive, Fairmont, MN 58031-1439 USA, (http://www.averyweigh-tronix.com/products/indicators/). Also at 28 d intervals, horse body measurements to include body length (BL), wither height (WH), hip height (HH), heart girth circumference (HG), cannon bone circumference (CB), forearm circumference (FA) and gaskin muscle circumference (GM) were also taken at 28 d intervals using a padded stock for handler and technician safety (Montana Westwood Inc., 50 Westwood Land, Trout Creek, Montana 59874, USA). With the exception of body weight, dual measurements were taken by two research technicians and the mean value of the two measurements was recorded.

At the end of the 84 d study, the hooves of each horse were inspected and given a laminitis score ranging from 1 to 5 with 1 being noticeable classic signs of laminitis and 5 no outward signs of laminitis. As each horse was led by a handler, a two-member panel scored each horse for painful gait or reluctance to move and the feet of each horse were held to feel for abnormal warmth and digital pulses [16,17].

Data was analyzed using the PROC GLM procedure of SAS [18]. Orthogonal contrasts were conducted for linear (L), quadratic (Q), and cubic (C) effects. Effects for L, Q, and C were only discussed when a significant *F*-test was detected. Individual horse served as the experimental unit. Differences between the supplement treatment groups were considered significant at $P \leq 0.05$.

RESULTS

The objectives of this study were to determine the acceptability and replacement value of FP, when FP replaced up to 66.7% of the oats in yearling growing horse supplements, to measure linear and circumference body measurements, and score horses in the feeding study for early process indicators of laminitis (prodromal stage).

The horses readily consumed the experimental supplements with no visible evidence of feed refusal or abdominal discomfort. The horses were weighed and measured individually initially and at 28 d intervals. Growth performance for the horses has been summarized in *Table 3*. There was no difference between treatments for horse ending BW ($P>0.10$); although there was a periodic 28 d linear effect for BW identified ($P<0.10$). ADG increased steadily from the start of the study to the end of the study for the 0PEA and 33.3PEA treatments. For the 66.7PEA treatment, there was a quadratic effect identified during the last 28 d period ($P<0.01$), indicating that ADG increased during the first 56 d and then declined during the last 28 d period. Despite the observed quadratic effect for ADG, horses fed the 66.7PEA supplement gained numerically more than horses fed the 0 and 33.3PEA supplements. Although speculative at best, the authors suggest that, perhaps, the horses receiving the 66.7PEA supplement may have been experiencing a very mild decline in hindgut pH during the last 28 d feeding period, although there was no indication based on the horses behavior that would indicate such a problem.

Length and height measurements for BL, HH, and WH are shown in *Table 4*. Experimental supplement treatment differences for BL ($P=0.93$), HH ($P=0.22$) and WH ($P=0.21$) did not differ statistically. Growth among horses within treatments and across treatments was similar resulting in a significant linear effect for BL, HH, and WH ($P<0.01$), when Orthogonal Contrast were analyzed [18]. The percent change between the starting and ending value for BL, HH, and WH was consistent across experimental treatments and when

Table 3. *Growth performance of yearling American Quarter horses[1] (2 Year)*
Tablo 3. *Bir yaşındaki Amerikan Quarter atlarının büyüme performansı (2 Yıl)*

Growth Performance	C*	33.3PEA*	66.77PEA*	P-Value
Body Weight, kg				
Initial	338.49	337.47	338.72	0.99
Day 28	345.32	341.56	346.23	0.89
Day 56	364.01	357.32	370.53	0.47
Final	385.44	383.17	390.09	0.77
ADG, kg				
Day 28	0.24	0.15	0.27	0.71
Day 56	0.67	0.56	0.87	0.29
Day 84	0.77	0.92	0.70	0.55
Total ADG	0.56	0.54	0.61	0.57
Orthogonal contrast**				
Linear	< 0.01	< 0.01	-	-
Quadratic	-	-	< 0.01	-

C- 100% oats and 0% pea; **33.3PEA- 66.7% oats and 33.3% pea; **66.7PEA-** 33.3% oats and 66.7% pea; ** Cubic orthogonal contrast was NS;[1] (n=48)*

Table 4. *Length and height measurements for yearling American Quarter horses[1] (2 Year)*
Tablo 4. *Bir yaşındaki Amerikan Quarter atlarının uzunluk ve boy ölçümleri (2 Yıl)*

Parameter	C*	33PEA*	67PEA*	P-Value
Body Length, cm				
Initial	137.62	136.01	138.26	0.78
Day 28	139.38	138.51	140.16	0.69
Day 56	141.17	140.41	142.54	0.38
Final	142.60	142.58	143.17	0.93
Orthogonal contrast**				
Linear	< 0.01	< 0.01	< 0.01	-
Hip Height, cm				
Initial	142.26	143.81	142.97	0.56
Day 28	143.79	145.58	144.66	0.53
Day 56	144.34	147.12	145.20	0.16
Final	145.97	148.23	146.41	0.22
Orthogonal contrast**				
Linear	< 0.01	< 0.01	< 0.01	-
Wither Height, cm				
Initial	137.64	139.29	137.44	0.37
Day 28	138.81	141.23	138.99	0.10
Day 56	139.96	142.40	139.96	0.14
Final	140.95	143.39	141.41	0.21
Orthogonal contrast**				
Linear	< 0.01	< 0.01	< 0.01	-

C- 100% oats and 0% pea; **33.3PEA- 66.7% oats and 33.3% pea; **66.7PEA-** 33.3% oats and 66.7% pea; **Quadratic and cubic orthogonal contrasts were NS;[1] (n=48)*

Table 5. *Circumference body conformation measurement for yearling American Quarter horses[1] (2 Year)*
Tablo 5. *Bir yaşındaki Amerikan Quarter atlarının vücut çevresinin ölçümü (2 Yıl)*

Parameter	C*	33.3PEA*	66.7PEA*	P-Value
Heart Girth Circumference, cm				
Initial	159.27	159.11	158.59	.94
Day 28	161.85	161.45	160.82	.84
Day 56	163.95	163.71	163.08	.84
Final	167.36	167.36	167.01	.97
Orthogonal Contrast**				
Linear	<0.01	<0.01	<0.01	
Cannon Circumference, cm				
Initial	17.56	17.23	17.58	.95
Day 28	17.86	17.90	18.02	.83
Day 56	18.02	18.04	18.08	.97
Final	18.37	18.18	18.37	.76
Orthogonal Contrast**				
Linear	<0.01	<0.01	<0.01	
Forearm Circumference, cm				
Initial	46.43	46.53	47.27	.60
Day 28	46.99	47.63	47.84	.58
Day 56	47.29	47.51	48.14	.64
Final***	47.45[a]	49.53[b]	47.79[a]	.05
Orthogonal Contrast				
Linear Quadratic	<0.01	<0.01	<0.01	
Gaskin Circumference, cm				
Initial	41.02	40.40	41.00	.72
Day 28	41.37	40.96	41.44	.71
Day 56	41.35	41.23	41.55	.84
Final	41.99	41.51	42.15	.44
Orthogonal Contrast**				
Linear	<0.01	<0.01	<0.01	

C- 100% oats and 0% pea; **33.3PEA- 66.7% oats and 33.3% pea; **66.7PEA**- 33.3% oats and 66.7% pea; ** Quadratic and cubic orthogonal contrasts were NS; *** Means with unlike superscripts differ significantly (P<0.01); [1] (n=48)*

averaged across treatments the average percent change was 4.0, 2.7, and 2.75% for BL, HH, and WH, respectively.

Physical circumference body measurements for HG, CB, FA, and GM are shown in *Table 5*. The anatomical circumference change due to supplement treatment was both uniform within and across measurements during the 84 d experimental supplement study. This parallels the consistent and repeating results reported for BL, HH, and WH. There was no statistical difference between circumference measurements for HG, CB, FA, and GM during each 28 d interval (P>0.10) or for the entire experiment (P>0.10). A significant orthogonal linear effect was identified for all of the circumference measurements (HG, CB, FA, and GM).

Safety is very important when feeding horses a supplement containing non-fibrous carbohydrate. Throughout the study, the horses were observed frequently after diets were consumed for signs of abdominal discomfort associated with the prodromal stage of laminitis. At the end of the study, each horse was given a physical evaluation and score for detectable evidence of laminitis or abnormal hoof development. There was no evidence of disease among the horses on test, especially horses that received the 66.7PEA supplement.

DISCUSSION

Variations in production and stage of horse growth result in nutrient requirements for the horse that exceed what can be supplied by pasture or harvested forages. Oats, barley, corn, and milo are the most common cereal

grains fed to horses, and oats are the preferred cereal grain among horsemen, because of the desirable groat to hull ratio.

FP grain is not a common grain fed to horses in the USA; however, this protein and energy dense ingredient may be a very valuable feed for horses. At least based on information in the scientific literature, FP is an excellent source of protein and energy in beef cattle and swine diets [19-22] and, although, FP contains 48-50% starch the starch component degrades more slowly than barley, wheat, or oats in ruminant diets and has a degradation rate similar to corn [7].

Feed grains are a significant source of energy from starch and the small intestine is the site of 55 to 85% of absorption [11]. Starch that is not absorbed in the small intestine passes to the caecum and large colon of the hindgut where anaerobic microbial digestion occurs producing absorbable volatile fatty acids, a process, which is similar to the digestive process in ruminants. The hindgut of the horse is very sensitive to digestive changes. When quantities of starch bypass the small intestine and disrupt hindgut digestive balance, an acidosis condition can develop due to declining pH leading to colic and, when severe, damage to the hoof [11].

Best horse management practice recommendations are to limit the amount of starch per meal to less than 2 g/kg BW [23] and to increase digestion of starch in the small intestine [24] by slowing passage rate or feeding multiple meals per day. Coarse feed texture passes more slowly than fine textured or pelleted feed, which is finely ground during preparation prior to the pelleting process [25]. Multiple meals per day (3 to 5) and mobile bag retention time were evaluated for oats, wheat, corn, horse bean, and barley [26]. Prececal digestibility was nearly 99% for oats and wheat, which are also known to be rapidly fermented in the rumen of cattle. Slower prececal degradation was measured for corn, barley, and horse bean suggesting that a greater portion of these substrates may pass on to the hindgut where alterations of the microbial community are associated with pH decrease and a corresponding increase in lactic acid concentration [27]. Accordingly, corn starch digestibility levels >200 mg/100 kg BW show smaller prececal digestibility [28] presumably due to starch characteristics that limit microbial degradation [29]. In our study, considering the digestibility constraints described for corn [27-29], we hypothesize that FP starch would not be completely digested in the small intestine. Therefore, assuming FP microbial degradation is similar to that of corn, FP could be considered a very useful non-structural carbohydrate energy source for the growing yearling horse provided supplements containing FP are fed using a multiple meal protocol such as the one used in this experiment.

Dietary safety and feeding management protocols are of utmost importance to protect horses of any age from unnecessary grain (starch/sugar) overload [30]. Grain overload is one of several causative events that can lead to laminitis. Laminitis is a very complex disease that begins in the digestive tract and ends with excruciating pain and irreparable damage to the feet and legs of the horse. Very simply, the disease begins with inflammatory gastrointestinal illness resulting in disruption of the intestinal mucosa due to acidosis and subsequent endotoxemia that initiates a cascade of metabolic and endocrine disturbances resulting in detachment of the coffin bone from the hoof basement membrane [10,31,32]. Alimentary gastrointestinal acidosis was avoided in our study by feeding individual supplement meals. Each supplement meal provided 1.79, 1.89, and 2.0 g of starch/kg of horse BW for the C, 33.3PEA, and 66.7PEA supplements, respectively. Dividing the daily amount of FP supplements into two meals daily separated by 6.0 hours resulted in no noticeable deleterious, negative, effects among horses in the study, especially, the 16 horses that received the highest 66.7PEA treatment.

Yearling horse growth and confirmation measurements improved linearly clearly suggesting that FP is a suitable replacement for oats in growing horse diets. Leg and hoof evaluation at the end of our study support feeding multiple meals with FP that provide ≤2 g of starch/kg of horse BW and, when feeding an oat-FP blended supplement, the oats in the mixed substrate may contribute to greater overall diet safety.

Finally, dietary supplements prepared with up to 66.7% FP replacement for oats that were fed in two meals per day were safe to feed and supported excellent yearling horse growth, conformation, and hoof and leg integrity.

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Research Article

The Effects of Yeast Culture Products on Fattening Performance, Rumen Papilla Morphology, Some Blood and Rumen Fluid Parameters in Saanen Male Kids [1]

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Abstract

The aim of this study was to investigate the effects of live yeast culture and the live yeast culture enriched with vitamin-mineral supplementation as a feed additive on fattening performance, some blood and rumen fluid parameters in male kids. Totally 18 male Saanen goat kids (average 8 weeks old) were divided into one control and two treatment groups each containing 6 kids. Rations were prepared as isonitrojenic and isocaloric. Live yeast culture (LYC) and the yeast-vitamin-mineral complex (YVM) (RumiSacc® and Intetotal® respectively, Integro food Industry and Trade Co., Istanbul, Turkey, Live yeast cell 344 x 1010 cfu/g,) were incorporated into concentrates at the level of 0% (C), 1% (YC) and 1% (YVM) on as-fed basis. During the study concentrate feed and fresh water were given ad libitum and the ration did not contain any roughages. Dietary yeast culture at the level of 1% increased final live weight (4.7% regarding control group) as numerically but not significantly. The fattening performance, rumen fluid and blood parameters were not statistically affected from the dietary treatments throughout the study. While the dorsal papillae length was not changed, ventral papillae length was decreased in the treatment groups. The length of papillae in YVM groups were similar to that of control groups.

Keywords: *Blood parameters, Fattening performance, Kid, Live yeast culture, Rumen parameters*

Saanen Erkek Oğlaklarında Maya Kültürü Ürünlerinin Besi Performansı, Rumen Papilla Morfolojisi, Bazı Kan ve Rumen Parametleri Üzerine Etkileri

Özet

Bu çalışmanın amacı erkek oğlaklarda rasyona canlı maya kültürü ve vitamin-mineral ile zenginleştirilmiş canlı maya kültürü ilavesinin besi performansına, bazı kan ve rumen parametleri üzerine etkilerini araştırmaktır. Toplam 18 erkek Saanen oğlak, her biri 6 adet oğlaktan oluşan bir kontrol ve iki deneme grubuna ayrılmıştır. Rasyon izonitrojenik ve izokalorik olarak hazırlanmıştır. Deneme yemlerinde canlı maya kültürü (LYC) ve maya-vitamin-mineral kompleksi (YVM) (Sırasıyla RumiSacc® ve Intetotal®, İntegro Gıda Sanayi ve Ticaret, İstanbul, Türkiye; Canlı maya hücresi 344 x 1010 cfu/g) %0 (C), %1 (YC) ve %1 (YVM) düzeyinde kullanılmıştır. Hayvanlara yem ve su ad libitum olarak sunulmuş, rasyonda kaba yem kullanılmamıştır. Rasyona %1 oranında canlı maya kültürü ilavesi araştırma sonu canlı ağırlığı rakamsal olarak (kontrol grubuna kıyasla %4.7) arttırmıştır ancak bu fark önemli bulunmamıştır. Çalışma süresince deneme gruplarına ait besi performansı, rumen sıvısı ve kan parametreleri farklı maya kültürleri ilavesinden istatistiksel olarak etkilenmemiştir. Deneme gruplarında dorsal papilla uzunluğu değişmezken, ventral papilla uzunluğu düşmüştür. YVM grubunun papilla uzunlukları kontrol grubuyla benzerdir.

Anahtar sözcükler: *Kan parametreleri, Besi performansı, Oğlak, Canlı maya kültürü, Rumen parametreleri*

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INTRODUCTION

Yeast and yeast products are often used in ruminant diets to manupulate rumen fermentation and improve animal performance. The benefits of live yeast culture are well understood. However, researches carried out on the effects of yeast products in small ruminants are limited. Studies on the effects of yeast cultures have reported variable results. These differences may depend on many factors such as diet composition, forage to concentrate ratio, type of forage feed, yeast dose, feeding strategy and stage of lactation [1]. *Saccharomyces cerevisiae* vary widely in efficiency, primarily because of differences in strain, the viability of yeast cells, and theirs dosage [2].

Studies reported that yeast supplementation increased growth, feed intake and nutrient utilization in Black Bengal kids [3], improved feed conversion ratio of Awassi lambs [4]. In contrast, yeast supplementation did not always improve the animal performance. One of the study on Awassi lambs and Shami goat kids reported that yeast supplementation had no effect on avarage daily gain and dry matter intake [5]. Another research conducted on Saanen dairy goats show that addition of live yeast into diet increased dry matter intake and milk yield in early lactation period [6].

Some of studies found that yeast culture supplementation did not affect some serological blood parameters in goats [7] and in dairy cows [1]. However, Galip [8] reported that dietary yeast culture altered serum total protein, urea, calcium concentrations, Ca/creatinine ratio and triglyceride concentrations in rams.

Addition of yeast culture to the ruminant diets has shown inconsistent effects on results of rumen volatile fatty acid (VFA) concentration. One of the reports on dairy cows with two different saccharomyces strains indicated that strain has an importance and that can modify ruminal ammonia, propionate and butyrate concentration. However, saccharomyces strains have no effect on productive performance [9]. Another study revealed that yeast culture addition to dairy cow diets had positive effects on ruminal VFA production [10]. Desnoyers et al.[11] reported that the positive effect of yeast supplementation on rumen pH was increased with the higher level of concentrate in the diet and the dry matter intake level. They also indicated that the positive effect of yeast supplementation on rumen was increased VFA concentration with higher dry matter and crude protein intake.Chaucheyras-Durand et al.[12] also mentioned that active dried yeasts in young ruminants has a stabilization function on rumen pH.

The goat population is about 5 million in Turkey and one of the prefered dairy goat species is Saanen. Male kids have less economic value for dairy farms in birth season when compared with females. Also there is growing concern about combination feed additives because of economic reasons. Combination of live yeast with vitamin-mineral is one of it. Effects of supplementing live yeast culture and the combination of live yeast culture with vitamin-mineral to the diets of fattening male Saanen kids have not been studied. Therefore, the objective of this study was to evaluate the effects of supplementation of these products to fattening diets of male Saanen kids on feed intake, growth performance, some blood parameters, papilla morphology and rumen organic acids.

MATERIAL and METHODS

The experiment was conducted in a commertial farm in Burdur, Turkey. Management protocols, animal care and protocols of the research were made in accordance with approved Local Ethical Committee on Animal Experiments of Mehmet Akif Ersoy University (01.04.2013- 93773921-30- verdict no: 29).

Animals, Housing, Feedstuffs, and Experimental Procedures

A total of 18 male, average 8 weeks old Saanen kids were used in the study. All animals were fed with milk replacer and concentrate (control group feed) before experiment. Rumisacc and Intetotal were not offered this period. All animals were treated for internal and external parasites using Ivomec (Novakim; active ingredient: 10 mg/ml Ivermectin; dose: 1 ml/50 kg live weight) 2 weeks before the experiment started. This study was conducted at the commercial feedlot for 11 weeks from May 2013 to July 2013. Kids were housed in individual cages (100x 150x120cm) under the shed with concrete floor with sawdust and dry manure bedding for the entire period of the experiment. Concentrates were prepared in a feedmill as a mash feed. The rations were formulated as isocaloric and isonitrogenous. The formulation and chemical composition of the concentrates are presented in *Table 1*.

Live yeast culture and its vitamin-mineral combination product (RumiSacc® and Intetotal® respectively, Integro food Industry and Trade Co., Istanbul, Turkey) were both included in the concentrates at 1.0%. Copper is completely removed from YVM (Intetotal®) by producer. During the study concentrate feed and fresh water were given *ad libitum* and the ration did not contain any roughages. Refused feeds were collected once in a week and weighed to accuretly determine the feed intake.

Nutrient composition of concentrates were determined according to the AOAC [13], crude fiber was determined by the methods of Crampton and Maynard [14]. The metabolizable energy levels of concentrate feeds were determined by using the following formula of TSI [15].

ME (kcal/kg OM) = 3260 + (0.455xCP) – (4.037xCF) + (3.517xEE) where CP (crude protein), CF (crude fibre) and EE (ether extract) were expressed as g/kg OM (organic matter).

Table 1. *The formulation and chemical composition of concentrate feeds*
Tablo 1. *Konsantre yemin kimyasal kompozisyonu ve formülasyonu*

Diet Formulation	**C**	**YC**	**YVM**
Corn	35	35	35
Barley	21	21	21
Wheat Bran	11	11	11
Full fat soy	11	11	11
Sunflower meal, 36% Crude Protein	10	10	10
Soybean meal, 48% Crude protein	7	6	6
DCP	1.7	1.7	1.7
Canola oil	1	1	1
DL-methionine	0.1	0.1	0.1
L-lysin hidroclorid	0.1	0.1	0.1
Live yeast culture[1]	-	1	-
Live yeast - vitamin, mineral complex[2]	-	-	1
Lime stone	1.5	1.5	1.5
Salt	0.4	0.4	0.4
Vitamin mineral premix[3]	0.2	0.2	0.2
Chemical Composition			
Dry matter, %	88.38	88.40	88.09
Crude protein, %	16.34	16.68	16.38
Crude fiber, %	7.03	6.78	6.46
Ether extract, %	5.31	5.94	6.06
Ash, %	6.68	6.94	6.71
ME, kcal/kg ME	2640.72	2664.70	2677.20

C: *Control group;* ***YC:*** *Group fed with diet containing live yeast culture;* ***YVM:*** *group fed with died containing the combination of live yeast culture with vitamin and mineral,* ***1:*** *RumiSacc, Integro Food Industry and Trade Co., İstanbul, Turkey (Live yeast cell 344 x 10^{10} cfu/g, Dry matter: 91.50%, Crude protein: 44.31%),* ***2:*** *Intetotal, Integro Food Industry and Trade Co., İstanbul, Turkey (Live yeast cell 344 x 10^{10} cfu/g, Dry matter: 91.50%, Crude protein: 40.51%),* ***3:*** *Each kilogram of vitamin-mineral mix contains 12.000.000 IU A vit, 20.000 mg E vit, 50.000 mg Mn, 50.000 mg Fe, 50.000 mg Zn, 10.000 mg Cu, 800 mg I, 150 mg Co, 150 mg Se*

Animals were weighed individually at the beginning of the experiment and every two weeks. The average daily weight gain over the duration of experiment was determined individually. Daily feed intake of the kids were determined and feed conversion ratio was calculated as kg feed per kg live weight gain of kids individually.

Slaughter Procedures, Sampling and Ruminal Fluid Evaluation

Animals were slaughtered at 11th weeks of the trial (avaregely 19th weeks old aged). Kids were sacrificed in a commercial slaughter house. Ruminal fluid and rumen wall samples were collected.

Hot carcasses were weighed, suspended through the achilles tendon, and then chilled at 4ºC for 24 h and weighted again. By this method hot and cold carcass yield were determined.

Rumen fluid samples were collected in two bottles from all kids in each group during the slaughtering process. Rumen fluid sample in one bottle was used for the measurement of pH, VFA and lactic acid. The pH was measured immediately by a pH meter (Hanna pH meter, model no: Hi917hN). Rumen fluid samples were filtered from cheese cloth before VFA analysis. After centrifugation (10.000 rpm, 10 min at +4ºC) concentrations of VFA in the supernatant were determined by HPLC system of Agillent 1260 series (Agillent Technologies, Waldronn, Germany) equipped with a Agilent-detector (1260 MVDVL) operated at 210 nm. Separation of acids was conducted using an organic acid analysis column (300 x 7.7 mm; Hi-plexH-organic acid column), with 0.005 M H_2SO_4 as eluent, at flow rate of 0.6 ml/min, and with the column temperature of 55°C. Rumen fluid samples were analysed in Integro lab. Concentrations of ammonia-N were determined by distillation (Gerhard, vapodest 2000) and titration, by using 5 ml of the rumen fluid which filtered by from cheese cloth [16].

Ruminal Wall Parameters

Samples were collected from the bottom of saccus ruminis dorsalis and ventralis for histological and morphometric examination after was slaughtered. The size of the samples was approximately 1x1x0.5 cm. The tissues were fixed for a period of 72 h after the collection in 10% formalin. Afterwards, the samples were processed using the common paraffin method, stained with haemotoxylin-eosin and evaluated by a light microscope. Ten papillae were measured in each section: the length of the papilla (from the base to the apex), the width of the epithelium (the distance between the external border of lamina propria mucosae and internal border of stratum corneum at the papillar base), the width of stratum corneum from the external end of stratum lucidum to the papillary surface and also keratinisation on the surface of the papillae [17].

Blood Analyses

At the first and last day of trial, blood samples were taken from jugular vein and collected in two tubes in the morning. In order to conduct hematological analysis the blood samples were transferred into tubes which contain EDTA and for biochemical analysis the blood samples were transferred into tubes which do not contain EDTA and centrifuged at 3.000 rpm at room temperature for 5 min and then serum was carefully harvested for determination of total cholesterol, triglyceride, glucose and blood urea nitrogen (BUN). Analysis was done with theVET TEST 8008 Autoanalyzer (IDEXX Laboratories, inc Westbrook ME 04092 USA). Other blood samples freshly used for hematological analyzes (WBC, RBC, HGB, HCT, MCV, MCH, MCHC, RDWc). Abacus Junior Vet Hematology Analyzer (Diatron MI PLC. Hungary) was used for hematological analysis.

Statistical Analyses

Statistical analysis was done by using computer programme. It is obtained that the means of data forming groups are normally distributed by using Shapiro-Wilk test One way ANOVA was performed to detect the differences between groups. The significance of mean differences between groups were tested by Duncan [18]. Values were given as mean ± standard error. Level of significance was taken as $P<0.05$.

RESULTS

Crude protein analysis of Rumisacc and Intetotal were found to be 44.31 and 40.51% respectively. Both of two dietary live yeast additives did not significantly affect final live weights of kids *(Table 2)*. Avarage body weight gain, feed intake, feed conversion ratio, hot and cold carcass yield *(Table 3)*, rumen pH, ammonia-N and VFA (total and individual) concentration *(Table 4)*, initial and final hematological and blood chemistry results *(Table 5* and *Table 6)* were not significantly affected (P>0.05) by treatments. Effects of dietary treatments on rumen papillae morphology has been showed in *Table 7*. While dorsal papillae lenghts did not change in all groups, ventral papilla lengths in YC group were shorter than the other groups (P<0.05). Thicknesses of epithelium were different among the groups (P<0.05).

DISCUSSION

Supplementation of YC increased final weight up to 1.44 kg numerically when compared to control group but this increase was not statistically significant. This situation may be attributed to low numbers of animals and individual

Table 2. *Effects of dietary treatments on body weight, kg, n=6*
Tablo 2. *Deneme rasyonlarının canlı ağırlığa etkisi, kg, n=6*

Days	C	YC	YVM	P
Initial (avarege of 8 weeks old) body weight, kg	16.45±0.82	16.80±0.35	16.82±1.22	0.939
Day 14 (10 weeks old)	19.39±0.86	20.03±0.45	19.29±1.26	0.808
Day 28 (12 weeks old)	22.16±1.21	22.12±0.35	22.41±1.49	0.866
Day 42 (14 weeks old)	24.20±1.44	25.57±0.54	24.44±1.43	0.683
Day 56 (16 weeks old)	27.67±1.64	29.02±0.47	27.43±1.48	0.654
Day 70 (18 weeks old)	30.15±1.71	31.68±0.52	29.91±1.70	0.629
Final day 77 (19 weeks old)	30.68±1.83	32.12±0.52	30.98±2.06	0.785

C: *Control group;* ***YC:*** *Group fed with diet containing live yeast culture;* ***YVM:*** *group fed with died containing the combination of live yeast culture with vitamin and mineral*

Table 3. *Effects of dietary treatments on performance and carcass parameters, n=6*
Tablo 3. *Deneme rasyonlarının performans ve karkas parametreleri üzerine etkisi, n=6*

Parameters	C	YC	YVM	P
Weight gain, g/d	175.67±16.59	187.59±12.66	181.30±22.65	0.881
Feed intake, g/d	952.06±50.57	1055.72±22.33	1013.05±68.96	0.325
Feed conversion ratio	5.53±0.27	5.78±0.48	5.79±0.53	0.889
Hot carcass weight, kg	14.30±0.83	14.70±0.28	13.80±1.00	0.706
Cold carcass weight, kg	13.86±0.81	14.33±0.29	13.48±0.96	0.717
Hot carcass yield, %	46.62±0.62	45.75±0.44	44.48±0.86	0.104
Cold carcass yield, %	45.21±0.77	44.61±0.50	43.46±0.75	0.236

C: *Control group;* ***YC:*** *Group fed with diet containing live yeast culture;* ***YVM:*** *group fed with died containing the combination of live yeast culture with vitamin and mineral*

Table 4. *Effects of dietary treatments on rumen organic acids (mg/l), pH and NH_3–N, n=6*
Tablo 4. *Deneme rasyonlarının rumen organic asit (mg/l), pH ve amonyak azotu üzerine etkisi, n=6*

Ruminal Fluid	C	YC	YVM	P
Lactic acid	185.50±84.08	89.00±53.69	162.66±76.47	0.720
Acetic acid	3146.00±320.38	2766.16±230.08	2719.00±340.23	0.558
Propiyonic acid	856.33±81.76	1055.50±112.13	893.33±146.41	0.456
Iso-butyric acid	159.50±60.59	91.00±18.45	37.33±13.36	0.101
n- butyric acid	795.50±104.61	748.83±86.76	899.00±132.50	0.621
pH	5.50±0.08	5.64±0.07	5.53±0.15	0.602
NH_3-N, mg/l	1079.33±190.90	991.00±53.31	992.00±50.10	0.101

C: *Control group;* ***YC:*** *Group fed with diet containing live yeast culture;* ***YVM:*** *group fed with died containing the combination of live yeast culture with vitamin and mineral*

Table 5. *Initial some blood parameters in kids, n=6*
Tablo 5. *Oğlaklarda deneme başı bazı kan parametreleri, n=6*

Fresh Blood Parameters	C	YC	YVM	P
WBC, 10 /L	11.82±1.14	12.54±1.85	12.10±1.17	0.937
RBC, 1012/L	17.31±0.39	17.60±0.42	17.85±0.49	0.697
HGB, g/dl	9.21±0.23	9.45±0.28	9.16±0.43	0.792
HCT, %	24.35±0.62	24.67±0.68	23.92±1.11	0.811
MCV, fl	14.00±0.44	14.00±0.44	13.40±0.50	0.607
MCH, Pg	5.31±0.13	5.36±0.14	5.14±0.20	0.487
MCHC, g/dl	37.86±0.70	38.21±0.34	38.32±0.50	0.829
RDWc, %	46.60±1.07	46.11±1.20	47.10±0.69	0.813
Blood Serum Parameters				
Total cholesterol, mmol/L	2.89±0.40	2.42±0.48	3.46±0.42	0.298
Glucose, mmol/L	5.77±0.24	5.73±0.52	6.53±0.50	0.395
BUN, mmol/L	3.28±0.38	5.90±0.98	4.48±1.23	0.143
Triglyceride, mmol/L	0.22±0.10	0.26±0.14	0.33±0.24	0.221

C: *Control group;* ***YC:*** *Group fed with diet containing live yeast culture;* ***YVM:*** *group fed with died containing the combination of live yeast culture with vitamin and mineral*

Table 6. *Final some blood parameters in kids, n=6*
Tablo 6. *Oğlaklarda deneme sonu bazı kan parametreleri, n=6*

Fresh Blood Parameters	C	YC	YVM	P
WBC, 10 /L	12.09±1.46	13.39±1.16	12.75±0.71	0.739
RBC, 1012/L	17.35±0.53	17.66±0.49	17.77±0.41	0.825
HGB, g/dl	9.21±0.25	9.66±0.22	9.54±0.63	0.685
HCT, %	23.21±0.55	24.11±0.76	22.95±1.41	0.655
MCV, fl	13.16±0.30	13.83±0.47	13.00± 0.70	0.474
MCH, Pg	5.31±0.60	5.46±0.13	5.36±0.27	0.797
MCHC, g/dl	39.71±0.43	40.11±0.43	41.44±0.76	0.107
RDWc, %	45.50±0.14	44.61±0.56	46.72±1.04	0.108
Blood Serum Parameters				
Total cholesterol, mmol/L	2.17±0.20	2.31±0.34	1.49±0.31	0.160
Glucose, mmol/L	3.64±0.14	3.67±0.10	3.65±0.14	0.989
BUN, mmol/L	7.38±0.53	7.43±0.66	7.54±0.56	0.983
Triglyceride, mmol/L	0.25±0.01	0.29±0.02	0.26±0.04	0.475

C: *Control group;* ***YC:*** *Group fed with diet containing live yeast culture;* ***YVM:*** *group fed with died containing the combination of live yeast culture with vitamin and mineral*

Table 7. *Effects of dietary treatments on rumen papillae length, rumen papillae epithelium and keratin thickness, µm*
Tablo 7. *Deneme rasyonlarının rumen papilla uzunluğuna, rumen epiteli ve keratin kalınlığı üzerine etkisi, µm*

Location	C	YC	YVM	P
Dorsal	3031.25±133.55 (n=60)	2773.40±168.56 (n = 20)	2912.78±77.86 (n=58)	0.522
Ventral	3365.00±160.98^{a} (n=50)	2053.96±97.74^{b} (n=50)	3075.00±157.72^{a} (n=40)	0.001
Epithelium	106.16±3.01^{a} (n=60)	97.21±2.79^{b} (n=50)	70.56±2.25^{c} (n=60)	0.001
Keratin	17.47±0.52 (n=60)	16.26±0.66 (n=50)	18.79±1.16 (n=60)	0.122

C: *Control group;* ***YC:*** *Group fed with diet containing live yeast culture;* ***YVM:*** *group fed with died containing the combination of live yeast culture with vitamin and mineral;* ^{a,b} *Means in a row with different superscripts are significantly different (P<0.01)*

differences in body weight of the animals in the groups. Interestingly, avarage daily weight gain was approximately two times higher in YVM group than in other treatment groups at the final week of the experiment. Related with the average body weight gain results of this study is similar with Titi et al.[5]. The investigators reported that yeast culture supplementation did not affect live weight, live weight gain and dry matter intake in Ivesi lambs and Shami goat kids. On the other hand significant increases in live weight gain associated with yeast supplementation have been reported in goats [7,19] and lambs [4]. In the present study kids fed diets containing either two yeast culture product consumed 10.89 and 6.41% more feed respectively than control group. Beside this result, Kamal et al.[19] reported that live yeast supplementation significantly improved dry matter intake (DMI) per kg liveweight gain. Investigators were also mentioned that the more DMI and relatively more avarage daily gain in live yeast fed groups subsequently lead to improvement in the feed conversion ratio at the same study. There are several studies which have mentioned improvement in feed conversion ratio due to yeast feeding in lambs [4] and in goats [20]. However Titi et al.[5] reported that yeast culture supplementation incresed digestibility with no effect on growth, feed intake or feed conversion ratio of fattening Awassi lambs and Shami kids.

Addition of YC and YVM did not alter hot or cold carcass weight. Very little published literature is available concerning effects of yeast culture supplemetation on carcass, especially with small ruminants. Titi et al.[5] reported that yeast culture supplementation significantly decreased cold dressing proportion and hot carcass weight of Awassi lambs however did not affect on Shami goat kids as our results.

Our ruminal pH results are similar with a series of study which have shown that ruminal pH was not affected by the supplementation of *Saccharomyces cerevisiae* [8,19,21]. However significant increases in ruminal pH associated with yeast supplementation, have been reported in goats [7,22]. In the present study kids were adapted to concentrate in early age, this situation may have influence this stability of ruminal pH. Also there are investigations which have similar result [23,24] are available related with *Saccharomyces cerevisiae* and ruminal fluid of ammonia-N concentration with our results. However Özsoy et al.[7] reported that dietary inclusion of 3.0 and 4.5% live yeast culture significantly increased ammonia-N concentration on goats. Similarly Galip [25] indicated that ruminal ammonia-N concentrations were significantly increased by dietary yeast culture supplementation whatever the ratio forage/concentrate of the diet. Related with VFA concentrations of ruminal fluid there is a series of study [7,21,23,26] which have similar results with ours. However Kamal et al.[19] indicated that total volatile fatty acid concentration was significantly higher in live yeast culture fed kids at 2 and 4 months.

Blood chemistry results and some hematological parameter results of present study had parallel with the of results Özsoy et al.[7] that plasma cholesterol and triglyceride concentrations were not altered by yeast culture supplementation on goats and Yalçın et al.[1] on dairy cows. On the other hand, dietary yeast supplementation decreased serum triglyceride level in rams [8].

Various studies were available recording to the effect of nutrition on rumen papillae size. The papillae size is directly related to food intake, digesta weight in rumen, rate of fermentation, and weigh of rumen. The diet influences rumen papillae surface area and eventually the rumen efficiency and animal health [27]. Butyrate has been shown to have potent effects on papillae size [27,28]. The results presented in this paper showed that ventral papillae lengths of YC groups shorter than the other groups. In YC group, in spite of the fact that papilla length was not different from the other groups, ventral papillae were shorter than the other groups. The cause of this depression of ventral papilla length may be rumen fluid accumulate in this area.

Addition of live yeast culture and its vitamin mineral combination to male kids fed with concentrate (without forage) did not affect fattening performance, some blood and rumen fluid parameters, but ventral papillae lengths of YC groups shorter than the other groups. Dietary yeast culture at the level of 1% increased 4.7% final live weight when compeared with control group as numerically. Differences in results from some literatures depend on animal traits (animal strain, animal sex, animal's race, health status), yeast traits (vaiability, amount of yeast) ration traits (rate of forage/concentrate, forage ang grain types). More reasearches with different doses and more replicates are required to be conducted to determine the affects of live yeast culture products.

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Research Article

Hereditary Osteochondrodysplasia in Scottish Fold Cats [1] [2]

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Abstract

In this study, the clinical progress and the treatment results of cases Istanbul University, Faculty of Veterinary Medicine, Department of Surgery with a complaint of stilted gait, pain, lameness, reluctance to move, kyphosis and miniature appearance have been evaluated. The study materials included eleven Scottish Fold cats. The cases underwent conservative and surgical treatment due to severity of osteochondrodysplasic lesions. The aim of this study is to draw attention to this illness of homozigot and heterozigot Scottish Fold cats whose population is increasing dramatically in our country, to stop the uncontrolled mating of this breed and also to share our foundings and results with our colleauges.

Keywords: *Scottish Fold osteochondrodysplasia, Cat*

Scottish Fold Kedilerde Kalıtsal Osteokondrodisplazi

Özet

Bu çalışmada, İstanbul Üniversitesi Veteriner Fakültesi Cerrahi Anabilim Dalı'na hareket etmede isteksizlik, tutuk yürüyüş, topallık, arka bacaklarında şişlik, kifozis ve minyatür görünüm gibi şikayetlerle getirilen, tanısı kalıtsal osteokondrodisplazi olan, onbir adet Scottish Fold kedinin klinik durumları ve sağaltım sonuçları değerlendirilmiştir. Olgulara osteokondrodisplaziye bağlı lezyonların şiddetine bağlı olarak konservatif ve/veya cerrahi sağaltım uygulanmıştır. Bu çalışmayla, ülkemizde sayıları giderek artmakta olan Scottish Fold kedilerindeki kalıtsal nitelikli bu hastalığa dikkat çekmek, kontrolsüz çiftleşmelerin engellenmesini sağlamak ve ilaveten sağaltım seçeneklerinin bulgu ve sonuçlarını meslektaşlarımızla paylaşmak amaçlanmıştır.

Anahtar sözcükler: *Scottish Fold osteokondrodisplazi, Kedi*

INTRODUCTION

The characteristic feature of Scottish Fold cats is their folded ears [1-4]. The reason for this morphological difference is an autosomal dominant gene mutation that affects cartilage development. However, this mutation not only creates a simple morphological difference but also presents itself as hereditary osteochondrodysplasia [1-3].

The Scottish Fold breed emerged for the first time in the 1960s as a result of the mating of naturally-mutated local farm cats and British Shorthair cats [2,3]. The osteochondrodysplasia developing in all cats with folded ears [2,3], is a developmental abnormality affecting all cartilaginous structures in the body and the disease is manifested by deformed bone structure and severe arthritis [1-4].

The abnormal ear structure of Scottish Fold cats is due to the insufficiency of cartilage support. The folded appearance of the ears may become apparent when the kittens are 2-4 weeks old [1,2]. Studies have concluded that, this malformation in the pinna is an incomplete dominant hereditary feature of Scottish Fold cats [4].

Osteochondrodysplasia is not seen in homozygote cats alone. Varying degrees of primary developmental deformities are also observed in heterozygote cats [1-4]. The earliest and most common finding seen in homozygote cats is a short, thick, unbending tail structure [2,4,5].

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Findings may emerge in later stages in heterozygote cats [2,3].

Progressive deformities in the tail and distal extremities emerge when the cats are approximately 7-weeks old and these remain throughout their lives. The metaphyses of bones forming the tail and extremities are wide and the bone length is shorter than normal. Deformed tail vertebrae are fused together and the tail loses its flexibility due to this fused structure [1,2,4].

On radiographs, a deformed and irregular structure is observed in the tarsus, carpus, metatarsus, metacarpus, phalanges and tail vertebrae [4-6]. Once the kittens have reached 7-weeks of age, the deformity which has developed in the metaphyses of the metacarpal and metatarsal bones is visible on radiographs. Widening, flattening and sclerotic areas may be observed in the physis. Lesions may be less severe in the phalanges. The tail vertebrae appear short and growth plates expand. After 6-months of age, progressive new bone formation, especially in the distal extremities, and exostosis formation on the plantar surface of the calcaneus are observed radiologically. These exostoses may lead to joint ankylosis. Widespread osteopenia also accompanies the lesions [2-6].

The aim of this study is to raise awareness of this disease observed in both homozygote and heterozygote Scottish Fold cats, whose numbers in Turkey are increasing, draw attention to the prevention of their breeding and to present to colleagues the findings and results obtained in the study.

MATERIAL and METHODS

The material of the study comprised eleven Scottish Fold cats brought to the Istanbul University, Veterinary Faculty, Surgery Department between the years of 2010-2014, with complaints such as; reluctance to move, a stilted gait, lameness, swelling in the hind legs, kyphosis and a miniature appearance.

Following obtaining the history of each case from patient owners, physical and radiological examinations of the cases were performed. In the radiological examination of the cases, craniocaudal (CC) and mediolateral (ML) radiographic views of the distal extremities and phalanges and ventrodorsal (VD) and laterolateral (LL) views of the tail vertebrae were obtained.

Apart from case numbers 1, 9, 10 and 11, radiological follow-ups of the patients still continue at 2-monthly intervals at the present time. Case numbers 2, 3, 4, 6, 7, 8, 9, 10 and 11 received conservative treatment while surgical treatment was performed on case numbers 1 and 5 in order to remove exostoses. Prior to commencement of treatment, full blood count and biochemical parameters of all cases were evaluated.

For treatment, meloxicam was recommended orally at a dose of 0.1 mg/kg on the first day and 0.05 mg/kg thereafter, glycosaminoglycan chondroitin sulphate at a dose of 0.5-1 mg/kg and 65 mg omega-3 oil acid containing tablets once daily.

RESULTS

Of the 11 cats constituting the study cases, 6 were female and 6 were male. Three of the females had been spayed and one of the males had been castrated. Ages of the cats varied between 5 months and 3.5 years and bodyweight changed between 1.6 kg and 3.8 kg.

During clinical examination, all of the cases had a placid temperament and presented with a stilted gait. With the exception of case numbers 1, 9 and 11, the patient owners reported that all of the cases, aged between 5-months old and 2-years old, had been reluctant to move since they had been homed and constantly displayed a desire to sit, had a miniature appearance together with kyphosis and gave a pain response when manipulated.

In case numbers 1, 9 and 11, patient owners expressed that there had not been any particular muscular complaint except for the lameness which had begun in the past few days, however, that the cats had never been very mobile.

In the physical examination of case numbers 1, 9 and 11, difficulty in walking and a bilateral plantar

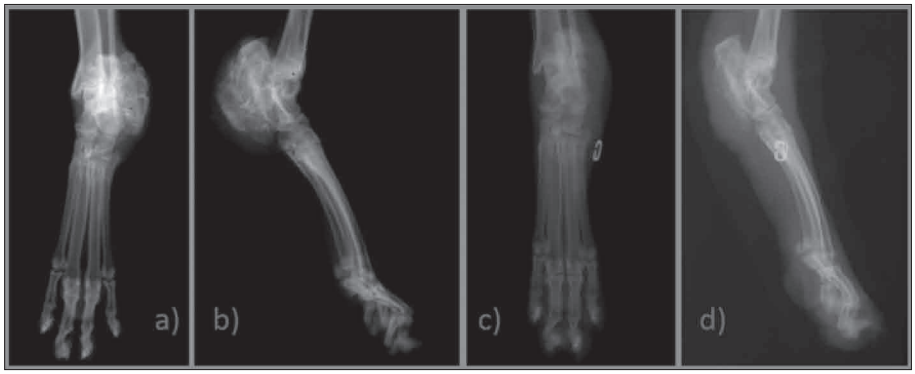


Fig 1. Case number 1: pre-operative (a, b) and immediately post-operative (c, d) radiographic view of left tarsal joint. Craniocaudal **a, c -** and mediolateral **b, d -** positions

Şekil 1. Olgu no: 1'e ait preoperatif (a, b) postoperatif hemen (c, d) sol tarsal eklem radyografik görüntüsü. **a, c-** kraniyokaudal, **b,d-** mediolateral pozisyonlarda alınan radyografiler

stance, together with bilateral, painful, hard masses of sizes ranging between a walnut and a tangerine were identified.

In case number 1, it was discovered that the range of motion in the tarsal joints had decreased and bilateral partial ankylosis had developed in the joints. In case number 9, there was partial ankylosis in the right tarsal joint while complete ankylosis was present in the left tarsal joint. Joint ankylosis in case number 11 had developed in the reverse order to those seen in case number 9. In all three cases, a clear pain response was elicited during physical examination of the tarsal joints. No abnormal findings were encountered in the length of the tail and distal extremities of these cases, however, in case number 11 there was severe pain during palpation of the tail region and difficulty in tail movement.

In radiographic assessments, distinct osteochondrodysplastic lesions were observed bilaterally in the tarsal joint areas. On radiographs, new bone formation with a cauliflower appearance was seen along the caudal surface starting from the proximal left calcaneous until the proximal metatarsus in case number 1 *(Fig. 1)*, and on the proximal plantar surface of the metatarsals in case numbers 9 *(Fig. 2)* and 11 *(Fig. 3)*. On the tail radiograph of case number 11, new bone formation was observed between the tail vertebrae *(Fig. 4)*.

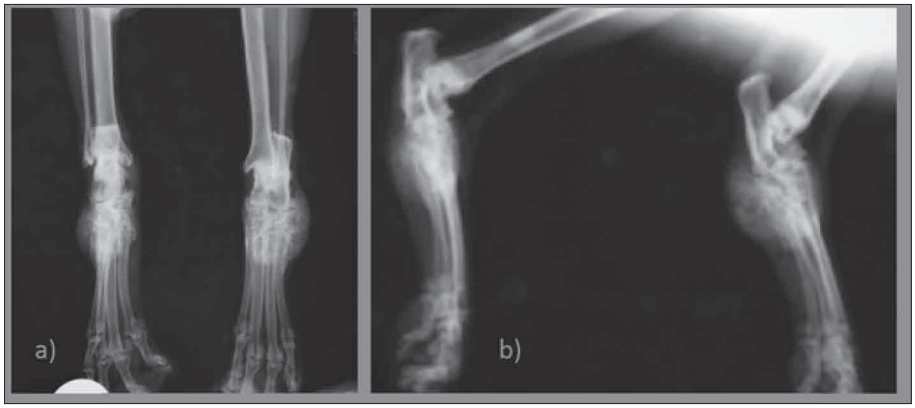
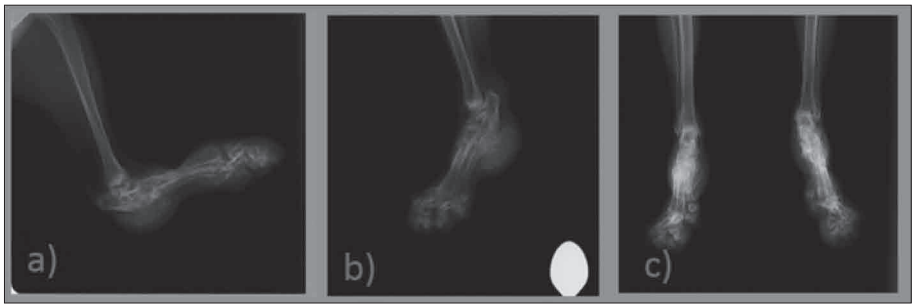


Fig 2. Case number 9: Radiographs taken at the time of first physical examination. **a-** craniocaudal view of bilateral tarsal joint, **b-** mediolateral view of bilateral tarsal joint

Şekil 2. Olgu no: 9'un muayeneye getirildiğinde alınan radyografileri. **a-** kraniyokaudal bilateral tarsal eklem görüntüsü, **b-** mediolateral bilateral tarsal eklem görüntüsü

Fig 3. Case number 11: Radiographs taken at the time of first physical examination. **a-** mediolateral view of left tarsal joint, **b-** mediolateral view of right tarsal joint, **c-** craniocaudal radiographic view of bilateral tarsal joint

Şekil 3. Olgu no: 11'in muayeneye getirildiğinde alınan radyografileri. **a-** sol tarsal eklem mediolateral, **b-** sağ tarsal eklem mediolateral, **c-** bilateral tarsal eklem kraniyokaudal radyografik görüntüsü



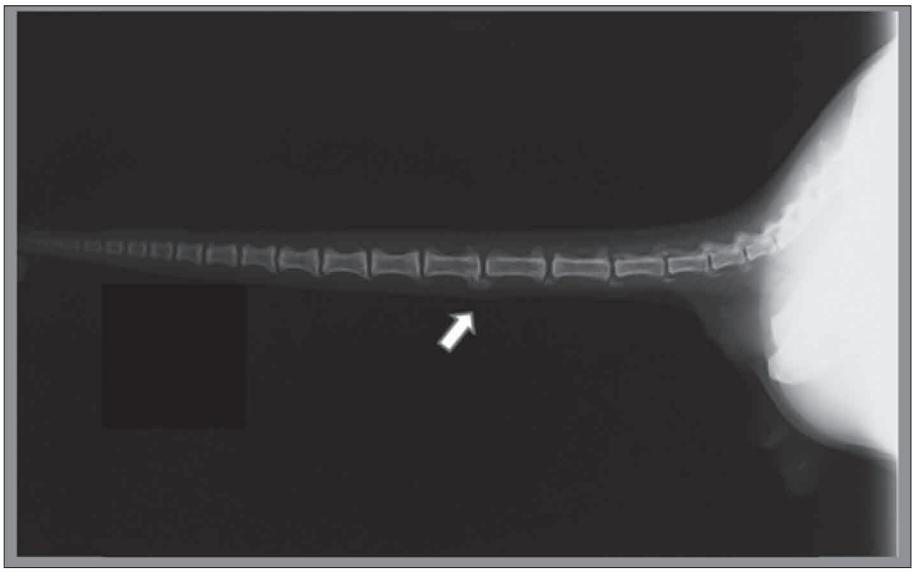


Fig 4. Case no: 11, laterolateral radiographic view of tail

Şekil 4. Olgu no: 11'e ait laterolateral kuyruk radyografisi

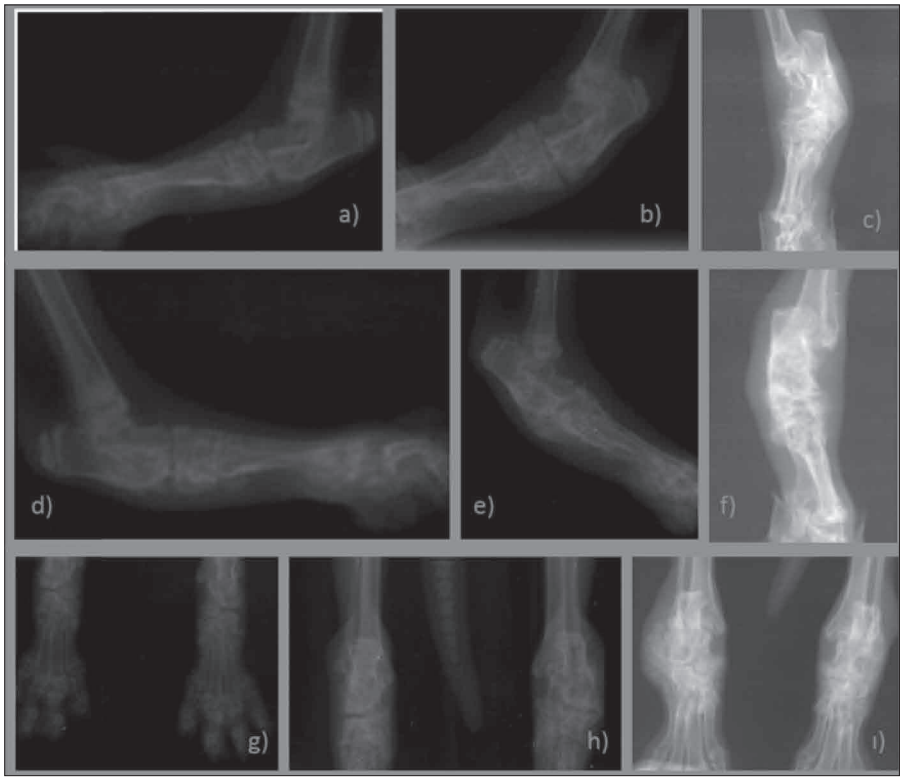
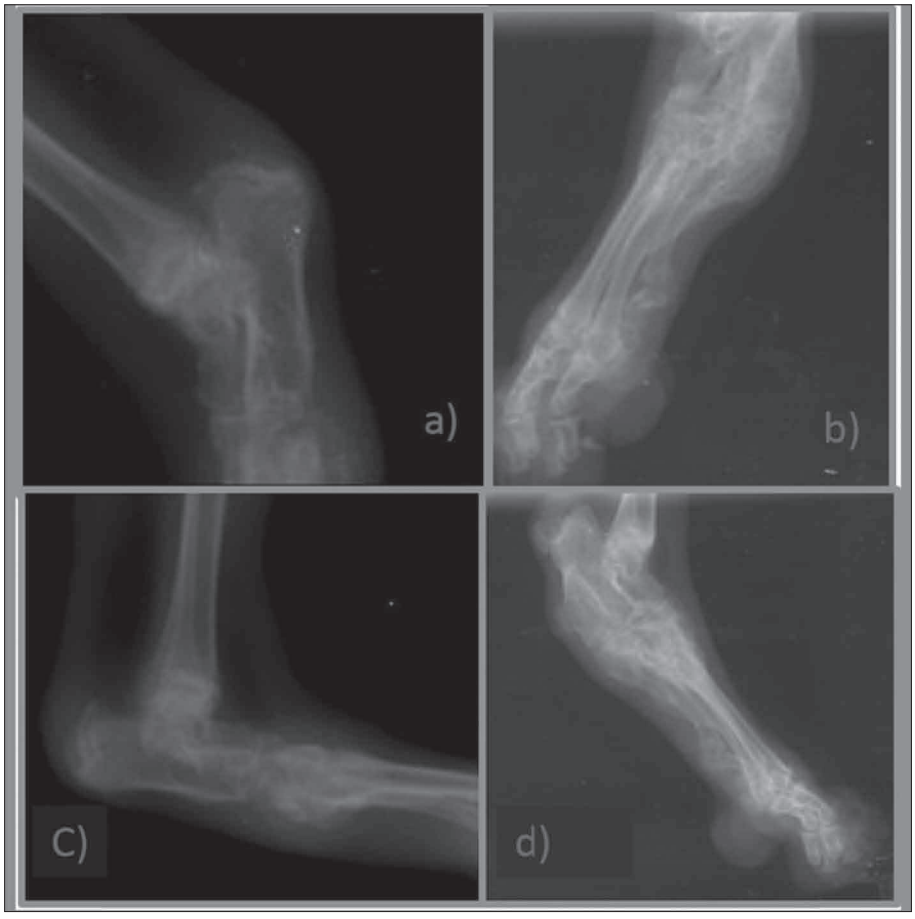


Fig 5. Case number 2, Radiographs of bilateral tarsal joints at time of first physical examination, 2 months later and 6 months later, respectively. **a, b, c-** right mediolateral tarsal joint, **d, e, f-** left mediolateral tarsal joint, **g, h, i-** craniocaudal bilateral tarsal joint

Şekil 5. Olgu no: 2'ye ait sırasıyla muayeneye getirildiğinde, iki ay sonra, altı ay sonraki bilateral tarsal eklem radyografik görüntüleri. **a, b, c-** sağ mediolateral tarsal eklem, **d, e, f-** sol mediolateral tarsal eklem, **g, h, ı-** kraniokaudal bilateral tarsal eklem

Fig 6. Case number 4, Radiographs of bilateral tarsal joints taken at time of first physical examination and 6 months later, respectively. **a, b-** right mediolateral joint, **d, e-** left mediolateral joint

Şekil 6. Olgu no: 4'e ait sırasıyla muayeneye getirildiğinde ve altı ay sonraki alınan bilateral tarsal eklem radyografileri. **a, b-** sağ mediolateral tarsal eklem, **d, e-** sol mediolateral tarsal eklem



In the physical examination of case numbers 2, 4 and 5, all under 1-year of age, the distal extremities were shorter than normal, thick and the joints were swollen and bulging. The tail structure of case numbers 2 and 4

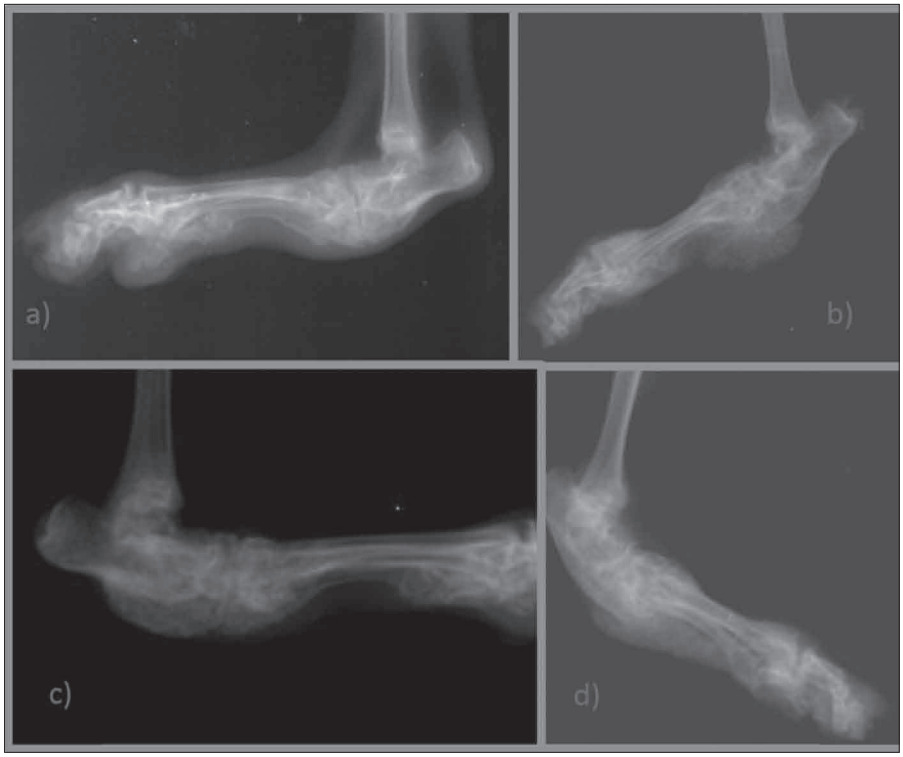
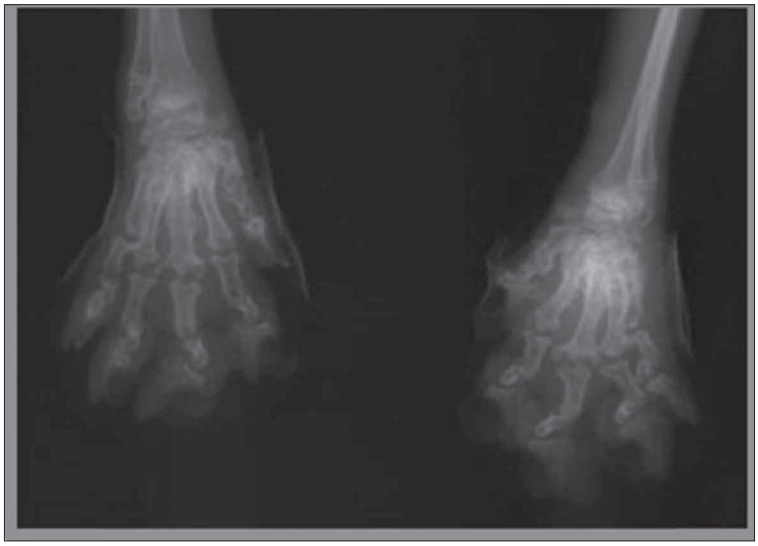


Fig 7. Case number 5, Radiographic view of bilateral tarsal joints obtained at time of first physical examination and 6 months later, respectively, **a, b-** right mediolateral tarsal joint, **c, d-** left mediolateral joint

Şekil 7. Olgu no: 5'e ait sırasıyla muayeneye getirildiğinde ve altı ay sonraki bilateral tarsal eklem radyografik görüntüleri. **a, b-** sağ mediolateral tarsal eklem, **d, e-** sol mediolateral tarsal eklem

Fig 8. Case number 2, Craniocaudal radiographic view of bilateral carpal joints

Şekil 8. Olgu no: 2'ye ait bilateral karpal eklem kraniyokaudal görüntüsü



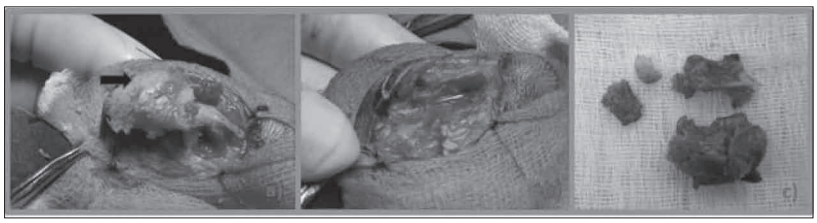


Fig 9. Case number 11, Operation views. **a-** new bone formation *(black arrow)*, **b-** after removal of new bone formation, **c-** removed bony parts

Şekil 9. Olgu no: 5'e ait operasyon görüntüleri. **a-** yeni kemik oluşumu *(siyah ok)*, **b-** yeni kemik oluşumunun uzaklaştırıldıktan sonraki hali, **c-** uzaklaştırılan kemik parçaları

was shorter than normal, thick and lacked flexibility. In radiographic images obtained at 2-monthly intervals, in case numbers *(Fig. 5)*, 4 *(Fig. 6)* and 5 *(Fig. 7)*, osteochondrodysplastic lesions first appeared as widening and proliferation on the plantar surface of the calcaneus and metatarsal bones and gradually became distinct exostoses. At the end of 6 months, it was seen that the epiphyseal and metaphyseal regions of distal extremities had flattened extensively, sclerotic areas had increased, intertarsal and tarsometatarsal joint spaces had become indiscernible and irregular bone structure was very advanced. Deformation in the

forelegs and metacarpal bones was seen only in case numbers 2 *(Fig. 8)* and 11. There were no obvious osteochondrodysplastic deformities in the forelegs of the other cases. In the palpation of distal extremities of all the cases, deformation was palpable and pain was present.

The severity of symptoms and radiographic lesions related to osteochondrodysplasia was relatively moderate in case numbers 3, 6, 7 and 8 compared to lesions in the remaining seven cases. The areas where proliferative lesions were observed were limited to the proximal part of the caudal surface of the metatarsals.

Following the assessment of case numbers 1 and 5, *(Fig. 9)* it was decided to perform surgical treatment in order to remove proliferative bone formations. Long-term clinical and radiological follow-up of case number 1 could not be done. In telephone conversations held with the owner of case number 5, it was established that the painful swellings in the hindlegs had recurred a few months after the operation. In case numbers 2 and 4, the lesions continue to advance. In case numbers 2, 4, 9 and 11, patient owners were explained that treatment was not curative and lesions could recur. Ostectomy was advised for ease of walking in the short-term, however, the patient owners declined this suggestion.

DISCUSSION

Despite being identified as a cat breed by The Governing Council of the Cat Fancy, the largest organisation for registering pedigree cats in the United Kingdom, the Scottish Fold cat was removed from the registered breed list for the first time in 1974, due to the deformities in its extremities and tail. Furthermore, the International Feline Federation (Federation Internationale Feline) deemed the reproduction of this breed unethical due to the distinct pain these cats suffer because of the genetic mutation and banned breeding of these cats carrying a hereditary disease [2,7].

The fact that the folded-eared appearance of this breed originates from generalised cartilage deformation [1,8-10] is either disregarded or not known and the endearing round faces and expressions of cats of this fenotype come across as likeable and attractive [11]. Regrettably, breeding of Scottish Fold cats continues in the United States and other countries around the world. This breed of cat used to be rare for Turkey at the beginning of the 21st Century, however, in recent years their numbers have been increasing.

All of the patient owners in this study expressed that they had chosen this breed of cat because of its folded ears. Also, it emerged that most of the patient owners were unaware of this disease while some patient owners explained that they knew about the disease but believed that they would not come across it since they thought their cat was a Scottish Fold-British Shorthair cross. The patient owners were strongly advised that the five unneutered cats should never be used for breeding.

Scottish Fold cats possess a miniature physiognomy. The distal extremities of affected cats may be insufficient to bear the bodyweight. In these cats, lameness, a stilted and strained walk together with reluctance to jump and move is observed in relation to the deformities [1,4,8-10].

In case number 1, a 3,5-year old cat, swelling had been noticed in the left hind leg several weeks before being presented to our clinic and it was established that, as well as the lesions progressing subclinically, the cat lived on a farm and the patient owner saw it very rarely. The owner of the other case (case number 9) with distinctive bilateral exostoses related to osteochondrodysplasia stated that they had also noticed the lesions at a late stage and had brought the cat in for physical examination once the mass on the left side had started obstructing the cat's walk. The fact that no abnormal findings were discovered in the tail structure and extremity length was compatible with data stating that heterozygote cats have less severe lesions [2,4,5,11] that emerge in later stages.

In heterozygote cats, radiologic follow-ups revealed new bone formation to be less widespread and of solitary character compared to homozygote cats. In the authors' opinion, the procedure for removal of new bone formation will present better results in heterozygote cats compared to homozygote cats.

The fact that there is no effective treatment procedure to eliminate osteochondrodysplasia in Scottish Fold cats causes treatment options to remain on a palliative level. Pentosan polysulfate [12] and chondroitin sulfate [6] have been recommended for cats affected by the disease. Furthermore, in cases accompanied by pain due to arthritis, meloxicam is a non-steriodal anti-inflammatory drug recommended for its safe long-term use [6,13]. In another study, the minimally invasive technique of radiotherapy was applied for its prevention of excessive bone formation and suppression of the inflammatory process [5,14]. Removal of exostoses can be suggested as a surgical treatment option. In one of the cases in this study, bilateral plantar exostoses were removed and pantarsal arthrodesis performed [15].

It is a fact that patients of this type will begin to suffer from chronic arthritic pain from a very early age. All the cats in this study, whether receiving surgical treatment or conservative treatment, were advised to use a long-term non-steriodal anti-inflammatory drug combined with Omega-3 fatty acid. In addition, the diet was altered by recommending commercial cat food containing joint and cartilage-protecting factors.

Setting out from these docile, attractive-faced cats' soft temperament and low-level physical activity, it is erroneous to assume that they can cope with the chronic pain caused by the disease or that they can adapt to an uncomfortable life. In the authors' opinion, the docile nature of these cats is due to the pain from which they suffer.

In conclusion, neither surgical nor conservative curative treatment is possible for osteochondrodysplasia in Scottish Fold cats and quality of life can only be improved using temporary methods.

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Research Article

Protection and Efficacy of Cell Culture Propagated Montanide Adjuvant Based Inactivated Vaccine Against Hydropericardium Syndrome in Poultry

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Abstract

The present study was designed to propagate field isolate of Fowl adenovirus-4 in chicken embryo liver (CEL) cell culture for development of efficient cell culture based inactivated vaccine. A pathogenic field isolate of fowl adenovirus-4 was propagated in chicken embryo liver cell culture. The liver homogenate virus ($10^{3.0}TCID_{50}$/ml) and cell culture propagated FAV-4 ($10^{3.0}TCID_{50}$/ml) was used for preparing water based and oil based inactivated vaccines. The post-vaccination antibody response to all vaccines was tested by Enzyme Linked Immunosorbant Assay (till 3 week post vaccination) and the chickens were subjected to challenge protection studies. The groups injected with oil base (Montanide) and water based conventional liver homogenate vaccines showed an average S/P (Sample/positive) value of 0.341 and 0.323 respectively, the groups given P-1 cell culture passaged oil (Montanide) based and water based vaccines showed an average S/P ratio 0.989 and 0.800 respectively. Cell culture based montanide adjuvanted vaccine showed highest antibody response (S/P) among all groups. Cell culture passaged vaccinated groups survived and provided 100% protection against challenge. Liver homogenate based vaccines provided 80% protection. As a whole the cell culture passaged vaccines qualified the known standards of safety, sterility and potency.

Keywords: *Fowl adenovirus-4, Hydropericardium Syndrome, Chicken embryo liver cell culture, Inactivated vaccine*

Kümes Hayvanlarında Hidroperikardium Sendromuna Karşı Hücre Kültüründe Üretilmiş Montanide Adjuvant Bazlı İnaktive Edilmiş Aşının Koruyucu Etkisi

Özet

Bu çalışma tavuk embriyo karaciğer (CEL) hücre kültüründe Fowl adenovirus-4'ün saha izolatını üreterek etkili hücre kültürü temelli inaktive edilmiş aşı geliştirmek amacıyla tasarlanmıştır. Fowl adenovirus-4'ün patojenik saha izolatı tavuk embriyo karaciğer hücre kültüründe üretildi. Karaciğer homojenat virus ($10^{3.0}TCID_{50}$/ml) ve hücre kültüründe üretilmiş FAV-4 ($10^{3.0}TCID_{50}$/ml) su bazlı ve yağ bazlı inaktive edilmiş aşılar hazırlamak amacıyla kullanıldı. Tüm aşılara karşı aşılama sonrası antikor cevabın oluşup oluşmadığı Enzyme Linked Immunosorbant Assay (aşılama sonrası 3 haftaya kadar) ile test edildi ve tavuklar etkene karşı koruma çalışmasında kullanıldı. Yağ bazlı (Montanide) ve su bazlı konvansiyonel karaciğer homojenat aşılar enjekte edilen gruplar sırasıyla 0.341 ve 0.323 ortalama S/P (Örnek/Pozitif) değeri gösterdi. P-1 hücre kültürü pasajlanmış yağ bazlı (Montanide) ve su bazlı aşılar enjekte edilen gruplar sırasıyla 0.989 ve 0.800 ortalama S/P değeri gösterdi. Montanide adjuvantlı hücre kültürü temelli aşı tüm gruplar içerisinde en yüksek antikor cevabı (S/P) gösterdi. Hücre kültürü pasajlanmış aşılı gruplar hayatta kaldılar ve etkene karşı %100 koruma gösterdiler. Karaciğer homojenat bazlı aşılar %80 koruma sağladı. Sonuç olarak, hücre kültürü pasajlı aşılar bilinen güvenlik, sterilite ve potansiyel standartlarını sağlamıştır.

Anahtar sözcükler: *Fowl adenovirus-4, Hidroperikardium sendromu, Tavuk embriyo karaciğer hücre kültürü, İnaktive aşı*

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INTRODUCTION

Hydrpericardium syndrome (HPS) is re-emerging disease caused by Fowl adenovirus serotype-4 resulting in huge economic losses to poultry industry in Pakistan since 1987. In affected chickens disease is characterized by accumulation of transparent straw colored watery fluid in the pericardium and swollen discolored fragile liver [1].

HPS has been controlled using autogeneous formalin inactivated vaccine prepared from infected liver homogenate [2]. Different vaccine formulations are being used in the field but none of them provides prompt, effective and long-lasting immune response against the natural outbreaks of HPS. Immunization of broiler chicks with a single dose of inactivated liver homogenate provides protection against hydropericardium syndrome although double shot of inactivated liver organ vaccine may be much more efficacious in breeders [3]. Infected liver homogenate based vaccines result in spreading of HPS and outbreaks even occur after vaccination. So extensive use of infected liver homogenate based vaccine should also be minimized by attempting to propagate the virus on eggs and cell cultures for the production of inactivated vaccines [4]. Adjuvants have been effective in enhancing antibody response of inactivated vaccines. Montanide ISA 70 adjuvant has been efficacious in different animal models [5]. Despite lot of work on HPS, literature regarding motanide adjuvant based cell culture passaged FAV-4 inactivated vaccines is scanty.

The present study was designed to develop an efficacious cell culture based inactivated vaccine against HPS.

MATERIAL and METHODS

Source of Virus

A field isolate Pak/NARC-3317/2008 of FAV-4 recovered from clinically affected birds with HPS was used as vaccine virus in this study. This virus was earlier stored in the repository of National Reference Lab for poultry Diseases (NRLPD), National Agricultural Research centre, Islamabad. The virus was confirmed through PCR using standard procedure [6]. Amplification of a 730 bp variable part of the hexon gene was done by PCR to confirm the presence of viral DNA. The PCR products were analyzed on 1% agarose gels containing 0.1% ethidium bromide along with 1 Kb DNA ladder. PCR product was visualized by placing the gel in Gel documentation system.

Propagation of FAV-4 in Cell Culture
Chicken Embryo Liver (CEL) Cell Culture

Primary chicken embryo liver cells (CEL) were prepared in 25 cm^2 cell culture flasks using the standard protocol [7]. CEL monolayer was first infected with 0.2 ml of FAV-4 virus suspension and incubated up to 72 h or till >75% CPE was noticed. The flasks were freeze thawed thrice and virus was clarified after centrifugation at 1.500 rpm at 4ºC for 10 min. This virus propagated in CEL cell culture was saved at -70ºC till used. The preparation was labeled as FAV-4-CEL cell culture.

Vaccine Preparation

Using liver homogenate FAV-4 and CEL cell culture propagated FAV-4 two types of vaccines with different combinations were prepared following the standard protocols of vaccine production [7]. The stock virus was inactivated for 48 h using formaldehyde to attain final concentration of 0.02%. To test viral inactivation the material was propagated in chicken embryo liver cell culture and tested for presence of FAV-4 by PCR. The inactivated virus stocks were saved at 4ºC till further used. This virus was used for preparing inactivated water based and oil based vaccines.

For preparing oil based liver homogenate inactivated FAV-4 vaccine the virus was blended with adjuvant using the following recipie.

A

FAV-4 liver homogenate (10^5 $TCID_{50}$/ml)	3 ml
Montanide ISA-70	7 ml

For preparing water based liver homogenate inactivated FAV-4 vaccine the virus was blended with adjuvant using the following recipie

B

FAV-4 liver homogenate (10^5 $TCID_{50}$/ml)	3 ml
Adjuvant (10% Aluminium hydroxide)	0.1 ml
Water	6.9 ml

For preparing cell culture propagated oil based inactivated FAV-4 vaccine the virus was blended with adjuvant using the following recipie

C

FAV-4/CEL/P1 stock (10^5 $TCID_{50}$/ml)	3 ml
Montanide-70	7 ml

D

For preparing cell culture propagated water based inactivated FAV-4 vaccine the virus was blended with adjuvant using the following recipie

FAV-4/CEL/P1 stock (10^5 $TCID_{50}$/ml)	3 ml
Adjuvant (10% Aluminium hydroxide)	0.1ml
Water	6.9 ml

Vaccination of Birds

100 day old broiler chicks were reared at the animal house of National Reference Laboratory for poultry

Diseases at NARC. For this purpose chicks were divided into five groups and kept in chicken isolators *(Table 1)*. In groups A and B a dose 0.2 ml per bird of inactivated liver vaccines were given subcutaneously at 8th day of their age in oil base and water base combination respectively. In group C and D birds were immunized with 0.2 ml of CEL cell culture propagated FAV-4. Group E was injected with Phosphate buffered saline (PBS).

Blood Sampling

Blood samples were collected weekly from birds of all groups including control up to 3 week post-vaccination. Sera were separated by centrifuging at 1.500 rpm for ten minutes and further tested for immune response by ELISA using protocol given below.

Challenge Studies

A field isolate of FAV-4 (Pak/NARC-3317/2008) was used for challenge studies. For this purpose birds in vaccinated and control groups were challenged three week post vaccination using 10^5 $TCID_{50}$ of field isolate. Clinical observations were recorded and postmortem was done upon death of any bird during the experiment. After two weeks of challenge all surviving birds were necropsied and checked for HPS lesions.

Indirect ELISA

Humoral immune response against FAV-4 was assessed by antibody detection using indirect ELISA in each group. ELISA was standardized by introducing some modifications in the procedure earlier reported in literature [8]. ELISA plate (96 well flat bottom polystyrene microtiter plate) was coated with 1:10 dilution of CEL cell culture propagated FAV-4 antigen in carbonate bicarbonate buffer (pH-9.6). 50 µl of diluted antigen was used for coating plates. After incubation of 90 min at 25°C plate was washed three times with PBS -Tween 20. Blocking of plate was done by addition of 100 µl of blocking solution (5% BSA) in each well. Plate was incubated for 1 h at 25°C and washed thrice with PBS-Tween-20 solution. Test samples were diluted (1.17) in dilution buffer (2% BSA) and 50 µl of diluted test sample was added in each well including positive (FAV-4 antiserum raised in chicken) a and negative control (Serum from Uninfected control chicken). Plate was incubated for 1 h at 25°C. After washing Plate 50 µl of 1:500 dilution of Horseraddish peroxidase conjugate was added and incubated for 40 min. 50 µl of Substrate (OPD 30% in phosphate citrate buffer pH 5.0 and 70% H_2O_2) was added in each well and incubated for 15 min. Reaction was stopped by adding 50 µl of 1M H_2SO_4 in each well. The absorbance values (OD) were read with ELISA reader at 492 nm. OD values from individual samples were used to calculate Mean sample-to-positive (S/P) ratio.

Ethical Committee Report

The study was approved by the Intuitional Ethical Committee of the Animal Sciences Institute, NARC, thorough letter No. 01521/ASI/NARC.

RESULTS

The virus was confirmed as Fowl adenovirus-4 through PCR (730 bp) *(Fig. 1)*. The virus inoculum from positive known FAV-4 serotype upon chicken embryo liver cells propagation showed cytopathic effects (CPE) after 48 h. CPE were characterized by rounding and clumping of cells *(Fig. 2)*. This was referred as passage 1 of FAV-4 and was used for cell culture based vaccine preparation. Confirmation of cell culture propagation of FAV-4 was done by PCR *(Fig. 3)*.

HPS specific antibody response was not detected at first week post vaccination by ELISA. HPS specific Antibody response of chicks in groups A, B, C, D, and E was detected positive by ELISA during second week post vaccination. Antibody response by ELISA was interpreted by finding the cut off value between negative and positive samples. Cut off value (0.116) was estimated as mean of known negative S/P ratio (0.026) plus two standard deviation [9].

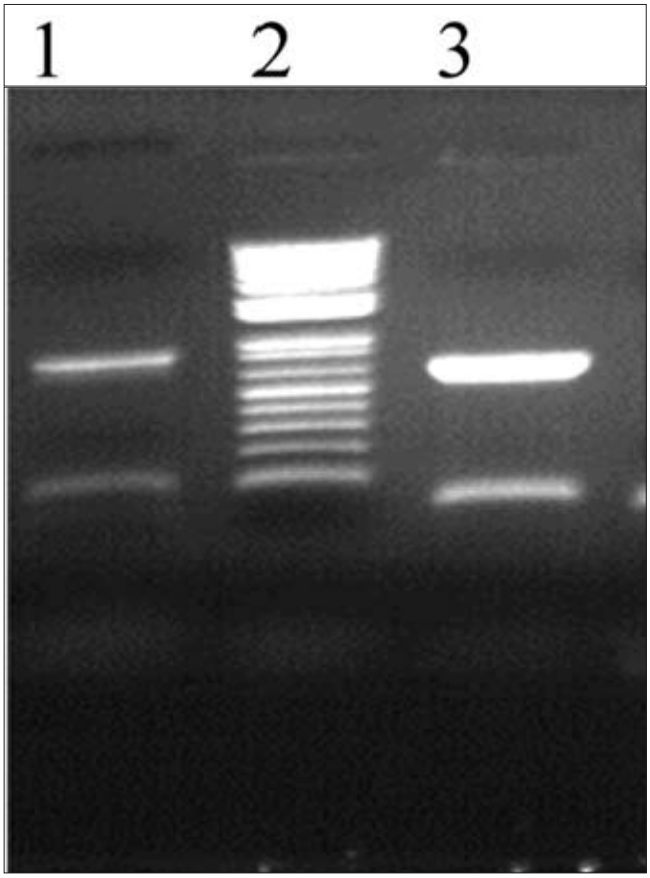

Fig 1. PCR amplification of hexon gene from field samples (Lane-1 FAV-4 positive sample 7 30bp hexon gene, Lane-2 Marker 1-kb plus, Lane-3 positive control)

Şekil 1. Saha örneklerinden hekzon geninin PCR amplifikasyonu (1. sıra FAV-4 pozitif örnek 7 30 bp hekzon geni, 2. sıra Markır 1-kb artı, 3. sıra pozitif kontrol)

Fig 2. CPE of P-1 CEL cell culture
Şekil 2. P-1 CEL hücre kültürünün sitopatik etkisi

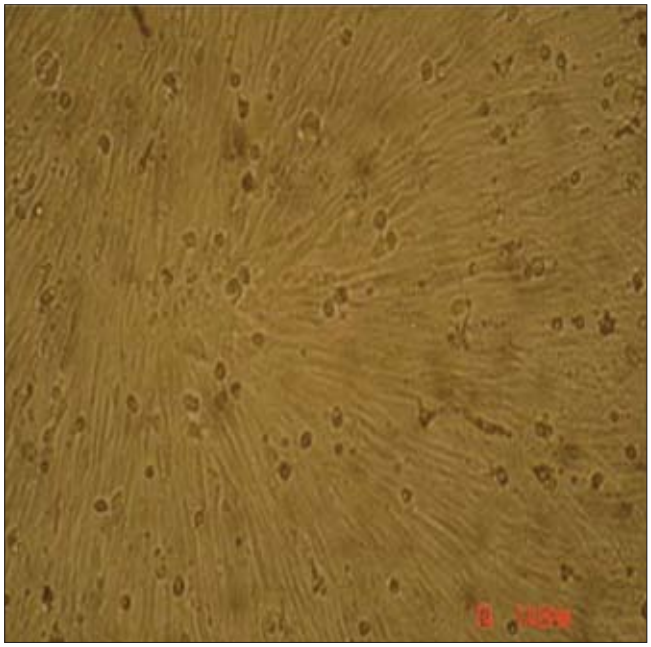

Fig 3. Uninfected Control monolayer
Şekil 3. Enfekte olmamış control monolayer

Samples giving OD higher than cut off values were considered positive and found protective.

The ELISA antibody response in groups C and D (Cell culture based montanide adjuvanted and water based vaccines) indicated mean S/P value of 0.665 and 0.496, respectively. These values were significantly higher ($P<0.05$) than those in groups A and B (mean S/P 0.287,C2, mean S/P 0.276) and were above cut off value. A significant

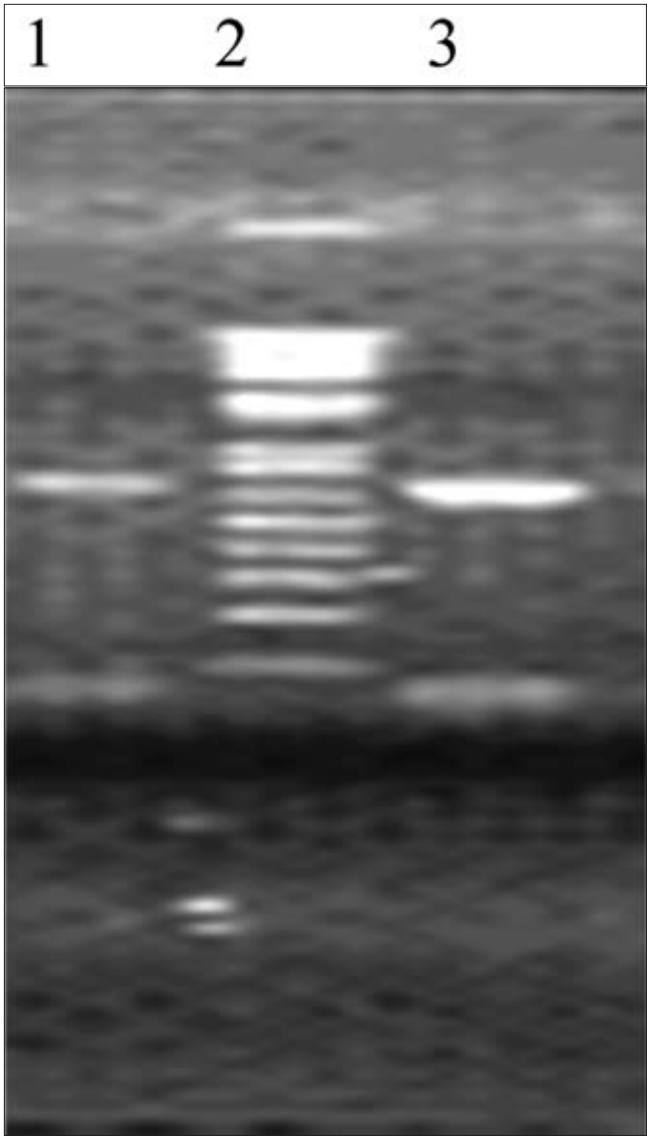

Fig 4. PCR data regarding FAV-4 grown in Chicken embryo liver cell culture **(**Lane-1 730 bp hexon FAV-4 P-1 CEL, Lane-2 Marker 1-kb plus, Lane-3 FAV-4 positive control)
Şekil 4. Tavuk embriyo karaciğer hücre kültüründe üretilmiş FAV-4 ilişkin PCR (1. Sıra 730 bp hekzon FAV-4 P-1 CEL, 2. sıra Markır 1-kb artı, 3. sıra FAV-4 pozitif kontrol)

increase ($P<0.05$) in ELISA S/P was recorded at 24th day PV in all groups *(Table 1)*.

Cell culture vaccine based on Montanide adjuvant showed highest antibody response (S/P 0.989). The chicks in unvaccinated control group did not indicate any seroconversion against HPS FAV-4 virus.

A challenge protection study was done for evaluation of the efficacy of cell culture based adjuvanted vaccine in comparison with liver homogenate based vaccines. Birds immunized with cell culture based adjuvanted vaccine having highest S/P (0.989) by ELISA showed 100% protection against hydropericardium syndrome. Birds in groups A and B immunized with liver homogenate vaccine

Table 1. *Comparitive antibody response of inactivated oil based and water based vaccines by ELISA in terms of S/P values*
Tablo 1. *ELISA ile belirleneni inaktive edilmiş yağ bazlı ve su bazlı aşıların karşılaştırmalı antikor cevapları (S/P değerine göre)*

ID	Vaccine Type	1 Week Post Vaccination Mean S/P	2 Week Post Vaccination Mean S/P	3 Week Post Vaccination Mean S/P
A	Tissue homogenate(Liver) Vaccine (Montanide ISA 70 based)	0.053	0.287	0.341
B	Tissue homogenate (Liver) Vaccine (Water based)	0.082	0.276	0.323
C	CEL Culture Propagated P-1 (Montanide ISA 70 based)	0.043	0.665	0.989
D	CEL Culture Propagated P-1 (Water based)	0.032	0.496	0.800
E	PBS (Control)	0.050	0.053	0.054

(commercially used in Pakistan) showed 80% protection. Birds in group E (negative control) showed 90% mortality. Dead birds showed typical signs of HPS (Watery fluid around heart).

DISCUSSION

Hydropericardium Syndrome (HPS) is re-emerging disease of broilers (3-6 weeks old) and breeders (6-20 weeks old) resulting in high mortality [10]. Formalin-inactivated liver organ vaccines are the only available source of vaccines against HPS in Pakistan which have been unable to control/eradicate disease [4]. For the development of an efficacious vaccine against HPS chicken embryo liver cell culture was used for continuous passages in this study. Protective efficacy of an oil adjuvanted cell culture adapted vaccine has been found superior to the liver homogenate vaccine [11].

In the present study CEL cell culture based montanide and water adjuvanted vaccines (C and D) were tested for efficacy in chicken. The results revealed that virus neutralizing antibody response rose at day 16 after the administration of vaccines. At 24th day post vaccination there was an increase in the antibody response. Cell culture montanide adjuvanted vaccine (C) at 16th and 24th day post vaccination showed significantly higher level ($P<0.05$) of antibody response (S/P) in birds as compared to cell culture water based vaccinated group (D).

In the present study the efficacy of cell culture based inactivated adjuvanted vaccines was tested and compared with liver homogenate vaccines (Commercially used in Pakistan) of HPS. The results revealed that in Group C (cell culture based montanide adjuvanted vaccine) and D (cell culture based water adjuvanted vaccine) the antibody response at week 2 post vaccination was significantly higher ($P<0.05$) than groups A and B (Tissue homogenate based inactivated vaccines). In general, the antibody response in Montanide adjuvanted cell culture based vaccine-inoculated birds (C) was significantly higher throughout the period of the experiment in comparison to the liver homogenate vaccine-administered groups (A and B) and cell culture based water adjuvanted vaccinated group (D). These observations suggested the superiority of Inactivated Montanide adjuvanted cell culture based vaccine over the commercial liver homogenate vaccines.

During earlier days of investigation many attempts were made for the control of hydropericardium syndrome in broilers by using formalin inactivated liver homogenate vaccines and there have been a lot of contrary findings regarding the efficacy of such liver homogenate vaccines [12,13]. The results of the present study suggested that chicken embryo liver cell culture based inactivated vaccines performed best in experimental conditions as compared to liver homogenate vaccine. Our results are in close agreement with already reported work [4].

The results of challenge protection study revealed that cell culture propagated montanide adjuvant and water based inactivated vaccines having higher ELISA S/P values gave maximum protection of 100% to chicks which is comparable with a recent study [5] who attained 94% protection of birds using Montanide adjuvanted egg adapted FAV-4 vaccine. The liver homogenate vaccine-inoculated groups A and B showed 80% protection.

The objective of the present study was to develop cell culture based efficacious vaccine against Hydropericardium syndrome in poultry. Cell culture based inactivated montanide adjuvanted vaccine performed best in experimental conditions and provided 100% protection. It is therefore recommended that Commercial tissue homogenate based vaccine should be replaced with cell culture based inactivated vaccines.

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Research Article

The Effects of High and Low Altitudes on Conjunctival Flora in Sport and Work Horses: A Field Study in the Northeast Anatolia Region of Turkey (Kars and Iğdır) [1]

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Abstract

The purpose of this study was to identify the conjunctival microflora in healthy horses living at various altitudes and used for both sports as well as work activities, and determine the predispositions they may cause in terms of ocular diseases. A total of 400 eyes of 200 horses (50 sports and 50 work horses living at high altitudes, and likewise, 50 sports and 50 work horses living at low altitudes) aged 6 year (min: 5, max: 13) of English and Arabian and native breed from both sexes were clinically and ophthalmoscopically examined, and swab samples were taken from the conjunctival recesses of horses with no eye problems. After microbiological examinations, bacteria was isolated in 125 (31.2%) of the 400 swab samples, while fungi was isolated in 194 (48.5%) of the samples. It was determined that the isolated bacteria were mostly Gram-positive (71.5%) with *Staphylococcus* spp. being the most frequently isolated and *Bacillus* spp. the second-most frequently isolated. In terms of fungi isolation, Yeast were the most isolated with a rate of 39.6%, *Penicillium* spp. were the second-most isolated with 22.6% and *Aspergillus* spp. were the third-most isolated with 17.5%. The most effective antibiotics on isolated bacteria were determined as cephalosporin, oxytetracycline, lincomycin/neomycin (L/N) and ceftiofur. It was concluded that the data collected from the study, taken into consideration as reference values, could help in the creation of a more effective treatment protocol in the case of an eye infection. It was also concluded that it is necessary to carefully evaluate the bacteria and fungi that are identified in horses used for these activities, both during check-ups and with regard to creating a treatment plan when an inflammatory eye problem occurs.

Keywords: *Horse, Conjunctival flora, High altitude, Low altitude, Antibiogram*

Yüksek ve Alçak Rakımın Spor ve Hizmet Atlarındaki Konjunktival Flora Üzerine Etkisi: Kuzeydoğu Anadolu'da (Kars ve Iğdır) Bir Saha Çalışması

Özet

Bu çalışmada gerek spor amaçlı gerekse hizmet faaliyetlerinde kullanılan ve farklı rakımlarda yaşayan sağlıklı atlara ait konjunktival mikrofloranın belirlenerek bunların oküler hastalıklar yönünden yaratabilecekleri predispozisyonların tesbit edilmesi amaçlanmıştır. Yaşları ortalama 6 olan İngiliz, Arap ve yerli ırklara mensup her iki cinsiyette toplam 200 ata (yüksek rakımlı bölgede yaşayan 50 spor ve 50 hizmet ile alçak rakımlı bölgede yaşayan 50 spor ve 50 hizmet atı olmak üzere) ait 400 gözün klinik ve oftalmoskopik muayeneyesi yapılarak herhangi bir göz problemi olmayan bu atlara ait konjunktival resesustan sürüntü örnekleri alındı. Mikrobiyolojik incelemeler sonucu 400 sürüntü örneğinin 125'inden (%31.2) bakteri, 194'ünden (%48.5) ise mantar izolasyonu gerçekleştirilmiştir. İzole edilen bakterilerin çoğunlukla Gram pozitif olduğu (%71.5) ve ilk sırada *Staphylococcus* spp, ikinci sırada ise *Bacillus* spp.'nin yer aldığı tespit edilmiştir. Mantar izolasyonu açısından en sık izole edilen %39.6 oranla Maya, ikinci sırada %22.6 ile *Penicillium* spp., ve üçüncü sırada %17.5 ile *Aspergillus* spp.'dir. İzole edilen bakteriler üzerine en etkili antibiyotikler sefalosporin, oksitetrasiklin, linkomisin/neomisin (L/N) ve seftiofur olarak belirlendi. Farklı amaçla kullanılan atların hem rutin sağlık kontrollerinde, hem de bakteri ve mantar nedenli göz yangılarında sağaltım planı oluşturulurken, çalışmamızla belirlenen konjunktival mikrofloranın dikkate alınması ve çalışmadan elde edilen verilerin referans değer olarak göz önünde bulundurulması, atlarda göz enfeksiyonu varlığında daha etkin bir sağaltım protokolü oluşturulmasına yardımcı olabilektir.

Anahtar sözcükler: *At, Konjunktival flora, Yüksek rakım, Alçak rakım, Antibiyogram*

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INTRODUCTION

Fungi and bacteria may be hosted in the ocular microflora of humans and animals. These microorganisms remain at a balanced state in the microflora depending on the immunity of the host. This resident flora also helps protect eye health by preventing potential pathogens from settling on the eye's surface layers. However, when the ocular defense mechanisms weaken, these members of the normal flora may become pathogenic and cause infection [1-3]. The bacterial and fungal flora of normal conjunctiva [4,5] has been studied in many animal species [6-10]. It has been reported in these studies that the animal's age, geographic changes experienced, environment and home range affect the conjunctival flora [3]. Upon literature review, no studies were found concerning the identification of the conjunctival flora of horses, including those used for javelin the traditional Turkish sport of *jeered* throwing on horseback. Studies report that conjunctivitis may develop mostly as a result of infection (viral, bacterial or fungal) and allergic reactions. In studies done on the conjunctival flora of healthy horses, what stands out is the abundance of Gram-positive bacteria. The most commonly isolated bacteria are staphylococcus and streptococcus [8]. The most commonly isolated fungi are *Aspergillus* [11], *Penicillium* and *Mucor*. In the occurrence of erosion or tissue damage caused by trauma or any other reason, the bacteria in the flora become active and cause infections [3].

The aim of this study was to identify the conjunctival microflora of horses used for work and sports living in two different areas with different altitudes, determine the predispositions they may cause in terms of ocular diseases, and set up tests to measure the resistance of conjunctival flora against microbial agents.

MATERIAL and METHODS

Study Area

The study was carried out in the Northeast Anatolia Region in the provinces of Kars and Iğdır/Turkey, which have completely different geographical and climatic characteristics even though they border each other.

Horses in this region are commonly used for both work and sports purposes. Both provinces have a significant potential for horses, and it is thought that there are around 30.000 horses at present.

1. High Altitude (Kars Region): The province of Kars has long, harsh winters and cool, rainy summers. It is located in the Northeast Anatolia Region at 40°26'N and 43°05'E. The region largely consists of plateaus with an average elevation of 1.747 meters [12].

2. Low Altitude (Iğdır Region): The province of Iğdır, a broad plain located at 39°55'N and 43°51'E with an average elevation of 858 meters, has a continental climate. It has short, warm winters and hot, dry summers [13].

Animals and Study Groups

The study material consisted of a total of 200 horses used for sports and work purposes, living in the two regions described above.

The animals in both study groups were examined in the regions in which they lived/worked, and cases with no eye problems were included in the study.

Sports Horses: This term usually describes riding horses and horses used in *jereed* games, including strong native breeds as well as purebreds (Arabian and English).

Work Horses: This term describes horses used for carrying cargo (kaşka [14]) and passengers (carriages and sleighs), as well as horses used for agricultural labor, and mostly consisting of native breeds.

The study took into consideration the animals' purpose of use and the elevation at which they lived, and was conducted in two main groups (Group I and Group II) and their corresponding subgroups (Group I-A, Group I-B, Group II-A and Group II-B). The study groups and their characteristics are shown in *Table 1*.

All samples were taken in the month of September.

Swaps Taken from Conjunctival Sac

Swab samples were taken from the conjunctival sac of the horses deemed healthy upon clinical and ophthalmological examinations. A sterile swab moistened with sterile saline was the applied to the anterior surface of the third eyelid, and rooled along the lower inferior conjunctival fornix, avoiding contact with eyelid margins or vibrissae. Swab samples were brought to Microbiology Laboratory of Veterinary Faculty, Kafkas University.

Microbiological Examinations

Each sample taken was planted on blood agar, MacConkey agar and Saboraud agar. The blood agar and MacConkey agar cultures were incubated at 37°C for 24-

Table 1. *Groups of the study*
Tablo 1. *Çalışma grupları*

Groups	Subgroups	The Number of Eyes Taken Swaps
Group I (n = 100) Kars region (High altitude)	Group I-A Sport horses (n = 50)	100
	Group I-B Work horses (n = 50)	100
Group II (n = 100) Iğdır region (Low altitude)	Group II-A Sport horses (n = 50)	100
	Group II-B Work horses (n = 50)	100

48 h, and the bacteria that multiplied were identified with Gram staining, morphology and biochemical tests [15]. The cultured Saboraud agars were incubated for 2 weeks at 20°C, and the fungi that multiplied were identified according to their species, taking into consideration their macroscopic and microscopic features [16].

Antibiotic Susceptibility Test

The Kirby-Bauer disk diffusion method was used in determining the sensitivity of the isolated bacteria to antibiotics [17], and cloxacillin, lincomycin/neomycin, oxitetracycline, cephalosporin, penicillin G and ceftiofur disks were utilized.

RESULTS

The average age of all horses used in the study was 6 years (min: 5, max: 13). Group I-A included 10 English, 15 Arabian and 25 native breeds, while all the horses in Group I-B were native breeds (n=50). Group II-A included 5 English, 20 Arabian and 25 native breeds, whereas all the horses in Group II-B were native breeds.

Despite the fact that the Kars and Iğdır regions have completely different geographical and climatic characteristics, it was observed that the horses bred in these regions had very similar purposes of use and types of shelter.

No abnormalities were detected during the clinical and ophthalmological examinations of any of the animals included in the study. In addition, no complications arose during the process of taking conjunctival swabs.

Bacteria were isolated in 125 (31.2%) of the 400 swab samples examined, while fungi were isolated in 194 (48.5%) of the samples. It was determined that the isolated bacteria were mostly Gram-positive (71.5%) with *Staphylococcus* spp. being the most isolated and *Bacillus* spp. the second-most isolated. In terms of fungi isolation, *Yeast* were the most frequently isolated with a rate of 39.6%, *Penicillium* spp. were the second-most isolated with 22.6% and *Aspergillus* spp. were the third-most isolated with 17.5%.

In terms of isolation results, a total of 32 bacterial species were isolated in the group of high elevation sports horses: 18 *Staphylococcus* spp., 6 *Bacillus* spp., 5 *Acinetobacter* spp. and 3 *Escherichia coli*. Thirty-two fungi were isolated in the fungal examination. Of these, 19 were identified as yeasts and 14 as moulds (8 *Aspergillus* spp., 5 *Penicillium* spp. and 1 *Mucor* spp.). It was observed that *Staphylococcus* spp. were the most isolated species. *Aspergillus* spp. were determined to be the most common type of mould. Microbiological test results of the groups are shown comparatively in *Table 2*.

The sensitivity rates of the isolated bacteria to antibiotics, measured using the Kirby-Bauer disk diffusion method, are shown in *Table 3*.

DISCUSSION

Activities such as games of *jereed* in particular, as well as flat racing and pacing horse races are done in the Eastern Anatolian Region, and horses are bred in the region by riding centers specifically for this purpose [18-20]. Sports horses raised in the Kars region for these purposes are becoming more and more common and gaining prominence by the day. For this reason our study aimed to comparatively evaluate the effects of the horses purpose of use as well as the different geographic elevations at which they lived on the normal conjunctival flora.

Studies have been done on the normal flora of the conjunctiva in horses. Of these, in the study carried out by Araghi-Sooreh et al.[1], it is stated that Gram-positive bacteria were detected at the rate of 59.51%, while *Bacillus*

Table 2. *Distribution of bacteria and fungi species and the number isolated from the samples according to the groups*
Tablo 2. *Örneklerden izole edilen bakteri ve mantar cins ve sayısı ile gruplara göre dağılımı*

Isolated Bacteria and Fungi	Group I High Altitude (Kars Province)		Group II Low Altitude (Iğdır Province)	
	Sports Horse	Work Horse	Sports Horse	Work Horse
Staphylococcus spp.	18	14	14	15
Bacillus spp.	6	5	8	6
Acinetobacter spp.	5	3	5	3
Eschericia coli	3	4	5	3
Aspergillus spp.	8	14	5	7
Penicillium spp.	5	10	16	13
Mucor spp.	1	-	8	7
Yeast	-	9	36	31
Alternaria spp.	-	-	5	3
Rhizopus spp.	-	-	3	3

Table 3. *Isolated bacteria and sensitivity rates (%) to antibiotics*
Tablo 3. *İzole edilen bakteriler ve antibiyotiklere olan duyarlılık oranları (%)*

Isolates	Antibiotic Sensitivity Rates of the Isolates (%)						
	Number of Isolates	Cloxacillin	Lincomycin/Neomycin	Oxytetracyclin	Cephalosporin	Penicillin G	Ceftiofur
Staphylococcus spp.	61	44	73	70	85	70	63
Bacillus spp.	28	28	53	82	71	46	53
Eschericia coli	20	60	85	85	85	80	80
Acinetobacter spp.	16	56	62	56	68	81	87

spp. were the most frequently identified species at 27.68%. Fungi was isolated at the rate of 96.85%, while the most common species were *Aspergillus* spp. at 48.03%. In another study done by Khosravi et al.[8], *Aspergillus spp*. were isolated as the most common fungus with a rate of 19.9%. In a study done in England [2], Gram-positive bacteria were detected at a rate of 52%, while the isolated species of fungi were detected to be 13%.

No comparative studies have been done on the Kars and Iğdır region in terms of altitude and the way in which the horses were raised. For this reason, our study is thought to contribute to the recording of the normal conjunctival flora of the horses in the region. This study, just like the rest of the literature [1-3,6], came across mostly Gram-positive bacteria in terms of the isolated bacteria group. The species of bacteria and fungi taken from the high elevation group for isolation have similar rates of isolation. These rates are in accordance with the literature [6-10] . For this reason, no difference was noticed in the microbial flora of the conjunctival recesses in terms of the horses purpose of use and their upbringing. On the other hand, the results obtained from horses at low elevations in especially hot and humid areas revealed that the species and isolation rates of the bacteria and fungi in the conjunctival sac samples of both the sports horse group and the work horse group were quite similar. Yet when the Iğdır region (low elevation) and Kars region (high elevation) are compared, significant differences in fungi species [4,5] and isolation rates become obvious when compared with the literature [1,2] . While *Aspergillus* spp.[11] were the most frequently isolated fungi in the first group, in the other group the ranking was Yeast followed by *Penicillium* spp. In addition, fungi species such as *Mucor* spp., *Alternaria* spp. and *Rhizopus* spp. were found in the second group at certain rates. This difference between the groups in the rates detected may linked to the different altitudes at which the horses live, as well as differences in climate conditions such as heat, humidity and rainfall.

While an antibiogram test done in England [2] showed gentamicin and chloramphenicol to be responsive, the antibiogram results obtained in this study revealed varying rates. For this reason, agents in eye infections occurring in horses must be isolated. In cases where this is not possible, depending on the agent, cephalosporin, oxytetracycline, lincomycin/neomycin (L/N) and ceftiofur could be considered options for empirical treatment.

In the spring and summer months, the animals in the Kars region were generally grazed in the area they were already in, while most of the animals in the Iğdır region, which has a very hot climate during those months, were sent to surrounding plateaus and brought back in the fall season. Taking this situation into consideration, in order to make a standard assessment, samples were taken from both regions in the month of September, as it is the end of summer and beginning of the fall season.

Based on the data obtained in this study, we are of the opinion that it is necessary to carefully evaluate the bacteria and fungi that are identified in horses used for these activities, both during check-ups and with regard to creating a treatment plan when an inflammatory eye problem occurs. Furthermore, it should not be forgotten that the normal flora of the eye may be affected by various factors [2], that therefore the microbiology of the eye must be constantly monitored and that knowing the normal flora of the eye will ease the assessment of potential eye infections.

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Research Article

Analysis of FecB, BMP15 and CAST Gene Mutations in Sakiz Sheep [1]

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Abstract

Fertility traits, such as the ovulation rate and the number of offspring at birth, are genetically regulated by fecundity genes. This study was performed to identify DNA polymorphisms in Booroola *(FecB)*, Galway *(FecX^G)*, Inverdale *(FecX^I)* and Calpastatin *(CAST)* genes in Sakiz sheep. A total of 71 ewes were genotyped for gene or allelic polymorphisms in the genes listed above using the PCR-RFLP method. The results obtained from this study indicated that all of the Sakiz ewes sampled were non-carriers for *FecB, FecX^G* or *FecX^I* mutations. However, genotypic frequencies in the *CAST* gene were 0.59, 0.36 and 0.05 for AA, AB and BB, respectively. A significant deviation from Hardy-Weinberg equilibrium for the *CAST* gene was not observed in the investigated breed (P>0.05). As a result, more extensive screening is required as tests for newly discovered mutations are developed. Additionally, this study is the first to report a genetic polymorphism in the *CAST* gene in Sakiz sheep.

Keywords: *Sakiz sheep, Fertility, FecB, BMP15, CAST*

Sakız Irkı Koyunlarda FecB, BMP15 ve CAST Genlerindeki Mutasyonların Analizi

Özet

Ovulasyon oranı ve bir batındaki yavru sayısı gibi dölverim özellikleri fekondite genleri tarafından regüle edilmektedir. Bu çalışma Sakız ırkı koyunlarda, Booroola *(FecB)*, Galway *(FecX^G)*, Inverdale *(FecX^I)* ve Calpastatin *(CAST)* genlerindeki DNA polimorfizmlerini belirlemek amacıyla yapılmıştır. Araştırılan gen veya aleller açısından toplam 71 koyun PCR-RFLP metoduyla genotiplendirilmiştir. Çalışmadan çıkan sonuçlar, Sakız koyunların *FecB, FecX^G* ya da *FecX^I*'ye ait mutasyonları taşımadığını göstermiştir. Ancak *CAST* geni açısından AA, AB ve BB genotiplerinin sırasıyla 0.59, 0.36 ve 0.05 frekans gösterdiği tespit edilmiştir. *CAST* geni açısından çalışılan sürünün Hardy-Weinberg dengesine uyduğu görülmüştür (P>0.05). Sonuç olarak yeni keşfedilen mutasyonların geliştirilmesi gibi daha yoğun tarama çalışmaları gerekmektedir. Ek olarak Sakız koyunlarında *CAST* genindeki polimorfizmin varlığı ilk defa bu çalışmada bildirilmektedir.

Anahtar sözcükler: *Sakız Koyunu, Fertilite, FecB, BMP15, CAST*

INTRODUCTION

The incorporation of a major gene for prolificacy into a flock using marker-assisted selection (MAS) promotes increased selection pressure on other traits, leading to increased genetic gain [1]. It is hypothesized that MAS using both the bone morphogenetic protein receptor IB *(BMPR-IB)* and the bone morphogenic protein 15 *(BMP15)* genes is warranted to increase litter size in sheep and will be of considerable economic value to sheep breeders.

Sakiz is a dairy sheep breed of considerable economic interest to Turkish farmers, mainly due to its high prolificacy and milk production. These sheep are raised in Cesme, the provinces of Izmir and Aydin, and coastal locations in the Marmara and Aegean regions. The average litter size is 1.7 to 2.3 lambs, and the ovulation rate is 2.9 to 3.3 [2,3].

In recent years, a number of natural genetic mutations have been associated with ovulation rates in sheep breeds, including one mutation in the *BMPR-1B* gene *(FecB)* and ten different mutations in the bone morphogenetic protein 15 gene *(FecX^I, FecX^H, FecX^G, FecX^B, FecX^R, FecX^L, FecX^{Gr}, FecX^{TT}, FecX^O* and *FecX^W)* [4-6]. These mutations are significantly associated with the ovulation rate of different sheep breeds.

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The *FecB* gene is a dominant autosomal gene responsible for the fecundity of Booroola Merino sheep, and its additive effect on the ovulation rate was first identified in the 1980s [4,7]. The *FecB* gene maps to chromosome 6 in sheep [8]. A point mutation that significantly increases the ovulation rate of ewes is located at base 746 of the coding region (746 A → G) in the highly conserved intracellular kinase signaling domain of the *BMPR-1B*; this mutation causes a glutamine to arginine amino acid substitution [9]. The gene dosage effect of the mutation is additive for the ovulation rate with an increase of 1.5 for each gene copy. These extra ovulations subsequently increased litter size by approximately 1.0 and 1.5, respectively [4,10]. *FecB* gene mutations have been reported in many other sheep breeds, such as Booroola Merino [7], Belclare, Cambridge, China Small Tailed Han, Hu [5], Romney [11], Kendrapada [12], Garole and Javanese sheep [13]. In contrast, *FecB* mutations were not detected in Thoka, Woodlands, Olkuska, Lacaune [13] and Sangsari sheep [14].

The *BMP15* located on the chromosome X [15]. Each *BMP15* gene significantly affects prolificacy [11]. Ewes with two inactive copies of the *BMP15* gene (homozygous animals) are sterile [15,16] and exhibit a similar ovarian phenotype. Ewes with a single inactive *BMP15* gene (heterozygous animals) are fertile and exhibit an increased ovulation rate and an increased incidence of twin or triplet births [4,5,13-18]. In general, heterozygous ewes with mutations in both *FecB* and *FecX*^{G} exhibited increased fertility compared with ewes harboring a mutation in only one of these genes [6]. The Galway mutation has been identified in Cambridge [16], Garole, Kendrapada [12] and Small Tailed Han sheep [19]. Hanrahan et al.[16] estimated the effect of *FecX*^{G} to be 0.77 and 1.18 in Belclare and Cambridge ewes, respectively. The *FecX*^{I} mutation was studied in Inverdale, Belclare, Cambridge, Romney, Hanna and Rasa Aragonese, Cambridge, Small Tailed Han, Sakiz [13,16,19] sheep. Recently, Gursel et al.[20] report that the Inverdale mutation was identified in Sakiz sheep in Turkey.

The *CAST* gene, which is located on sheep chromosome 5, acts on growth characteristics, such as muscle development, birth weight of the lamb, weight at weaning, and postmortem meat qualities, such as tenderness [21-24]. In addition, the *CAST* gene affects cataract development and the reproductive performance of the animals [23-25]. In a study by Garcia et al.[26], calpastatin exerted a significant impact on fertility and longevity on cattle. Based on these results, the use of *CAST* markers together with increased predicted transfer ability (PTA) potential in selection programs for dairy parameters is projected to increase milk yield together with fertility [26].

FecB, FecX^{G} and *FecX*^{I} mutations have been studied in local sheep breeds, such as Akkaraman, Morkaraman, Daglic, Awassi, Tuj, Karakas and Bafra (Sakiz x Karayaka cross) in Turkey as well as in Greek Sakiz ewes; however, these mutations were not identified [10,27,28]. In addition, the *CAST* gene has not been studied in Sakiz or other Turkish domestic sheep breeds.

We tested the hypothesis that a major gene was segregating in the Sakiz breed. Therefore, the aim of this study was to investigate *FecB, FecX*^{G}, *FecX*^{I} and *CAST* gene mutations in the Sakiz sheep breed and the potential introgression of these genes into these breeds to enhance their reproductive rate.

MATERIAL and METHODS

Animals

A total of 71 ewes were used in the study. Ewes were randomly selected from five unrelated herds. These herds were managed within the scope of the Protection of Indigenous Genetic Resources Project under the supervision of the Ministry of Agriculture.

DNA Isolation

Initially, wool samples were prepared by cleaning with EtOH (ethanol). The CTAB (hexa-decyltrimethylammonium bromide) method of DNA isolation was performed using wool samples obtained from selected animals [29]. The amount of DNA obtained and its purity were determined by spectrophotometry (Nanodrop-2000c, Thermo Scientific). The quantified DNA was stored at -80°C until PCR-RFLP (Polymerase chain reaction and Restriction fragment length polymorphism) was performed.

PCR-RFLP Genotyping

DNA was amplified by PCR using the primer sets provided in *Table 1*. The amplification products were cut with restriction enzymes, and the alleles or mutations were determined. The primer sets used for the amplification of *FecB, FecX*^{G}, *FecX*^{I}, and *CAST*; the size of the PCR products; the restriction enzymes used and their product sizes and references for PCR-RFLP are provided in *Table 1*.

For the PCR amplification of *FecB, FecX*^{G}, *FecX*^{I} and *CAST*, 33.5 µl of dH_2O, 5 µl of 10X buffer, 5 µl of $MgSO_4$, 1 µl of dNTPs (2.5 mM), 2.5 U Taq DNA polymerase (Biomatik Corp., Cambridge,Canada) and 1 µl of (0.025 µM) forward primer and 1 µl of (0.025 µM) reverse primer from each primer sets presented in *Table 1* were used. The final volume was 50 µl after the addition of 3 µl of 100 ng/mL DNA sample.

The PCR conditions for *FecB* [5,13], *FecX*^{G} [16,27], *FecX*^{I} [5,15,27] and *CAST* [21,30] were as follows: initial denaturation at 94 to 95°C for 0.30 s to 5 min followed by 33 to 35 cycles of denaturation at 94 to 95°C for 0.30 s to 3 min, annealing for 40 s to 1 min at 60 to 61°C, elongation at

Table 1. *Primer sets and enzymes for PCR-RFLP of FecB, BMP15 (FecX^G,FecX^I) and CAST genes*
Tablo 1. *PCR-RFLP için FecB, BMP15 (FecX^G, FecX^I) ve CAST genlerinin primer set ve enzimleri*

Gene-Allele	Primer Sets*	PP (bp)	RE	RP (bp)		References
FecB	**F:**5′-CCAGAGGACAATAGCAAAGCAAA-3′ **R:**5′-CAAGATGTTTTCATGCCTCATCAACAGGTC -3′	190	*AvaII*	AA	190	[5,13]
				BB	159, 31	
				AB	190, 159, 31	
FecX^G	**F:**5′CACTGTCTTCTTGTTACTGTATTTCAATGAGAC-3′ **R:**5′-GATGCAATACTGCCTGCTTG-3′	141	*Hinf I*	AA	141	[16,27]
				BB	112, 29	
				AB	141, 112, 29	
FecX^I	**F:**5′-GAAGTAACCAGTGTTCCCTCCACCCTTTTCT–3′ **R:**5′-CATGATTGGGAGAATTGAGACC-3′	154	*XbaI*	AA	154	[5,15,27]
				BB	124, 30	
				AB	154, 124, 30	
CAST	**F:**5′-TGGGGCCCAATGACGCCATCGATG - 3′ **R:**5′- GGTGGAGCAGCACTTCTGATCACC - 3′	622	*MspI*	AA	336, 268	[21,30]
				BB	622	
				AB	622, 336, 268	

** **GenBank accession no:** BMP15(FecX^G, FecX^I)(AH009593), FecB (AF312016), CAST (AF016006-8); (**PP:** PCR Product, **RE:** Restriction Enzyme, **RP:** Restriction Product)*

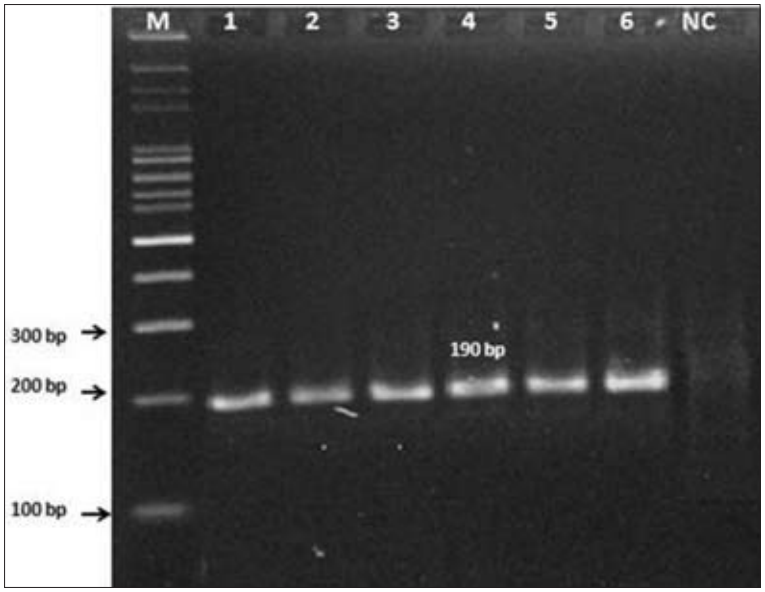
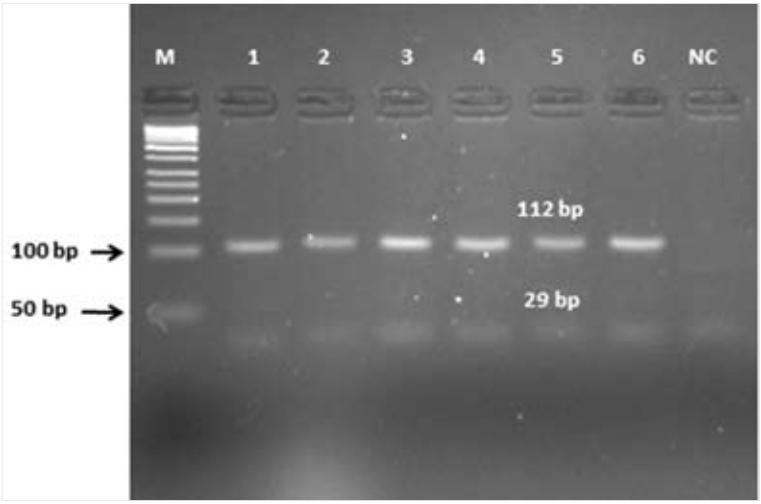


Fig 1. The *FecB* gene cut with the *AvaII* restriction enzyme. The products were electrophoresed on a 2.5% agarose gel. No mutations were detected in any of the individuals (M: Marker, Columns 1 to 6: 190 bp band, NC: Negative Control)

Şekil 1. *FecB* geninin *AvaII* restriksiyon enzimi ile kesimi (%2.5'lik agaroz jel- tüm bireylerde mutasyon yok) (M: Markör, Sütun1-6: 190 bç'lik bant, NK: Negatif Kontrol)

Fig 2. The *FecX^G* allele cut with the *HinfI* restriction enzyme. The products were electrophoresed on a 4% agarose gel. No mutations were detected in any of the individuals (M: Marker, Columns 1 to 6: 29 and 112 bp bands, NC: Negative Control)

Şekil 2. *FecX^G* allelinin *Hinf I* restriksiyon enzimi ile kesimi (%4'lük agaroz jel- tüm bireylerde mutasyon yok) (M: Markör, Sütun1-6: 29 ve 112 bç'lik bant, NK: Negatif Kontrol)



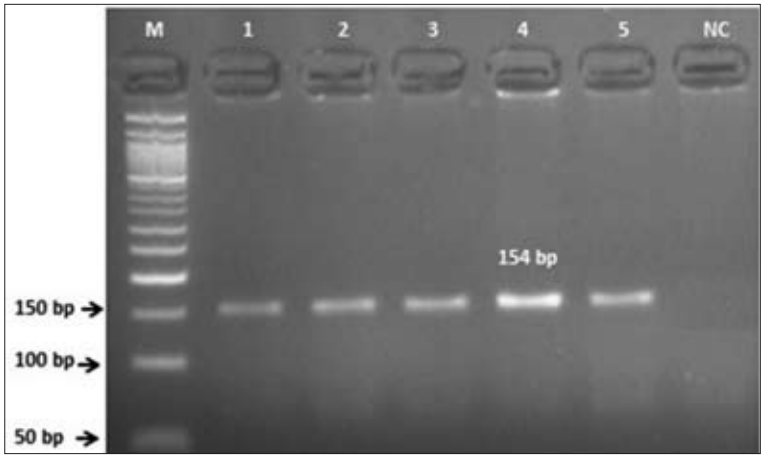


Fig 3. The *FecX*^{I} allele cut with the *XbaI* restriction enzyme. The products were electrophoresed on a 3.5% agarose gel. No mutations were detected in any of the individuals (M: Marker, Columns 1-5: 154 bp band, NC: Negative Control)

Şekil 3. *FecX*^{I} allelinin *XbaI* restriksiyon enzimi ile kesimi (%3.5'luk agaroz jel- tüm bireylerde mutasyon yok.) (M: Markör, Sütun1-5: 154 bç'lik bant, NK: Negatif Kontrol)

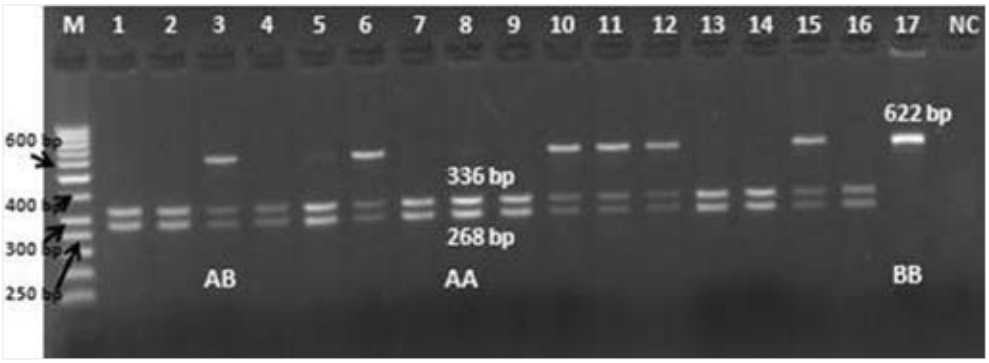


Fig 4. The 622-bp PCR product from the *CAST* gene cut by the *MspI* enzyme. The products were electrophoresed on a 2.5% agarose gel. AA, AB and BB genotypes were detected (M: Marker, Columns 1, 2, 4, 5, 7, 8, 9, 13, 14, 16: 268 and 336 bp bands, Columns 3, 6, 10, 11, 12, 15: 268, 336 and 622 bp bands, Column 17: 662 bp band, NC: Negative Control)

Şekil 4. *CAST* geninin 622 bç'lik PCR ürününün *MspI* enzimi ile kesimi (%2.5'lik agaroz jel görüntüsü - AA, AB, BB genotipi) (M: Markör, Sütun 1,2,4,5,7,8,9,13,14,16: 268 ve 336 bç'lik bant, Sütun3,6,10,11,12,15: 268-336-622 bç'lik bant, Sütun17: 662 bç'lik bant, NK: Negatif Kontrol)

70 to 72°C for 30 s to 2 min, and a final extension at 72°C for 4 to 8 min.

The PCR products for the amplification of the *FecB* gene (product size 190 bp), *FecX*^{G} allele (product size 141 bp), *FecX*^{I} allele (product size 154 bp) and *CAST* gene (product size 622 bp) were subjected to electrophoresis using 2%, 3%, 2.5% and 1.5% agarose gels, respectively.

For RFLP, 15 µl of the PCR products were incubated with 4 µl buffer, 4 µl dH_2O and 15 U of restriction enzymes at 37°C for 16 h. The restriction digestion products *(FecB, FecX*^{G}*, FecX*^{I} and *CAST)* were visualized in agarose gels stained with 2.5%, 4%, 3.5% and 2.5% ethidium bromide, respectively. The PCR and RLFP product bands were by visualized using the DNr Minilumi imaging system *(Fig. 1, 2, 3, 4)*.

Statistical Analysis

Data were processed by POPGENE V1.32 software to calculate genotypic frequencies and Hardy-Weinberg equilibrium [31].

RESULTS

In the present study, *FecB*, *FecX*^{G}, *FecX*^{I} and *CAST* mutations were investigated in Sakiz sheep breeds. The agarose gel images of PCR-RFLP products are presented in *Fig. 1, 2, 3, 4* respectively. Mutations in the *FecB gene* and at the *FecX*^{G} *and FecX*^{I} loci in the *BMP15* gene were assessed in the samples. Based on the PCR-RFLP analysis, no digested fragments were observed for *FecB*, *FecX*^{G} *and FecX*^{I} at 190, 141 and 154 bp, respectively, if the herd did not carry the mutations [5]. All of the 71 individuals were homozygous for *FecB* and *BMP15*. None of the samples harbored the *FecB* mutation, and similarly no *FecX*^{G} *and FecX*^{I} mutations were noted in the *BMP15* gene. Hardy-Weinberg equilibrium and χ^2-values were not estimated due to the genotypic frequency.

A total of 71 Sakiz individuals were genotyped. Of these sheep, 42 were AA, 25 were AB and 4 were BB for the *CAST* gene. Genotypic frequencies were estimated as 0.59, 0.36 and 0.05 for AA, AB and BB, respectively, for the *CAST* gene *(Table 2)*. Significant deviation from Hardy-Weinberg

Table 2. *Genotypic frequencies of FecB, BMP15 (FecX^G,FecX^I) and CAST genes*

Tablo 2. *FecB, BMP15 (FecX^G, FecX^I) ve CAST genlerinin genotipik frekansları*

Regions	Allele	n	Frequency	Chi²	P
FecB	AA	71	1.00	NE	NE
	AB	0	0.00		
	BB	0	0.00		
FecX^G	AA	0	0.00	NE	NE
	AB	0	0.00		
	BB	71	1.00		
FecX^I	AA	71	1.00	NE	NE
	AB	0	0.00		
	BB	0	0.00		
CAST	AA	42	0.59	0.01	0.91
	AB	25	0.36		
	BB	4	0.05		

NE: *Not Estimated*

equilibrium was not observed for the *CAST* gene in the investigated breed (P>0.05).

DISCUSSION

The results of our study indicate that *FecB, FecX^G* and *FecX^I* mutations, which have a major effect on litter size, are not present in Sakiz sheep.

These results are consistent with reports in Romanov, Finn, Thoka, Woodlands, Olkuska, Lacaune [13], Sangsari [14], Akkaraman, Morkaraman, Daglıc, Tuj, Karakas [10], Awassi, Imroz, Kivircik [20] and Bafra [28] sheep breeds wherein *FecB* mutant alleles do not segregate in these breeds. In contrast, *FecB* mutations are reported in several sheep breeds, such as Booroola Merino [7], Belclare, Cambridge, China Small Tailed Han, Hu [5], Garole, Javanese [13], Kendrapada [12] and Romney sheep [17].

The *FecX^G* mutation was analyzed in Garole, Kendrapada [12], Malpura, Deccani, Baluchi [32] and Sangsari sheep [14]; however, none of these sheep breeds carried the *FecX^G* mutation in the *BMP15* gene. Additionally, the *FecX^G* mutation was identified in Small Tailed Han, Belclare and Cambridge sheep [1,33]. In contrast with results from Gursel et al.[20], our results indicate that the point mutation in *FecX^G* might not serve as a major gene that influences prolificacy in Sakiz sheep. Galway and Inverdale gene polymorphisms were analyzed as likely candidate genes influencing high prolificacy in Sakiz breeds of Greece, but no polymorphism has been identified [27]. The *FecX^I* mutation was first identified in the Romney sheep breed, which exhibits high fertility traits [1]. Studies indicate that the Small Tailed Han, Hu [33], Olkuska, Garole, Javanese, Woodland, Lacaune [13], Egyptian [34], East Friesien, Finn, Romanov [5] and Greek Sakiz [27] sheep do not carry the *FecX^I* alleles. Our results demonstrate that the *FecX^I* mutation we examined was not present in Sakiz sheep. In addition our results are consistent with reports by Gursel et al.[20].

In this study, we analyzed a *CAST* gene polymorphism. The genotypic frequencies of the AA, AB and BB alleles for the *CAST* gene were calculated as 0.59, 0.35 and 0.56, respectively. These frequencies are similar to those reported in Arabic [21], Iranian Zel [23] and Makoi sheep [24]. The *CAST* gene, which encodes calpastatin, regulates the activity of calpain as a protease inhibitor [35]. In addition to its role in determining the quality of meat, the *CAST* gene has also effects on reproductive activity [23]. However, studies on reproductive activities are very limited. Garcia et al.[26] reported that the *CAST* gene was strongly correlated with fertility and longevity. However, Byun et al.[35] reported that the *CAST* gene did not affect fertility or longevity in Romney, Corriedale, Merino, Polwarth, Kelso and Coopworth ewes. Proof of the relationship between the *CAST* genes and reproductive activity may explain the variety of litter sizes in Sakiz ewes.

These results suggest that the high prolificacy of the Sakiz breed does not result from *FecB*, *FecX^G* and *FecX^I* mutations. Further investigation should be directed at other loci of the *BMP15* gene or other genes and involve larger sample sizes. Furthermore, the effect of *CAST* gene alleles on reproductive parameters should be investigated. Studies on the genomic aspects of fertility, while improving the accuracy of selection, will allow for more economical and efficient use of resources.

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Research Article

Genetic Polymorphism of Five Genes Associated with Meat Production Traits in Five Cattle Breeds in Turkey

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Abstract

There are many potential genes that are known to be related to meat production and that can be used for selection to improve meat quality and production include Calpain (CAPN1), Myostatin (MSTN), Calpastatin (CAST), Osteopontin (SPP1) and Thyroglobulin (TG). This study evaluated Zavot (n=60), Anatolian Black (n=59; AB), South Anatolian Red (n=53; SAR), Turkish Gray (n=60; TG) and East Anatolian Red (n=49; EAR) cattle breeds, with the primary goal of investigating DNA polymorphisms of the CAST, TG, SPP1, MSTN and CAPN1 genes. Polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) was employed to genotypes. Restriction enzymes revealed polymorphisms with 2 alleles and three genotypes of each CAST, TG, SPP1 and CAPN1 genes were determined in all breeds, while the MSTN locus was found to be monomorphic. Deviation from Hardy-Weinberg equilibrium (HWE) was not observed in the five cattle breeds on SPP1 locus. Significant deviation was observed from HWE in the TG and AB breeds on TG locus. Except for the TG and SAR breeds, genotype frequencies were not consistent with the HWE on CAPN1 locus. Four breeds were found at Hardy-Weinberg equilibrium except AB breed for CAST locus. Consequently, this study has shown that genetic polymorphisms do not exist in the MSTN gene but do exist in the remaining four genes that were examined in five Turkish native cattle breeds.

Keywords: *Cattle, CAST, TG, SPP1, MSTN, CAPN1*

Türkiye'deki Beş Yerli Sığır Irkında Et Verim Özellikleri ile İlişkili Beş Genin Polimorfizmi

Özet

Et verimi ile ilişkili olduğu bilinen birçok potansiyel gen vardır. Bu genler içerisinde yer alan; Calpain (CAPN1), Myostatin (MSTN), Calpastatin (CAST), Osteopontin (SPP1) ve Thyroglobulin (TG) genleri seleksiyonda et kalitesi ve verimini geliştirebilmek için kullanılabilir. Bu çalışmada, Zavot (n=60), Yerli Kara (n=59, YK), Güney Anadolu Kırmızısı (n=53, GAK), Boz Irk (n=60, BI) ve Doğu Anadolu Kırmızısı (n=49, DAK) ırkı sığırlarda; CAST, TG, SPP1, MSTN ve CAPN1 genlerinin DNA polimorfizmleri değerlendirilmiştir. Genotiplerin belirlenmesi için polimeraz zincir reaksiyonu ve restriksiyon parça uzunluk polimorfizmi (PZR-RFLP) kullanılmıştır. Restriksiyon enzimleri ile CAST, TG, SPP1 ve CAPN1 genlerinin her birinde 3 genotip ve 2 allel belirlenmiştir. MSTN geni ise monomorfik olarak bulunmuştur. SPP1 lokusunda beş sığır ırkında Hardy-Weinberg dengesinden (HWD) sapma gözlemlenmemiştir. TG lokusunda, Boz ve Yerli Kara ırklarında HW dengesinden önemli sapma gözlenmiştir. Boz ve Güney Anadolu Kırmızısı ırkları dışındaki ırklarda CAPN1 lokusunda HW dengesinden sapma olduğu belirlenmiştir. CAST lokusunun Yerli Kara dışında kalan dört ırkta HW dengesinde olduğu bulunmuştur. Sonuç olarak; bu çalışmada incelenen beş yerli sığır ırkında MSTN geni hariç, diğer dört gende polimorfizm olduğu belirlenmiştir.

Anahtar sözcükler: *Sığır, CAST, TG, SPP1, MSTN, CAPN1*

INTRODUCTION

Molecular genetics techniques make it possible to describe genetic variation at different loci and to investigate the relationships between productivity and the variation at a Quantitative Trait Locus (QTL). The ultimate goal in selection is to estimate an animal's genetic worth with greater accuracy, thereby increasing the genetic gain achieved through selection. For example, studies have reported that variations in genes that affect physiological

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events associated with the phenotype can affect quantitative variations in the phenotype [1]. Contemporary molecular genetics technology makes it possible to employ genetic markers or traits which exhibit a high correlation with the traits being researched in order to identify very productive animals. These genetic markers can also help to identify these traits when the animal is young, regardless of its gender. The rate of genetic progress using traditional selection methods is quite slow for animals such as cattle, sheep and goats, which have a long generation interval. Marker assisted selection (MAS) used together with traditional selection methods can be very effective for complex traits because it shortens the generation interval, accelerates the genetic progress and achieves improvement in the right direction [1,2]. Recently there has been a significant increase in the number of studies done in Turkey on polymorphism in genes that affect the economic traits of cattle breeds [3-6]. One of the most important economic traits of farm animals is meat production. There are many potential genes that are known to be related to meat production and that can be used for selection. Potential genes that can be used for selection to improve meat quality and production include Calpain (CAPN1), Myostatin (MSTN), Calpastatin (CAST), Osteopontin (SPP1) and Thyroglobulin (TG). Calpain activity is a cytoplasmic cysteine protease that requires the presence of calcium [7]. Suzuki and Sorimachi [8] identified two genes for calpain (CAPN1 [μ-calpain] and CAPN2 [m-calpain]). Prior studies have identified a correlation between the regulation of CAPN1 activity and variations in meat tenderness as well as a quantitative trait locus on chromosome 29 where CAPN1 is located which affects meat tenderness [9]. Myostatin, which is also known as Growth Differentiation Factor-8 (GDF-8), is a member of the Transforming Growth Factor-B (TGF-B) family. GDF-8 is primarily synthesized in developing skeletal muscles and plays a role in skeletal muscle growth and differentiation. Muscular hypertrophy, a heritable trait commonly known as 'double muscling', is defined as excessive development of muscle mass [10,11]. Mutations occurring in the DNA sequences that encode the myostatin gene cause an increase in the muscle mass that is referred to as double muscling, which occurs due to inactivation of the myostatin gene. Senger et al.[12] were the first to describe osteopontin (OPN) as 60-kDa transformation-specific phosphoprotein that is secreted and that mediates cell matrix interactions and cellular signals by binding with integrin and CD44 receptors. Denhardt and Guo [13] reported that this protein is expressed in a number of different tissues. For this reason, the term 'secreted phosphoprotein 1' (SPP1) was proposed to reflect the factor's extensive functional role. The bovine SPP1 gene is made up of 7 exons that span about 7 kb of the genomic DNA. Schnabel et al.[14] and Khatib et al.[15] have identified the SPP1 gene on bovine chromosome 6 as a candidate for influencing milk and beef production traits in dairy cattle. Thyroglobulin (TG), which is a glycoprotein precursor of the thyroid hormones T3 and T4, is only synthesized in the thyroid gland. The SPP1 gene is located at the centromere of chromosome 14 and contains 37 exons. Calpastatin (CAST) specifically inhibits the calcium-dependent neutral protease μ-calpain, which is found in the tissue of mammals. Not only does calpastatin inhibit μ- and m-calpain activity, it also regulates post-mortem proteolysis. Koohmaraie [16] identified an association between increased post-mortem calpastatin activity and reduced meat tenderness. μ-calpain (CAPN1) and m-calpain (CAPN2) and their inhibitor calpastatin (CAST) are three enzymes that have a significant influence on this process.

The Anatolian Black (AB) breed of cattle is raised in the central Anatolian region. This breed has the smallest body size of all the Turkish native cattle breeds. However, this breed and its crossbreeds, together with the East Anatolian Red (EAR) breed, is the most important source of beef in Turkey. The Turkish Gray (TG) breed is raised in the regions of Marmara and Thrace. Similar breeds are bred in Ukraine, Romania, Hungary and Italy. The South Anatolian Red (SAR) breed is raised in southern Anatolia. It is bred for high milk yield and yields more milk than other Turkish native cattle breeds. The Zavot cattle breed was developed by crossbreeding the Simmental, Brown Swiss and EAR cattle breeds. The Zavot breed yields more milk and meat than EAR cattle, which is another cattle breed native to Turkey.

The number of genetically pure individuals from these native cattle breeds has been greatly reduced because of uncontrolled mating to improve yields, unconscious breeders, not exercising timely protection, and migration from rural to urban centers. Consequently, these breeds are faced with the threat of extinction. In order to survive, these breeds must become more productive. Yield traits related to the use of genetic markers is a good option to achieve this end. However, primarily genetic characterizations of known markers for these breeds are needed.

In the present study, the restriction fragment length polymorphism for the polymerase chain reaction (PCR-RFLP) technique was used to detect genetic polymorphism within five genes associated with meat production traits; CAST, TG, SPP1, MSTN and CAPN1 in EAR, SAR, TG, AB and Zavot cattle.

MATERIAL and METHODS

Samples and DNA Isolation

A total of 281 blood samples were collected from Zavot (n=60, Ardahan), AB (n=59, Ankara and Çankırı), SAR (n=53, Şanlı Urfa and Adana), TG (n=60, Edirne and Balıkesir) and EAR (n=49, Erzurum and Kars) cattle breeds. Genomic DNA was isolated with the phenol chloroform isoamlyalcohol (25:24:1) method and examined using a NanoDrop 2000 (Thermo Scientific) for quantity and quality control.

Polymorphism Detection and Genotyping

Table 1 shows the primer sequences used in amplification the relevant gene regions of the DNA that were obtained as well as the primer annealing temperatures, $MgCl_2$ concentrations and the restriction enzymes used to cut the DNA. All PCR reactions were performed on an Amplitronyx Series 6 thermal cycler. The PCR amplification reaction mixtures were performed in a total volume of 25 µl including ddH_2O, 1X buffer, $MgCl_2$ *(Table 1)*, dNTP (200 µM), primers (5 pmol) and Taq DNA polymerase (1 U/µL). This mixture was aliquoted into test tubes with approximately 100 ng DNA. The PCR reactions were cycled with routine procedures (denaturation, annealing and elongation). The annealing temperature for each primer was optimized *(Table 1)*. The PCR products were electrophoresed on 2% agarose gel stained with ethidium bromide. The PCR products of five genes were digested with fast digest enzymes *(Table 2)* according to the manufacturers' instructions (Fermentas, Vilnius, Lithuania). Restriction products were electrophoresed on 3% (CAPN1, TG, SPP1, CAST) and 5% (MSTN) agarose gel, and then displayed under a UV-transilluminator.

Statistical Analysis

Direct counting was used to estimate genotype and allele frequencies of genetic variants of the CAST, TG, SPP1, MSTN and CAPN1 genes. A chi-square statistic was used to check whether the populations were in Hardy-Weinberg equilibrium. PopGene32 software was used [17].

Table 1. *Primer sequence, PCR product size, $MgCl_2$ concentration and primer annealing temperature (Ta)*
Tablo 1. *Primer dizisi, PZR ürün büyüklüğü, $MgCl_2$ konsantrasyonu ve primer bağlanma sıcaklığı*

Gene	Primer	PCR	$MgCl_2$	Ta
CAPN1	5′-TTCAGGCCAATCTCCCCGACG-3′ 5′-GATGTTGAACTCCACCAGGCCCAG-3′	670bp	1mM	58.4°C
MSTN	5′-CCAATTACTGCTCTGGAGGAT-3′ 5′-GGAGACATCTTTGTAGGAGTACAGC-3′	124bp	1.5mM	56.8°C
TG	5′-GGGGATGACTACGAGTATGACTG-3′ 5′-GTGAAAATCTTGTGGAGGCTGTA-3′	545bp	1.5mM	60.7°C
SPP1	5′-GCAAATCAGAAGTGTGATAGA C-3′ 5′-CCAAGCCAAACGTATGAGTT-3′	290bp	2mM	60°C
CAST	5′-CCTCGACTGCGTACCAATTCCGAAGTAAAGCCAAAGGAACA-3′ 5′-ATTTCTCTGATGGTGGCTGCTCACT-3′	523bp	2mM	55.4°C

Table 2. *Restriction endonucleases and restriction profiles*
Tablo 2. *Restriksiyon endonükleazlar ve restriksiyon profilleri*

Gene	RE	Restriction Profiles (bp)		
CAPN1	*Fok*I	TT	CT	CC
		670	670/530/140	530/140
MSTN	*BstF5*I (*Fok*I)	-/-	+/-	+/+
		124	124/100/24	100/24
TG	*Psu*I	TT	CT	CC
		545	545/370/175	370/175
SPP1	*Bsr*I	TT	CT	CC
		290	290/200/90	200/90
CAST	*Rsa*I	CC	CG	GG
		523	523/266/257	266/257

RESULTS

MSTN Locus

The MSTN locus was found to be monomorphic, and all cattle breeds examined were of the +/+ (100/24bp) genotype *(Fig. 1)*.

SPP1 Locus

The allele and genotype frequencies of the SPP1 locus obtained for five different cattle breeds have been shown in *Table 3*. Digestion of the 290 bp fragment of the SPP1 gene with the *Bsr*I restriction enzyme revealed a polymorphism with two alleles *(Table 3)*. Three genotypes, CC (200 bp/90 bp), TT (290 bp) and CT (290 bp/200 bp/90 bp), were found in the SPP1 gene *(Fig. 2)*. Results showed that the (T) allele had higher frequency in all breeds *(Table 3)*. More animals had the TT genotype than any other genotype. Deviation from HWE was not observed in the five cattle breeds *(Table 3)*.

Heterozygosity of the SPP1 locus varied from 0.17 to 0.37 in the five Turkish native cattle breeds. The heterozygosity, allele and genotype frequencies in these five Turkish native cattle breeds have been shown in *Table 3*.

TG Locus

The allele and genotype frequencies of the TG locus obtained for five different cattle breeds have been shown in the *Table 4*. Digestion of the 545 bp fragment of the TG gene with the *Psu*I restriction enzyme revealed a polymorphism with two alleles *(Table 4)*. Three genotypes, TT (545 bp), CC (370 bp/175 bp) and CT (545 bp/370 bp/175 bp), were found in the TG gene *(Fig. 3)*. Results showed that the (C) allele had higher frequency in all breeds *(Table 4)*. More animals had the CC genotype than any other

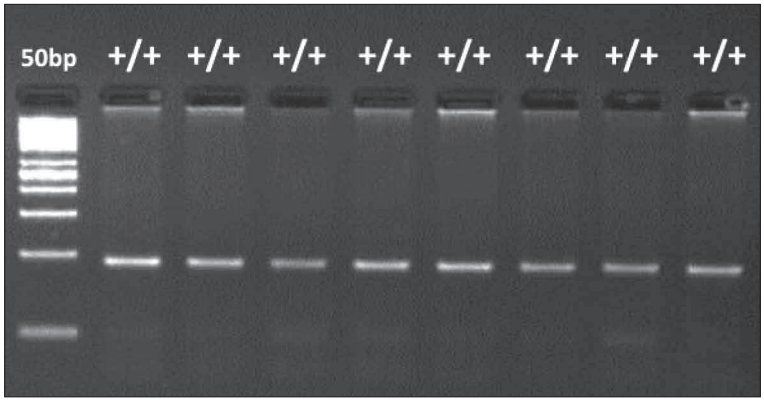

Fig 1. MSTN genotyping after digestion with *Fok*I restriction enzyme

Şekil 1. *Fok*I retriksiyon enzimi ile kesilen MSTN genotiplendirmesi

Table 3. *Heterozygosity, allelic genotypes frequency of SPP1 alleles C, T in five cattle breeds in Turkey*
Tablo 3. *Türkiye'deki 5 sığır ırkında SPP1 geninin C, T allelik genotip frekansları, heterozigotlukları*

Gene	Breed	n	Allele Frequency		Genotype Frequency (%)			Heterozygosity		χ2 (df=1)
			C	T	CC	CT	TT	Ho	He	
SPP1	TG	60	0.17	0.83	3.3	26.7	70.0	0.27	0.28	0.14^{NS}
	AB	59	0.20	0.80	3.4	33.9	62.7	0.34	0.33	0.08^{NS}
	Zavot	60	0.21	0.79	8.3	25.0	66.7	0.25	0.33	3.83^{NS}
	EAR	49	0.24	0.76	8.2	32.7	59.1	0.33	0.37	0.81^{NS}
	SAR	53	0.09	0.91	1.9	15.1	83.0	0.15	0.17	0.91^{NS}

NS: *nonsignificant*

Fig 2. SPP1 genotyping after digestion with *Bsr*I restriction enzyme

Şekil 2. *Bsr*I retriksiyon enzimi ile kesilen SPP1 genotiplendirmesi

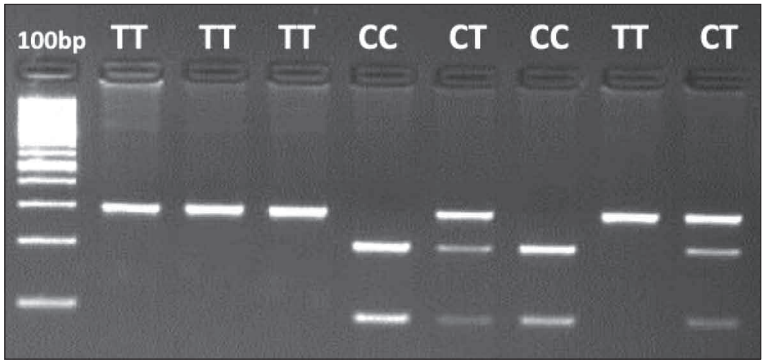

Table 4. *Heterozygosity, allelic genotypes frequency of TG alleles C, T in five cattle breeds in Turkey*
Tablo 4. *Türkiye'deki 5 sığır ırkında TG geninin C, T allelik genotip frekansları, heterozigotlukları*

Gene	Breed	n	Allele Frequency		Genotype Frequency (%)			Heterozygosity		χ2 (df=1)
			C	T	CC	CT	TT	Ho	He	
TG	TG	60	0.84	0.16	75.0	18.3	6.7	0.18	0.27	6.36*
	AB	59	0.86	0.14	78.0	16.9	5.1	0.17	0.24	5.01*
	Zavot	60	0.80	0.20	61.7	36.7	1.6	0.37	0.32	1.16^{NS}
	EAR	49	0.77	0.23	61.2	30.6	8.2	0.31	0.36	1.25^{NS}
	SAR	53	0.91	0.09	83.0	15.1	1.9	0.15	0.17	0.91^{NS}

** (P<0.05);* ***NS:*** *nonsignificant*

genotype. Significant deviation was observed from HWE in the TG and AB breeds *(Table 4)*.

CAPN1 Locus

Digestion of the 670bp fragment of the CAPN1 gene with the *Fok*I restriction enzyme revealed a polymorphism with two alleles *(Table 5)*. The CAPN1 gene had three genotypes: TT (670 bp), CT (670 bp/530 bp/140 bp) and CC (530 bp/140 bp) *(Fig. 4)*. Except for the TG and SAR breeds, genotype frequencies were not consistent with the HWE

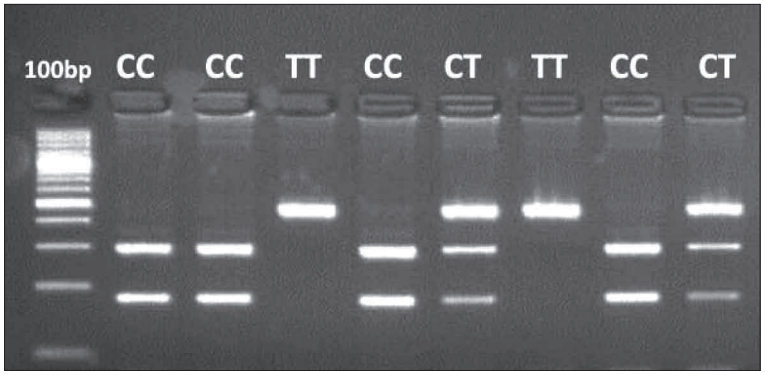

Fig 3. TG genotyping after digestion with *Psu*I restriction enzyme

Şekil 3. *Psu*I retriksiyon enzimi ile kesilen TG genotiplendirmesi

Table 5. *Heterozygosity, allelic genotypes frequency of CAPN1 alleles C, T in five cattle breeds in Turkey*
Tablo 5. *Türkiye'deki 5 sığır ırkında CAPN1 geninin C, T allelik genotip frekansları, heterozigotlukları*

Gene	Breed	n	Allele Frequency		Genotype Frequency (%)			Heterozygosity		χ2 (df=1)
			C	T	CC	CT	TT	Ho	He	
CAPN1	TG	60	0.77	0.23	61.7	30.0	8.3	0.30	0.36	1.76^{NS}
	AB	59	0.75	0.25	66.2	16.9	16.9	0.17	0.38	18.82*
	Zavot	60	0.74	0.26	63.3	21.7	15.0	0.22	0.39	11.89*
	EAR	49	0.75	0.25	61.2	26.5	12.3	0.26	0.38	4.84*
	SAR	53	0.87	0.13	75.5	22.6	1.9	0.23	0.23	0.03^{NS}

** (P<0.05);* ***NS:*** *nonsignificant*

Fig 4. CAPN1 genotyping after digestion with *Fok*I restriction enzyme

Şekil 4. *Fok*I retriksiyon enzimi ile kesilen CAPN1 genotiplendirmesi

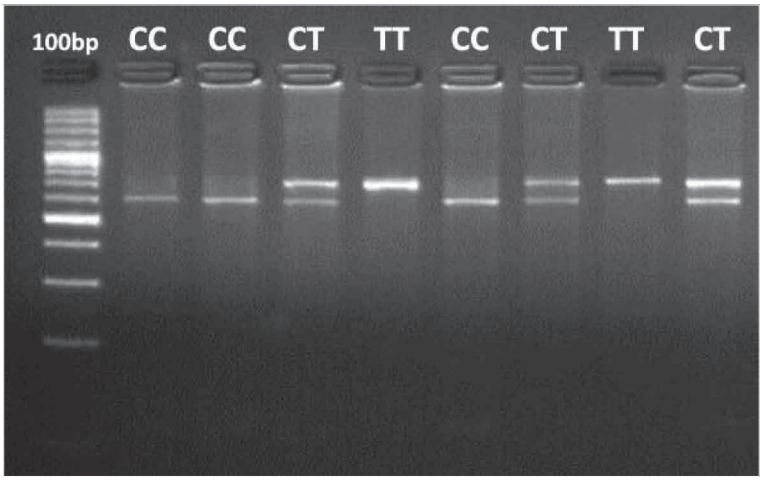

Table 6. *Heterozygosity, allelic genotypes frequency of CAST alleles C, T in five cattle breeds in Turkey*
Tablo 6. *Türkiye'deki 5 sığır ırkında CAST geninin C, T allelik genotip frekansları, heterozigotlukları*

Gene	Breed	n	Allele Frequency		Genotype Frequency (%)			Heterozygosity		χ2 (df=1)
			C	G	CC	GC	GG	Ho	He	
CAST	TG	60	0.60	0.40	35.0	50.0	15.0	0.50	0.48	0.07^{NS}
	AB	59	0.67	0.33	38.9	56.0	5.1	0.56	0.45	3.86*
	Zavot	60	0.68	0.32	46.7	43.3	10.0	0.43	0.44	0.01^{NS}
	EAR	49	0.61	0.39	38.8	44.9	16.3	0.45	0.48	0.21^{NS}
	SAR	53	0.66	0.34	47.2	37.7	15.1	0.38	0.45	1.51^{NS}

** (P<0.05);* ***NS:*** *nonsignificant*

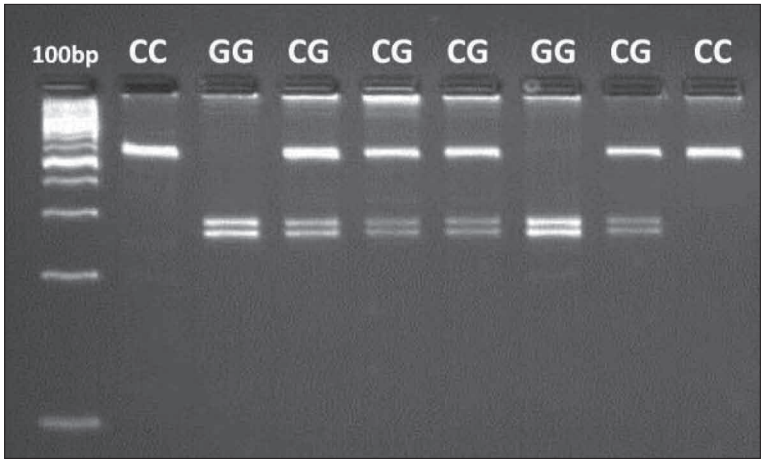

Fig 5. CAST genotyping after digestion with *Rsa*I restriction enzyme

Şekil 5. *Rsa*I retriksiyon enzimi ile kesilen CAST genotiplendirmesi

(P<0.05). The frequency of the (C) allele was found to be higher than the (T) allele *(Table 5)*.

CAST Locus

The 523bp fragment of the CAST gene was digested with the *Rsa*I restriction enzyme and a polymorphism with two alleles was detected. The CAST gene had three genotypes: CC (523 bp), CG (523 bp/266 bp/257 bp) and GG (266 bp/257 bp) *(Fig. 5)*. Except for the AB breed, genotype frequencies were consistent with the HWE (P<0.05) *(Table 6)*.

DISCUSSION

In the last 50 years, the world has experienced very rapid population growth. In addition, the Food and Agriculture Organization of the United Nations reported that food demand will double around the world in the next 50 years. Moreover, it has been predicted that global meat consumption will increase 68% and global milk consumption will increase 57% by 2030 [18]. Outside Europe and North America, in countries like Turkey that have a large population land area, the demand for meat cannot be met only by European breeds such as the Holstein. Therefore, it is important to improve the productivity of native breeds. To this end, the use of molecular markers associated with meat and milk production provides new opportunities to improve native breeds.

In animal breeding, some genes have been proposed as potential candidates with respect to traits that enhance economic value, such as milk yield, meat productivity traits and beef tenderness [19]. In the present study, the genetic polymorphisms of the CAPN1, MSTN, TG, SPP1 and CAST genes were evaluated in the TG, AB, Zavot, EAR and SAR cattle breeds. We used PCR-RFLP analyses to identify the different genotypes of each gene in these five Turkish native cattle breeds.

The frequency of the CAPN1-T allele was found to be higher than the frequency of the C allele in the Nellore breed *(Bos indicus)* [20,21] and its crossbreeds, where the different breeds originated from *Bos taurus* [20]. Additionally, the CC genotype was not found or was found to be lower than other genotypes in breeds that originated from Zebu [20,21]. Similarly, in purebred Brahmans *(Bos indicus)*, the TT genotype was found to be most frequent, while the CC genotype was not found at al.[22]. On the other hand, the T allele was found to be more frequent in beef cattle breeds such as Red Angus, Charolaise and Limousin breeds [23]. At the same time, in a study that examined more animals, the frequency of the C allele (0.577) was found to be higher than that of the T allele (0.423) in a population that consisted of *Bos Taurus* (Hereford, Angus, Red Angus, Limousin, Charolais, Gelbvieh and Simmental). In the same study, the frequency of the C allele (0.64) was found to be higher than that of the T allele (0.36) in a population that consisted of *Bos taurus* and *Bos indicus* (Hereford, Angus, Brangus, Beefmaster, Bonsmara, and Romosinuano) [22]. In the present study, the C allele was found to be more frequent than the T allele in five Turkish local cattle breeds when compared with the same European cattle breeds which originate from *Bos Taurus*. Furthermore, the CC genotype was found to be more frequent than other genotypes, while the TT genotype was found to be less frequent in five Turkish local cattle breeds. Therefore, it is thought that the TT genotype could be used to improve meat yield and flavour in Turkish local cattle breeds. To this end, the TT genotype frequency could be increased in Turkish local cattle breeds because the TT genotype is associated with favourable meat production traits that are important [22,24]. Furthermore, the CT and TT genotypes were associated with the best meat tenderness when compared with the CC genotype [24,25]. Despite the fact that the TT genotype frequency is low in Turkish local cattle breeds, this genotype was found to be have the lowest frequency in the SAR breed (0.019). This cattle breed is bred for milk and has a higher milk yield than that of other Turkish native cattle breeds. The second lowest genotype frequency was found in the TG breed (0.083), and in terms of milk yield, this breeds ranks second in Turkish native cattle breeds. Consistent with these

findings, the C allele was found to be higher in dairy cattle breeds such as Simmental, Black-and-White and Polish Red cattle breeds [23].

It has been determined that a mutation in the MSTN gene was associated with the double-muscle phenotype in the Piemontese cattle breed [26]. Subsequently, this mutation was found in the Belgian Blue [27] and South Devon [28] cattle breeds. However, this mutation was not reported in other cattle breeds including Turkish native cattle breeds [5]. This mutation was not found in the five Turkish native cattle breeds that were examined in this study. This provides strong evidence that this mutation is not present in Turkish native cattle breeds. Despite the fact that these mutations are associated with high carcass weight, they were found to be associated with increased calving difficulty and reduced meat flavour [29]. Therefore, this situation can be considered to be good for Turkish native cattle breeds, and leads to the conclusion that these cattle breeds have maintained their purity.

The TG gene in cattle was found to be not associated with the traits of daily weight gain, weight at slaughter, rib eye fat and rib eye area [30]. However, the TG genotypes were associated with the marbling score in beef cattle breeds such as Aberdeen Angus, Shorthorn and Waghu cattle [31], and the T allele was found to be associated with a higher marbling score [32]. On the other hand, the TT genotype of this gene was found to be associated with the thickness of back fat in beef cattle [30]. Additionally, it was reported that the TT genotype of this gene may be used in marker assisted selection (MAS) for higher intramuscular fat content as a desirable trait [33,34]. Therefore, it is thought that the detection of TG gene polymorphism can be used for efforts to improve beef quality traits and for characterizing Turkish native cattle breeds.

The frequency of the TG-TT genotype was either not found or found to be very low in many cattle breeds such as Holstein, indigenous Polish Red, Hereford, Limousin [35,36], Charolais, Simmental, Angus, Belgian Blue [36], Red Angus [34], indigenous Japan Wagyu cattle [37] and Korean native cattle [38]. The current study also found the TG-T allele frequency in five Turkish native cattle breeds to be lower than the C allele frequency. In addition, the TT genotype frequency was found to be lower than the frequency of other genotypes in the five Turkish native cattle breeds evaluated in this study. This study and other studies suggest that the TG-C allele can be considered to be predominant in cattle.

Osteopontin (OPN, also called secreted phosphoprotein 1 or SPP1) plays a role in different processes such as the regulation of growth and development of the fetus, initiation and maintenance of pregnancy, and proliferation and differentiation of the mammary gland in mammals [39,40]. Because it has so many tasks, it is thought that the OPN gene could be used as a marker gene for milk performance traits [35], and growth [41]. It has been reported that the OPN (SPP1)-C allele has a positive effect on milk fat yield and milk protein content, while others report that it has a negative impact on milk yield [42]. In general, the frequency of the OPN (SPP1)-T allele was found to be higher than that of the C allele in different cattle breeds such as the Jersey [43], Holstein, Polish Red, Hereford and Limousin breeds [35]. In a study that examined EAR and SAR breeds, the T allele frequency was found to be higher than that of the C allele [44]. Similarly, the present study also found that the T allele frequency was higher than that of the C allele in the five Turkish native cattle breeds that were examined. Even in the SAR breed, which is known to be the most prevalent dairy breed of Turkish native cattle breeds, this rate was found to be over ninety percent.

The CAST gene has been associated with the trait of meat tenderness [45] and this gene has been suggested as a marker for tenderness [21,46]. The CAST-CC genotype has been found to be a desirable genotype for tenderness in many cattle breeds such as the Brahman, beef cattle crossbreeds [22,45], Nellore [21], Hanwoo (Korean cattle) [47], Red Angus, Charolaise, Limousin, Simmental, Hereford, Polish Friesian and Polish Red [23]. Therefore, the CAST gene is important for work done to improve the quality of meat. The CAST-C allele frequency was found to be higher than that of the G allele in many cattle breeds which originated from *Bos taurus* [Hanwoo (Korean cattle) [47], Angus, Limousin, Charolais [45], Simmental, Hereford and Friesian [23]], *Bos indicus* [Nellore [21,48] and Brangus [48]], and *Bos taurus-Bos indicus* crosbreeds [Angus x Nellore, Rubia Gallega x Nellore and Canchim [48]]. In a previous study, the C allele frequency was found to be higher in the TG and TG x Brown Swiss crossbreeds [49]. Similarly, the CAST-C allele frequency was found to be higher in all Turkish native cattle breeds that were examined in the current study, as is the case with other cattle breeds. However, the CG genotype was found to be high, thus it is thought that the CAST gene could be used to improve the tenderness trait of Turkish native cattle breeds.

Many indigenous livestock breeds are still bred due to their adaptation traits and lower maintenance costs. However, the productivity of these breeds is lower than many European breeds. Despite this, the conservation and survival of the different genotypic variants in the genetic pool of a species is very important for genotyping livestock for selection against various infections, effects and environmental conditions. Further studies such as association analysis and QTL analysis are required to investigate the connection with productivity traits in native farm animal populations. In conclusion, this study has shown that genetic polymorphisms do not exist in the MSTN gene but do exist in the remaining four genes that were examined in five Turkish native cattle breeds.

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Research Article

Effects of Different *Juncus acutus*: Maize Silage Ratios on Digestibility and Rumen Cellulolytic Bacteria [1]

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Abstract

The objectives of this study were to estimate the digestibility of different ratios of *Juncus acutus* and maize silage and to investigate the effects of them on rumen bacteria. Three different ratios of *Juncus acutus* and maize silage 100:0 (A), 50:50 (B) and 0:100 (C) were prepared and their gas productions were determined at 0, 3, 6, 12, 24, 48, 72 and 96 h incubation times by ANKOM ^{RF} gas production system. OMD%, ME_{OMD}, ME_{GP}, and b values of A, B, C were 42.06, 51.06 and 60.21%; 6.72, 8.16 and 9.63 MJ/kg DM; 5.15, 6.28 and 7.55 MJ/kg DM; 20.85, 35.24 and 48.11 mL respectively. There were significant variations between the chemical composition, gas production, OMD%, ME_{GP} and ME_{OMD} values of A, B and C ($P<0.05$). Abundance of ruminal bacteria were as following *Fibrobacter succinogenes*>*Ruminococcus flavefaciens*>*Ruminococcus albus* values at all incubation times. In conclusion, mixing of *Juncus acutus* with maize silage in 50:50 ratio increased the amount of rumen cellulolytic bacteria and 22% of both OMD and ME of *Juncus acutus*. Supplementation of maize silage to *Juncus acutus* in ruminant diet may improve the utilization of *Juncus acutus* through providing of nitrogen and fermentable carbohydrates to rumen bacteria.

Keywords: *Cellulolitic bacteria, Juncus acutus, Maize silage, Metabolizable energy, Organic matter digestibility*

Juncus acutus ve Mısır Silajının Farklı Oranlarının Sindirilebilirlik ve Rumen Selülolitik Bakterileri Üzerine Etkisi

Özet

Bu çalışma ile farklı oranlarda karıştırılan *Juncus acutus* ve mısır silajının sindirilebilirliğinin ve rumendeki selülolitik bakteriler üzerine etkisinin belirlenmesi amaçlandı. *Juncus acutus* ve mısır silajı üç farklı oranda (100:0 (A), 50:50 (B), 0:100 (C)) karıştırılarak kaba yem örnekleri hazırlandı ve 0, 3, 6, 12, 24, 48, 72 ve 96 saatlik inkübasyonlarda gaz üretim (GÜ) değerleri belirlendi. A, B ve C örneklerinin % organik madde sindirilebilirliği (OMS), (metabolik enerji) ME_{OMS} ve $ME_{GÜ}$, potansiyel gaz üretimi (b) değerleri sırasıyla %42.06, 51.06 ve 60.21; 6.72, 8.16 ve 9.63 MJ/kg KM; 5.15, 6.28 ve 7.55 MJ/kg KM; 20.85, 35.24 ve 48.11 mL bulundu. A, B ve C örneklerinin kimyasal kompozisyonları, gaz üretimi, %OMS, $ME_{GÜ}$ ve ME_{OMS} değerleri arasında önemli farklılıklar tespit edildi ($P<0.05$). Bakteri miktarlarındaki artış *Fibrobacter succinogenes*>*Ruminococcus flavefaciens*>*Ruminococcus albus* şeklinde tespit edildi. Sonuç olarak *Juncus acutus* ile mısır silajının 50:50 oranında karıştırılması rumen bakterilerinin oranını ve *Juncus acutus*'un OMS ve ME değerlerini %22 oranında artırdı.

Anahtar sözcükler: *Juncus acutus, Metabolik enerji, Mısır silajı, Organik madde sindirilebilirliği, Selülolitik bakteri*

INTRODUCTION

Nowadays, one of the most important problems of the livestock sector is finding roughage without considering quality in Turkey. Mainly crop residues like wheat, barley and rice straw have been used to meet roughage requirement. A large proportion of crop residues consists of indigestible lignin [1]. Therefore, the use of straw as roughage in ruminant feeding should be used in conjunction with other easily digestible high quality roughages which will have a positive effect on the digestive system. Maize silage is a high energy roughage with high dry matter yield relative to the other roughage crops. Maize silage has low concentrations of protein and some minerals, but high concentrations of fermentable carbohydrates. Energy value of maize silage is mostly

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estimated from chemical composition and *in-vitro* organic matter digestibility (OMD) [2]. Therefore, maize silage are often preferred together with straw and hay in rations. In Kizilirmak Delta in Turkey, farmers mix maize silage with straw for cattle and buffalo nutrition.

Juncus acutus is the most abundant plant in wetlands. There are about 2549.22 ha of natural grassland in the Kizilirmak Delta [3]. *Juncus acutus* presents mainly in Yorukler, Doganca and Sarikoy districts having 519.843 ha land and its *Juncus acutus* production capacity is 8.650 tons. This amount corresponds to 3.719 tons on dry matter basis. Total *Juncus acutus* production capacity of 23 wetlands in Turkey is approximately 85.537 tons. *Juncus acutus* are consumed by water buffaloes which is part of the natural habitat of Kizilirmak Delta. *Juncus acutus* has been proposed as an alternative roughage to cereal straw and also in term of CP % to medium-quality roughage [4].

The *in-vitro* gas production method have been widely used to estimate organic matter digetibility and metabolisable energy values in feed evaluation for ruminants [5]. Advantages and disadvantages of *in-vitro* gas methods are discussed by Gatechew *et al.*[6]. A simple *in vitro* approach is described by Menke *et al.*[7] which is convenient and fast, and allows a large number of samples to be handled at a time. Makkar [8] highlights the potential of a novel approach using an *in-vitro* gas prodution methods for evaluation of nutritional quality of feed resources. Recently, *in-vitro* gas production technique for feed evaluation well reviewed by Singh *et al.*[9].

Rumen microbial ecosystem consist of bacteria, archaea, protozoa, fungi, and bacteriophages [10]. Bacteria are the most numerous of these microorganisms and play major role in the biological degradation of dietary fiber. Cellulose is the major component of forages, and its digestion and subsequent fermentation by ruminal microbes provide much of the energy for forage-fed ruminants [11]. Ruminal degradation of cellulose is mediated primarily by cell-associated enzymes produced by a few predominant cellulolytic bacteria [12]. The rate and extent of fiber digestion in the rumen in large measure are dependent on the population size of these cellulolytic bacteria. *Fibrobacter succinogenes, Ruminococcus flavefaciens* and *Ruminococcus albus* are presently recognized as the major cellulolytic bacterial species found in the rumen [13-15].

Recent advances in molecular biology techniques allow the analysis of such bacteria without cultivation, there by many functional but uncultured, bacteria as new targets for basic and aplied research [16]. Real-time PCR has been successfully used for quantifying protozoa, cellulolytic fungi and cellulolytic bacterial species [4,17-19].

The objectives of this study were to estimate the digestibility of different ratios of *Juncus acutus* and maize silage and to investigate the effects of them on rumen bacteria.

MATERIAL and METHODS

The study was approved by the Local Ethics Committee on Animal Experiments of Ondokuz Mayis University, Turkey (OMU, 18.12.2012, HADYEK 2012/70). Chemical analyses and *in-vitro* gas production experiments were carried out in the Ruminant Feed Evaluation Laboratory of Departmant of Animal Nutrition and Animal Diseases, OMU Faculty of Veterinary Medicine. Real-time PCR analyses were conduted in Samsun Public Health Laboratories, Ministery of Health.

Animals and Feeds

Rumen fluid was obtained from three fistulated Karayaka rams (2 years old, BW = 50±5 kg) fed twice daily at the maintanence level with a diet containing 65% alfaalfa hay and 35% concentrate (Samsun feed processing factory; 1 3% CP, 10% CS, 4% EE, 9% Ash) after three weeks adaptation period. Twenty *Juncus acutus* samples were collected from Kizilirmak Delta. Twenty maize silage samples were taken from dairy cattle enterprise in Doganca Bafra, Turkey. Cut roughage samples were dried in oven at 105°C over-night [20], ground in a mill to pass a 1 mm mesh screen, and kept at room temperature till laboratory analysis.

Chemical Analysis

All roughage samples were milled through a 1 mm sieve then three different ratios of *Juncus acutus* and maize silage 100:0 (A), 50:50 (B) and 0:100 (C) were prepared. Prepared roughage samples A, B and C were used for chemical analysis, gas production and real-time PCR methods. Dry mater (DM), ash, ether extract (EE) and nitrogen (N) contents of samples were analysed according to AOAC methods [20]. Neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were determined by Van Soest *et al.*[21].

In-Vitro Gas Production

The ANKOM^{RF} gas production method was used for the incubation [22]. Each experimental unit consisted of 250 mL glass jar with attached module top. The module tops having the communication system were used. Gas accumulating in the headspace of bottle was automatically released when the pressure inside the units reached 1.5 kPa above ambient pressure. Pressure was measured every 10 min. Approximately 1 g of the milled feed samples was weight into 250 mL glass jar and incubated at 39°C overnight.

They were fed at least 3 h before the rumen fluid was collected. The fluid was collected into pre-heated thermos-flask. The buffer was prepared according to Menke and Steingass [5], and buffer mixed with rumen fluid 4:1. A mixture of 100 mL of this media was added to preheated units containing feed samples. The glass jar were then closed and put into an incubator. Media and incubation preparation were done under anaerobic conditions by

constantly flushing CO_2, at a temprature of about 39°C and pH of about 6.5-6.8. The incubation procedure was repeated three times. The samples were incubated for 0, 3, 6,12, 24, 48, 72, 96 h. The average cumulative pressure measured for each sample. Pressure was converted to mL of gas at standard temprature and pressure. Then after gas produced per gram DM incubated substrat was calculated. Cumulative gas production data at 24 h was fitted to the model of Ørskov and McDonald [23]. Gas (Y) = b (1-e^{-ct}), where; b = the gas production from the insoluble fraction (mL), c = the gas production rate constant for the insoluble fraction (mL/h), t = incubation time (h). OMD%, ME_{GP}(MJ/ kg DM), and ME_{OMD} (MJ/ kg DM) values of roughage samples A, B and C were estimated from measured pressure by *in-vitro* method at 24 h by using below equations [5].

ME (MJ/kg DM) = 2.2 + 0.136 GP + 0.057 CP + 0.0029 EE

OMD (%) = 57.2 + 0.365 GP + 0.304 CP-1.98 ADL

GP (mL/200 mg DM)

ME (MJ/kg DM) = 0.16 OMD

Real -Time PCR Analysis

The effects of roughages A, B and C on rumen cellulolytic rumen bacteria *Fibrobacter succinogenes* > *Ruminococcus flavefaciens* > *Ruminococcus albus* were determined by real-time PCR method. DNA isolation of rumen fluids obtained from 0, 3, 6, 9, 12, 24, 48, 72 and 96 h incubations were carried out by applying bacterial DNA isolation procedure by using Chelex-resin [24]. Real-time PCR assays of isolated DNA samples were performed on C 1000 Bio-rad real-time PCR device. Assays were set up using the EVA Green PCR Master Mix (2X) (Seegene Technologies;Taewon Bldg., 91, Ogeum-ro, Songpa-gu, Seoul, 138-828, Korea).

The targeted bacteria were 3 predominant cellulolytic bacteria *Fibrobacter succinogenes, Ruminococcus flavefaciens* and *Ruminococcus albus.* Primer for *Fibrobacter succinogenes* Forward(Fs219f):5´-GGTATGGGATGAGCTTGC-3´,

Reverse(Fs654r):5´-GCCTGCCCCTGAACTATC-3´,

Ruminococcus albus Forward(Ral281f):5´-CCCTAAAA GCAGTCTTAGTTCG-3´,

Reverse(Ral439r):5´-CCTCCTTGCGGTTAGAACA-3´and

Fibrobacter flavefaciens Forward (Rf154f):5´TCTGGAAA CGGATGGTA-3´,

Reverse(Rf425r): 5´-CCTTTAAGACAGGAGTTTACAA-3´. Those primers were chosen from previously published sequences that demostrates species-specific amplication [13].

PCR conditions for *Fibrobacter succinogenes* was as follows: 30 sec. at 94°C for denaturing, 30 sec. at 60°C for anneling and 30 sec. at 72°C for extension (48 cycles), except for 9 min of denaturation in the first cycle and 10 min of extention in the last cycle. Amplification of 16 sec. rDNA for *Ruminococcus flavefaciens* and *Ruminococcus albus* was carried out similarly except an annealing temperature of 55°C.

The relative abundance of three predominant bacteria in rumen fluids obtained from 0, 3, 6, 12, 24, 48, 72 and 96 h incubations of *Juncus acutus* samples which were collected from three different stations was quantified using the relative quantification Δ C_T [25]. The mean values of each bacteria at 0, 3, 6, 12, 24, 48, 72 ve 96 h incubation time of *Juncus acutus* which were collected from three different station.

Statical Analysis

One-Way analysis of variance and multiple comparisons among treatment means were performed by Duncan's new multiple range [26]. Means differences were considered significant at P<0.05.

RESULTS

Chemical composition of different ratio of *Juncus acutus*: maize silage samples A, B and C collected from Kizilirmak Delta is presented in *Table 1*. There was significant differencies between roughages in terms of chemical composition (P<0.05). Roughage A was very rich in DM, OM, CP, NDF, ADF and ADL contents and higher than that of the others roughages B and C, however roughage C was the lowest. Besides, ash, EE and ME_{ADF} values, the highest was found in roughage C, but the lowest was in roughage A.

Cumulative GP_{mL}/200 mg DM, OMD%, ME_{OMD} (MJ/ kg DM), ME_{GP} (MJ/kg DM) and potential gas production (b) mL of roughages A, B and C at 24 h are presented in *Table 2*. Cumulative GP_{mL}/200 mg DM, OMD%, $ME_{OMD,}$ $ME_{GP,}$ and

Table 1. *Chemical composition and ME_{ADF} (MJ/kg DM) values of roughages A, B and C*

Tablo 1. *A, B ve C kaba yemlerinin kimyasal kompozisyonu ve ME_{ADF} (MJ/ kg KM)* değerleri

%	Roughage Sample		
	A (n=20) X±Sx	B (n=20) X±Sx	C (n=20) X±Sx
DM (105°C)	97.36±0.21^a	95.43±0.18^b	94.51±0.06^c
ASH	4.11±0.02^c	5.15±0.04^b	6.30±0.08^a
OM	93.25±0.05^a	90.28±0.03^b	88.21±0.09^c
CP	10.13±0.06^a	8.41±0.05^b	6.55±0.06^c
EE	1.53±0.05^c	1.69±0.06^b	1.94±0.05^a
NDF	73.14±0.08^a	60.66±0.06^b	47.62±0.03^c
ADF	45.84±0.04^a	37.95±0.03^b	31.45±0.04^c
ADL	12.43±0.04^a	9.23±0.04^b	6.19±0.06^c
ME_{ADF} (MJ/kg DM)	8.65±0.01^c	9.67±0.02^b	10.52±0.01^a

A: *100% Juncus acutus,* ***B:*** *50% Juncus acutus + 50% maize silage,* ***C:*** *100% maize silage. n: number of samples; Means with in a row with different superscripts differ (P<0.05)*

b values of roughages A, B, C were 17.56, 26.57 and 36.63 mL; 42.06, 51.06 and 60.21%; 6.72, 8.16 and 9.63 MJ/kg DM; 5.15, 6.28 and 7.55 MJ/kg DM; 20.85, 35.24 and 48.11 mL respectively.

Fibrobacter succinogenes, Ruminococcus flavefaciens and *Ruminococcus albus* values calculated from threshold (C_T) values in rumen fluids obtained from 0, 3, 6, 12, 24, 48, 72 and 96 h incubations of roughages A, B, C collected from Kizilirmak Delta by real-time PCR method are shown in *Table 3*.

DISCUSSION

Chemical Analysis

Chemical composition of roughages A, B and C collected from Kizilirmak Delta are presented in *Table 1*.

Table 2. *Cumulative gas production volume at 24 h (GP), potantial gas production volume (b), organic matter digestibility (OMD), metabolic energy (ME_{OMD} and ME_{GP}) of roughages A, B, and C*

Tablo 2. *A, B ve C kaba yemlerinin 24 saatlik kümülatif gaz üretim hacmi (GÜ), potansiyel gaz üretim hacmi (b), organik madde sindirilebilirliği (OMS) ve metabolik enerji (ME_{OMS} ve $ME_{GÜ}$)*

Parameter	Roughage Sample		
	A (n=20) X±Sx	B (n=20) X±Sx	C (n=20) X±Sx
GP_{mL}(GP_{mL}/ 200mg DM)	17.56±0.41^c	26.57±0.35^b	36.63±0.39^a
OMD (%)	42.06±0.07^c	51.06±0.15^b	60.21±0.16^a
ME_{OMD} (MJ/kg DM)	6.72±0.03^c	8.16±0.02^b	9.63±0.02^a
ME_{GP} (MJ/kg DM)	5.15±0.07^c	6.28±0.05^b	7.55±0.05^a
b (mL)	20.85±0.26^c	35.24±0.25^b	48.11±0.45^a

A: *100% Juncus acutus,* ***B:*** *50% Juncus acutus + 50% maize silage,* ***C:*** *100% maize silage. n:number of samples; Means with in a row with different superscripts differ (P<0.05)*

There was considerable variation between roughages in terms of chemical composition (P<0.05). The crude protein content of roughages changed from 6.55 to 10.13%. Roughage A was very rich in crude protein and higher than that of the other silages. Roughage C was very poor in crude protein. The crude protein content of roughage A was similar to that reported for *Juncus acutus* by Erdem [4]. The crude protein content of roughage B was similar to that reported for maize silage by Nkosi *et al.*[27]; for orange pulp by Akinfemi *et al.*[28]. The crude protein content of roughage C was similar to that reported for maize silage by Ozturk *et al.*[29], Karakozak and Ayasan [30] and Podkowka and Podkowka [31].

There were statistically significant differences between of roughages A, B and C in terms of NDF, ADF and ADL (P<0.05). The NDF contents of roughage A, B and C was found 73.14%, 60.66% and 47.62% respectively. The NDF content of roughage A was similar to that reported for *Juncus acutus* by Erdem [4]; for rice straw by Rahman *et al.*[32]. The NDF content of roughage B was similar to that reported for bromegrass by Doane *et al.*[33]. The NDF content of roughage C was similar to that reported for pea hay by Canbolat *et al.*[34]; for tomato pomace by Mirzaei-Aghsaghali *et al.*[35].

The ADF contents of roughages A, B and C was found 45.84%, 37.95% and 31.45% respectively. The ADF content of roughage A was similar to that reported for *Juncus acutus* by Erdem [4]. The ADF content of roughage B was similar to that reported for *Convoivuius arvensis* by Canbolat [36]. The ADF content of roughage C was similar to that reported for *Onobrychis sativa* hay by Canbolat [37]; for tomato pomace by Mirzaei-Aghsaghali *et al.*[35]; for *Eucalyptus camaldulensis* leaves by Akcil and Denek [38].

The ADL contents of roughages A, B and C samples

Table 3. *The mean fold changes of Fibrobacter succinogenes, Ruminococcus flavefaciens and Ruminococcus albus in rumen fluids obtained from 0, 3, 6, 12, 24, 48, 72 and 96 h incubations of roughages A, B and C*

Tablo 3. *A, B ve C kaba yemlerinin 0, 3, 6, 12, 24, 48, 72 ve 96 saatlik inkübasyonlarından elde edilen rumen sıvısındaki Fibrobacter succinogenes, Ruminococcus flavefaciens ve Ruminococcus albus bakterilerinin ortalama kat artışlarının değişi*mi

t(h)	***Fibrobacter succinogenes*** (mean fold *)				***Ruminococcus flavefaciens*** (mean fold *)				***Ruminococcus albus*** (mean fold *)			
	Roughage Sample				Roughage Sample				Roughage Sample			
	A	B	C	SEM	A	B	C	SEM	A	B	C	SEM
0	1	1	1		1	1	1		1	1	1	
3	1.08^b	1.10^b	1.17^a	0.01	1.05^b	1.07^b	1.11^a	0.02	1.01^b	1.03^b	1.06^a	0.01
6	1.20^b	1.19^b	1.29^a	0.05	1.11^b	1.12^b	1.19^a	0.03	1.05^b	1.08^a	1.09^a	0.03
12	1.32^b	1.36^b	1.56^a	0.05	1.21^b	1.28^a	1.30^a	0.04	1.12^b	1.21^a	1.22^a	0.02
24	1.99^c	2.63^b	2.92^a	0.04	1.55^c	2.39^b	2.43^a	0.04	1.47^c	1.68^b	1.95^a	0.04
48	2.32^c	3.48^b	3.87^a	0.05	1.92^c	2.65^b	2.72^a	0.03	1.73^c	2.01^b	2.23^a	0.05
72	2.49^b	3.51^b	3.90^a	0.05	2.21^c	2.87^b	2.96^a	0.05	1.92^c	2.20^b	2.28^a	0.04
96	2.53^b	3.56^b	3.92^a	0.06	2.27^c	3.05^b	3.20^a	0.05	2.00^c	2.24^b	2.31^a	0.03

A: *100% Juncus acutus,* ***B:*** *50% Juncus acutus + 50% maize silage,* ***C:*** *100% maize silage; t: incubation times (h);* ***SEM:*** *Mean of Standard eror. Means within a row with different superscripts differ (P< 0.05);* **** fold:*** *amount of microbial population at each incubation time over 0 h (control) which was taken as 1*

was found 12.43%, 9.23% and 6.19% respectively. The ADL content of A roughage was similar to that reported for *Juncus acutus* by Erdem [4]; for wheat straw by Kalkan and Filya [39]. The ADL content of roughage B was similar to that reported for good quality alfalfa hay by Gungor *et al*.[40]. The ADL content of roughage C was similar to that reported for maize silage by Gungor *et al*.[40]; for cereal roughages from corn and wheat by Canbolat [41].

In-Vitro Gas Production

The cumulative volume of gas production increased with increasing incubation time. A statistically significant difference was observed between roughages A, B and C samples of gas production at all incubation times (P<0.05). It may be due to different ADL content of roughages A, B and C. Mertens *et al*.[42] repoted that high ADL level of feedstuffs adversly affect gas production however NDF content increase gas production. The ADL contents and cumulative volume of gas production of roughages A, B and C were 12.43, 9.23 and 6.19%; 17.56, 26.57 and 36.63 mL at 24 h of incubation respectively. At all incubation time, gas production of roughage C was significantly higher than the others (P<0.05) and gas production of roughage A was significantly lower than the others (P<0.05).

In-vitro gas production, kinetic parameters, ME_{GP}, ME_{OMD} and OMD% are significantly affected by nutrient content of roughages A, B and C *(Table 2)*.

ME_{GP} and ME_{OMD} values of roughages A, B and C were 6.72, 8.16 and 9.63 MJ/kg DM; 5.15, 6.28 and 7.55 MJ/kg DM respectively. The OMD% value of roughages A, B and C was found 42.06%, 51.06% and 60.21% respectively. There were statistically significant differences between of roughages in terms of OMD% (P<0.05). Obtained differences among OMD% of roughages A, B and C were associated with gas production. The OMD% value of roughage A was similar to that reported for *Juncus acutus* by Erdem [4]; for rice straw by Rahman *et al*.[32]. ME, OMD and gas production values of *Juncus acutus* were the significantly improved by treatment maize silage due to maize has low concentrations of protein and some minerals, but high concentrations of fermentable carbohydrates. The OMD% value of roughage B was similar to that reported for corn cobs and guinea corn threshed tops by Akinfemi *et al*.[28]. The OMD% value of roughage B was similar to that reported for *Convoivuius arvensis* by Canbolat [36].

There were significant differences between roughages in terms of estimated ME_{GP}, ME_{OMD} and OMD% levels (P<0.05). It may be due to the major causes of the differences in the amount of CP and ADL. The lag time for all roughages was very low and very close to zero. Therefore, lag time was ignored. However, potential gas production (b) value may be affected in the presence of secondary metabolites in *Juncus acutus*. Potential gas production of roughage C was higer than the other roughages. Potential gas production value of roughage A was similar to that reported for *Juncus acutus* by Erdem [4]. Potential gas production value of roughage C was similar to that reported for Mirzaei-Aghsaghali *et al*.[35].

Positive associative effects occured when *Juncus acutus* was mixed with maize silage in 50:50 ratio which increased the OMD and ME values of *Juncus acutus*. This observed effect maybe due to providing energy and protein for rumen microorganisms in required ratio from a mixture of *Juncus acutus* and maize silage.

Real-Time PCR Analysis

Fibrobacter succinogenes, Ruminococcus flavefaciens and *Ruminococcus albus* values calculated from threshold (C_T) values in rumen fluids obtained from 0, 3, 6, 12, 24, 48, 72 and 96 h incubations of roughages A, B and C by real-time PCR method showed an increases as FS > RF > RA *(Table 3)*. This ranking is in agreement with reported values by Polyorach *et al*.[43]; Hung and Wanapat [44]; Erdem [4]; Wanapat and Cherdthong [18]; Koike and Kobayyashi [13]. The population of *Fibrobacter succinogenes* compared to *Ruminococcus flavefaciens* and *Ruminococcus albus* was highest in all roughages A, B and C. Furthermore *Ruminococcus albus* was the lowest compared with *Fibrobacter succinogenes* and *Ruminococcus flavefaciens* in all roughages. Our obtained results showed that supplementation of maize silage to *Juncus acutus* provides nitrogen and fermentable carbohydrates to rumen cellulolitic bacteria and this caused to increase in the following order of *Fibrobacter succinogenes, Ruminococcus flavefaciens* and *Ruminococcus albus* growth. Apparently because *F. succinogenes* and *R. flavefaciens* can colonize the cellulose more rapidly than *R. Albus* [44,45]. *R. albus,* always was less abundant than was *F. succinogenes* and *R. flavefaciens* because it was less effective in colonizing cellulose and was probable reduced to growing on soluble products released by the other species during cellulose hydrolysis [46].

Gas production values of roughage samples A, B and C at 3, 6, 12, 24, 48, 72, 96 h of incubations were compatible with *Fibrobacter succinogenes, Ruminococcus flavefaciens* and *Ruminococcus albus* values calculated from threshold (C_T) values in rumen fluids obtained from 0, 3, 6, 12, 24, 48, 72, 96 h of incubations. There is a strong relationship between the OMD of feedstuffs and the rate of gas production [47]. Feedstuffs should contain at least 10% CP for optimum microbial activity in the rumen [48]. Mixing of *Juncus acutus* with maize silage is being a good combination for rumen bacteria because of high protein content of *Juncus acutus* (10% CP).

Mixed *Juncus acutus* with maize silage in 50:50 ratio may be used as medium quality roughage source in ruminant nutrition. It may be suggested to do further study on *in-vivo* condition to explore more about *Juncus acutus* and its potential effects on animal performance.

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Research Article

Spoligotyping of *M. tuberculosis* Strains from Cattle in Turkey

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Abstract

Although it is generally accepted that *M.bovis* leads to tuberculosis in cattle, there are statements given from the different regions of the world, referring to the fact that *M. tuberculosis*, which is known as the human tuberculosis agent, causes tuberculosis in cattle as well. The material of the study consisted of 13 *M. tuberculosis* isolates which were isolated and identified from the organ pieces of 95 cattle with the culture methods; these organ pieces had been taken from the cattle with granulomatous lesion detection after the slaughtering in slaughterhouses located in Çukurova region and brought to the laboratory under sterile conditions. It was determined in the genotyping conducted by using the Spoligotyping method that 13 of the 55 isolates were *M. tuberculosis* and they belonged to the T1 family (SIT53) by becoming dense in one cluster (100%). Consequently, it was shown with this study that *M. tuberculosis*, which leads to tuberculosis in humans, could be transmitted from humans to animals and from animals to humans again, and researching the human and epidemiological tuberculosis cases by using molecular epidemiology-based methods such as spoligotyping might provide useful information about explaining the ways of transmission of tuberculosis.

Keywords: *Cattle, Mycobacterium tuberculosis, Spoligotyping*

Türkiye'de Sığırlardan İzole Edilen *M. tuberculosis* Suşlarının Spoligotiplendirmesi

Özet

Tüberkülozise sığırlarda, *M. bovis*'in sebep olduğu genel olarak kabul edilmesine rağmen insan tüberkülozis etkeni olarak bilinen *M. tuberculosis*'in de sığırlarda tüberkülozise neden olduğuna dair dünyanın farklı bölgelerinden yapılmış bildirimler bulunmaktadır. Çalışmanın materyalini Çukurova bölgesinde bulunan mezbahalarda kesim sonrası granülamatöz lezyon tespit edilen sığırlardan alınan ve steril şartlarda laboratuvara ulaştırılan 95 adet sığıra ait organ parçalarından kültür yöntemleri ile izole ve identifiye edilen 13 adet *M. tuberculosis* izolatı oluşturdu. Spoligotyping yöntemi ile yapılan genotiplendirmede 55 izolattan 13 tanesinin *M. tuberculosis* olduğu ve bunların tek bir küme içerisinde yoğunlaşarak (%100) T1 ailesine (SIT53) ait olduğu belirlendi. Sonuç olarak bu çalışma ile insanlarda tüberkülozise neden olan *M. tuberculosis*'in insanlardan hayvanlara, hayvanlardan da tekrar insanlara bulaşabileceği, insan ve hayvan kaynaklı tüberkülozis vakalarının spoligotyping gibi moleküler epidemiyolojik temelli yöntemlerle araştırılmasının tüberkülozisin bulaş yollarının açıklanması konusunda faydalı bilgiler verebileceği gösterilmiştir.

Anahtar sözcükler: *Sığır, Mycobacterium tuberculosis, Spoligotyping*

INTRODUCTION

Tuberculosis is a chronic disease which is comprised of *M. tuberculosis*, *M. bovis, M. canettii*, *M. microti*, *M. africanum, M. caprae* and *M. pinnipedii* included in the *Mycobacterium tuberculosis* complex (MTC) and has a wide host range including humans, fish, reptiles, birds, wild animals and domestic mammals. *Mycobacteria* are the acid-resistant bacteria known with their abilities to settle in almost all the tissues of the body like lungs, kidneys, bones, liver, skin and brain and the lymph nodules belonging to these tissues, and with their formation of typical granulomas in the tissues they settle in [1,2].

Although it is generally accepted that *M. bovis* leads to tuberculosis in cattle, there are statements from studies made in different regions, referring to the fact that *M. tuberculosis*, which is known as the human tuberculosis agent, causes tuberculosis in cattle as well. It was reported by many researchers that tuberculosis caused by the *M. tuberculosis* was seen in domestic and wild animals after

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short and long-term contacts with humans and existed especially in cattle [3-5]. Although there are researches in Africa stating that the incidence rate of the *M. tuberculosis* in cattle is under 1% [3,6,7], it was stated that the *M. tuberculosis* dependent tuberculosis rate in cattle is 7.4% in countries like Sudan where the human tuberculosis incidence is high [8]. It was reported in another study conducted in India that the 30.8% of the tuberculosis occurrences identified in cattle were the tuberculosis cases that develop depending on the *M. tuberculosis* [9].

People with tuberculosis spread the agents with their urine, sputum and stools. There are reports stating that the *M. tuberculosis* infection develops in cattle through eating the feeds that have agents or the inhalation of the contaminated respiration air [5,10]. Direct transmission of the *M. tuberculosis* among cattle is doubtful.

The *M. tuberculosis* complex genome includes 36-base-pair direct repeat (DR) loci at different numbers and there are sequences called *spacer* at the length of 35-41-base pairs between these loci. The genetic relation between the strains can be determined by using the spoligotyping method on basis the number of DR copies and the existence or absence of the spacer sequences. The spoligotyping method has been used frequently over the last years to put forth the molecular epidemiology of the tuberculosis in humans and animals. Spoligotyping is a PCR-based reverse dot blot hybridization method and it is fast, simple and repeatable [11].

Simeon Cadmus et al. stated in the spoligotyping they conducted on human and cattle tuberculosis isolates in Nigeria that 51 of the 60 human MTC-member isolates were *M. tuberculosis*, these isolates had 18 different spoligo-patterns and the most observed pattern was the NH1 pattern, which belongs to LAM 10-CAM family including 35 isolates. Same researchers stated that 15 of the 17 MTC members they obtained from cattle were *M. bovis*, one member was *M. tuberculosis*, another member was *M. africanum* and the *M. tuberculosis* isolate belonged to the NH1 (LAM10-CAM) pattern, which is most frequently seen in humans [4].

It was aimed in this study to perform a molecular characterization of the *M. tuberculosis* strains by using the spoligotyping method, which were isolated from the lesions with the suspicion of tuberculosis obtained from the cattle slaughtered in the slaughterhouses located in Çukurova region.

MATERIAL and METHODS

The material of this study consisted of 13 *M. tuberculosis* isolates which were isolated and identified from the organ pieces of 95 cattle by using the culture methods; these organ pieces had been taken from the cattle with granulomatous pneumonia detection after the slaughtering in slaughterhouses located in Çukurova region and brought to the laboratory under sterile conditions. These 95 cattle were chosen out of 6.800 cattle slaughtered for meat production.

Culture

The tissue samples, which were taken from the lesions with the suspicion of tuberculosis observed in the lung and lymph nodes in the macroscopic examination carried out after the slaughtering and brought to the laboratory, were decontaminated in accordance with the protocol specified by Petroff [12].

Samples were inoculated onto the LJ medium with (4 gr/l) and without pyruvate and left for incubation at 37ºC [12,13]. EZN-stained preparates were prepared from the bacterial growth. Biochemical tests were applied to the bacterial growth, which were positive in terms of the ARB [14]. The strains which had morphological eugonic growth in the LJ medium, positive niacin accumulation test, nitrate reduction reaction, TCH reaction and negative catalase activity at 68ºC were evaluated as *M. tuberculosis* [5,15] .

Spoligotyping

DNA extraction was made by using the Mickle device from the colonies multiplied in the LJ medium of the 13 isolates, which were evaluated as *M. tuberculosis* as a result of the biochemical tests (Mickle tissue disintegrator). The spoligotyping method was applied after the extraction by using the DRa and DRb primer pairs, array of which is given below [16,17].

DRa: 5'-GGT TTT GGG TCT GAC GAC-3' (biotin labelled at the 5' end)

DRb: 5'-CCG AGA GGG GAC GGA AAC-3'

DRa and DRb primer pairs targeting DR area is synthesized. As DRa primer is labelled with biotin, it is preserved at +4ºC. DRb primer is splitted into small amounts and preserved at -20ºC. In each process, positive (*M. bovis*, *M. bovis* BCG, *M. tuberculosis* H37Rv, or clinical isolate whose genotype is known) and negative control (dH_2O) are used. For each strain; dH_2O 8.5µl, DMSO 1.0 µl, 2x PCR Master Mix (Fermantas) 12.5 µl, DRa (25 pmol/µl) 0.25 µl, DRb (25 pmol/µl) 0.25 µl, template DNA 2.50 µl are used. Tubes containing PCR reaction mixture are placed in Thermal Cycler device (Applied Biosystem AB) and heat cycle as: 95ºC 5 min, 40 cycle 94ºC 1 min, 55ºC 1 min, 72ºC 45 sec and then 72ºC 10 min 4ºC ∞. By using below mentioned octal coding key, results are converted to "Octal code" made of 15 characters between 0 and 7. By using databases, groups and clades are determined by obtained data (http://www.pasteurguadeloupe.fr:8081/SITVITDemo/outils Consultation.jsp, http://www.mıru-vntrplus.org, http://www.mbovis.org).

□□□ = 0 □□■ = 1 □■□ = 2 □■■ = 3
■□□ = 4 ■□■ = 5 ■■□ = 6 ■■■ = 7
■ = 1 □ = 0 Spacer 43

RESULTS

It was determined that isolated strains belonged to three different farms and the age of cattle were determined to vary between 3 to 6. It drew attention that farms which arrival of cattle isolated *M. tuberculosis* had aproximately 30-50 Holstein or Holstein hybrid breed and small dairy cows.

In the genotyping of the 13 *M. tuberculosis* isolates conducted by using the spoligotyping method, which were isolated and identified by using the culture methods from the organ pieces of 95 cattle with granulomatous pneumonia detection after the slaughtering and brought to the laboratory for bacteriological isolations under sterile conditions, it was determined that all the isolates belonged to the T1 family included in one profile (100%). The spoligotyping patterns of the *M. tuberculosis* isolates are given in *Table 1* and seen in *Fig. 1*.

DISCUSSION

In this study genotyping were carried out 13 *M. tuberculosis* isolates which were isolated and identified from the organ pieces of 95 cattle were chosen out of 6.800 cattle slaughtered for meat production and had been taken with granulomatous pneumonia detection after the slaughtering by spoligotyping methods.

Tuberculosis, which is thought to cause the death of approximately 2 million people every year, is one the significant zoonotic diseases that has reached so far. It is estimated that 9 million people got sick, 1.5 million people died and 360.000 of those dead people had HIV positive disease in 2013 [18]. Almost all the deaths resulting from tuberculosis are preventable and the application of tuberculosis control programs is necessary to decrease the deaths resulting from tuberculosis. Beside the early and right diagnosis of the disease, determination of the disease origin, infection-carriers and the type of the Mycobacterium leading to the disease is quite important to achieve success in the tuberculosis control programs. There are studies showing that the intra-dermal skin test (IDDT), which is used in most of the control programs to determine the prevalence of the disease, is not a reliable indicator in the determination of the active disease in cattle and IDDT results and clinical findings related to tuberculosis do not match up with the molecular diagnosis results [5]. It is obvious that supporting the fieldwork with laboratory work by using molecular methods like the spoligotyping method, which was used in this study together with the conventional methods applied in the tuberculosis diagnosis, will make important contributions to the eradication efforts of tuberculosis.

Sensitivity period of the IDDT, which is used for the diagnosis of tuberculosis in livestock, is short and reaction may not be observed when the infection source disappears in the herd. While a negative IDDT does not show that the disease does not exist in places where the tuberculosis incidence is high, a positive IDDT does not mean that the disease always exists [19]. Infection caused by the *M. tuberculosis* must be considered if the animal in the herd, which had a negative tuberculin dermal test before, is young in the first positive reaction determined with the tuberculin dermal test and if the farm staff has tuberculosis.

Ocepec Matjaz et al.[20] reported in the screening, which they made in a facility consisting of 78 animals in Slovenia, that they did not come across granulomatous lesions pointing at tuberculosis in 3 animals after the slaughtering, which they assessed as strongly positive with the IDDT, but reproduction was seen following the 28th day in the inoculations of the LJ medium belonging to a sample from the mediastinal and portal lymph nodes of a 2-year-old cow, however, there was no reproduction in the MGIT, stonebrink medium and Middle Brook 7H10 medium despite the 8-week incubation, and the isolate was *M. tuberculosis* as a result of the biochemical tests conducted with the colonies they defined in the LJ medium. It should not be forgotten that the cattle can be a potential reservoir in terms of not only *M. bovis*, but also

Table 1. *Spoligotyping pattern of 13 M. tuberculosis isolates*
Tablo 1. *13 M. tuberculosis izolatının spoligotyping paterni*

Strain Number(N)	Octal Code	Spoligotyping	Spoligotyping Family	
			SpoIDB4	
13	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■■	777777777760771	T1	SIT53

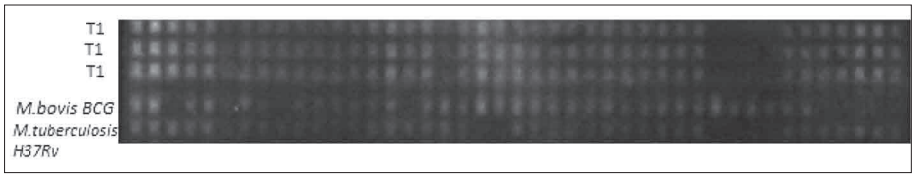

Fig 1. Spoligotyping image of 13 *M. tuberculosis* isolates and control strains

Şekil 1. 13 *M.tuberculosis* izolatının ve kontrol suşların spoligotyping görüntüsü

M. tuberculosis in places where the tuberculosis incidence is high in humans and animals.

In another study including the analysis of 768 samples, which were taken from the cattle with the suspicion of tuberculosis after the slaughtering in Northern India, 54 MTC isolates were determined and it was shown in the biochemical tests conducted on the isolated MTC strains that 14 of the isolates were *M. tuberculosis* [5]. The fact that 13 MTC isolates isolated from 95 cattle with the detection of granulomatous lesions were determined as *M. tuberculosis* in this study resembles the results of Srivastava et al.[5]. Besides, this result reveals that the cattle tuberculosis resulting from the *M. tuberculosis* must also be taken into account in the determination of the tuberculosis eradication strategies, which will be applied in our country.

Researchers stated at the end of the study they conducted between 2005-2007 that the cattle-isolates, which were among the 19 isolates isolated from the animals, were included in the T1 family (SIT53) by reporting in the spoligotyping of the 74 tuberculosis agents isolated from humans, who did cattle business with the animals slaughtered in the southwest of Nigeria and had the diagnosis of tuberculosis, that 32 agents were *M. tuberculosis*. The fact that all the *M. tuberculosis* isolates obtained from the cattle with granulomatous lesion detection after the slaughtering belonged to the T1 family in this study matches up with the results of Jenkins et al.[21].

It is stated in the studies related to the molecular epidemiology of the *M. tuberculosis* belonging to the people in our country that the *M. tuberculosis* Beijing strains have started to be observed at growing rates [17,22,23]. With the spoligotyping method, Zozio et al.[24] stated in their study, where they assessed 245 clinical *M. tuberculosis* isolates from Ankara and Malatya regions, that 206 isolates gathered in 33 clusters and 39 isolates were in specific clusters with single members, the LAM7 TUR family constituted the biggest cluster by 21% and the T1 family followed it with the rate of 16.3% and the Haarlem 3 family with 5.3%. Durmaz et al.[23] reported in the spoligotyping they conducted with 145 human MTC isolates in Malatya that LAM7-TUR was the most common pattern (23.96%) and the T1 family (SIT53) was the second most common pattern (22.5%) in Malatya. The fact that the T1 family (SIT53), which was defined as the second density by the researchers in the molecular typing of the *M. tuberculosis* isolates isolated from the tuberculosis cases, included all the *M. tuberculosis* isolates isolated in this study puts forth the necessity to definitely consider the cattle for presenting the transmission dynamics of the tuberculosis seen in humans.

This study has a critical and important role in the control programs that will be applied in our region due to the fact that it is the first molecular epidemiological study conducted on the *M. tuberculosis* infection in the cattle of our region.

Consequently, the results obtained through this study showed that *M. tuberculosis*, which leads to tuberculosis in humans, could be transmitted from humans to animals and from animals to humans again, animals may have significant roles in the transmission chain of the *M. tuberculosis*, and researching the human and animal-origin *M. tuberculosis* isolates with molecular epidemiology-based methods such as spoligotyping might give useful information about determining the ways of transmission of tuberculosis in the development of struggle strategies against tuberculosis.

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Research Article

Antibiotic Susceptibility and Molecular Identification of Antibiotic Resistance Genes of Staphylococci Isolated from Bovine Mastitis in Algeria

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Abstract

The study aimed to investigate the phenotypic and genotypic identification of *in vitro* antimicrobial susceptibility of 21 Staphylococci (10 *Staphylococcus aureus* and 11 Coagulase Negative Staphylococci) isolated from bovine mastitis to 12 antimicrobial drugs frequently using in veterinary medicine in Algeria. Isolates of staphylococci from bovine mastitis were tested for antibiotics with disc-diffusion method according to the National Committee for Clinical Laboratory Standards guidelines in the Mueller-Hinton agar, and resistant genes *mecA, blaZ, aac-aph, ermA, ermC, tetK* and *tetM* were detected by PCR. Staphylococci isolates showed high resistance to penicillin (95.23%), oxacillin (80.95%), clindamycine (80.95%), and erythromycin (76.19%) but, no resistance in all these strains was detected for gentamicin. Among 21 isolates of Staphylococci, 20 were found to be methicillin and multidrug resistant. Multidrug resistant strains exhibited several antibiogram patterns (antibiotic I to XIII). The distribution of antibiotic-resistant genes was *mecA* (100%) and *tetM* (100) followed by *blaZ* (42.85%). In the present study, the significant determination was the high prevalence of methicillin-resistant Staphylococci, which were resistant to multiple antibiotics. The finding of methicillin-resistant staphylococci from bovine mastitis is the first report in Algeria and revealed the status of resistant isolates in herd that might be helpful in treatment, controlling of resistant strains and for deciding culling of cows.

Keywords: *Antimicrobial susceptibility, Bovine mastitis, Methicillin-resistant staphylococci, Resistance genes*

Cezayir'de İnek Mastitislerinden İzole Edilen Stafilokokların Antibiyotik Direncinin Fenotipik ve Moleküler Yöntemlerle Belirlenmesi

Özet

Bu çalışma, Cezayir'de süt sığırlarındaki mastitis vakalarından izole edilen 21 stafilokok (10 *Staphylococcus aureus* ve 11 Koagulaz Negatif Stafilokok) suşunun Cezayir'de veteriner sahada sıklıkla kullanılan 12 antibiyotiğe karşı *in vitro* fenotipik ve genotipik direncinin belirlenmesi amacıyla yapıldı. Stafilokok izolatları disk difüzyon yöntemiyle test edildi. *mecA, blaZ, aac-aph, ermA, ermC, tetK* ve *tetM* direnç genleri ise PCR ile araştırıldı. Stafilokok izolatları penisilin (%95.23), oksasilin (%80.95), klindamisin (%80.95) ve eritromisine (%76.19) karşı yüksek oranda dirençli bulundu. 21 stafilokok izolatından 20 tanesinin metisilin dirençli ve çoklu antibiyotik direncine sahip olduğu belirlendi. Çoklu antibiyotik direncine sahip suşların bir çok antibiyotiğe karşı direnç paterni belirlendi antibiyotik I-XIII). Antibiyotik direnç genlerinin oranları ise *mecA* (%100), *tetM* (%100), *blaZ* (%42.85) şeklinde gerçekleşti. Bu çalışmada çoklu antibiyotik direncine de sahip metisilin dirençli stafilokokların yüksek prevalansı dikkat çekici idi. Bu çalışma Cezayir'de sığır mastitislerinden izole edilen stafilokoklarda metisilin direncini ortaya koyan ilk çalışmadır. Bu direncin ortaya konulması sürü bazında hastalığın tedavi, kontrol ve mastitis nedeniyle sürüden ayrılacak hayvanlar için karar verilmesine yardımcı olabilir.

Anahtar sözcükler: *Antibiyotik direnci, Sığır mastitisi, Metisilin dirençli Stafilokoklar, Direnç genleri*

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INTRODUCTION

Mastitis or the inflammation of mammary gland has been recognized as a complex and the most costly disease in dairy herds [1,2]. It imposes serious economic losses for the farmers and the dairy industry [3-5]. Among the various pathogens isolated as causative agents of bovine mastitis, *Staphylococcus aureus* is a predominant etiological agent of both subclinical and clinical forms of mastitis [6-8]. Coagulase Negative Staphylococci (CNS) have traditionally been considered as minor pathogens but, during the last years, their importance has clearly increased and they have become the predominant pathogens isolated from subclinical mastitis in several countries [9-12]. These bacteria can cause mainly subclinical mastitis [6], but some authors reported high percentage of clinical cases evoked by CNS [13,14]. The disease is the most frequent reason for the use of antimicrobial agents on dairy farms [15]. In numerous locations worldwide, cure rates of staphylococci infections are poor after antibiotic treatment [16-18]. In addition, multi-antimicrobial resistance was often seen in staphylococci [19]. In fact, the main reason of low efficacy of antibiotic treatment of staphylococcal mastitis is among others the resistance of bacteria. On the other hand, during the past decade, bacteria that cause human diseases have developed resistance to many of the antibiotics commonly used for treatment. Furthermore, the number and proportion of MRS (Methicillin-Resistant Staphylococci) infections in different countries has increased. Similar results were reported for many countries in the world [6,20-30] but, in Algeria a little information was available on diversity of bovine staphylococcal mastitis isolates and their anti-bacterial resistance, because this problem is not well investigated before. Furthermore, there is dearth of information on MRS from food products including milk in Algeria.

The aim of the present study was to identify and determine the *in vitro* activity of 12 different antimicrobial drugs against staphylococci isolated from bovine mastitis and identify the antibiotic resistance genes by PCR.

MATERIAL and METHODS

Sample Collection and Microbiological Analysis

The antibiotic susceptibility test was carried out on 21 staphylococcal strains isolated from bovine mastitis during the years 2011-2013 in Algeria. Before sampling the teat ends were cleaned with alcohol swabs and allowed to dry. The first few streams were discarded and then 5 ml of secretion was collected in sterile tubes. Samples were immediately transported to laboratory by cooled container. Bacteriological examinations were performed according to the commonly accepted principles [31]. Briefly, from each milk sample, 0.1 ml was plated on Columbia Agar medium (Merck, Germany), containing 5% sheep blood, and incubated at 37°C for 48 h. The isolates were identified by conventional methods, including Gram staining, colony morphology, haemolysis, catalase and coagulase tests and anaerobic fermentation of mannitol. All the tests were performed as described by Koneman et al.[32]. The identification of the isolates was confirmed subsequently by PCR. To make PCR, all isolates were stored at -20°C in trypticase soy broth containing 20% of glycerol. Prior to the testing, the isolates were twice serially cultured on columbia agar medium, containing 5% of sheep blood, for 24 h at 37°C under aerobic conditions.

Antibiotic Susceptibility Test

Ten colonies from the Columbia blood agar medium, incubated at 37°C for 18 h, were suspended in 2 ml of sterile saline to a density approximately equal to McFarland Opacity Standard No. 0.5. A dry cotton wool swab was placed in the suspension and excess liquid was expressed against the inside of the tube. The bacterial suspension was inoculated onto Mueller-Hinton agar (Merck, Germany) with the swab in such a way that the whole surface of the agar was covered. The antibiotic disks, containing the antibiotics were dispensed on the surface of the medium and incubated aerobically at 37°C for 18 h.

Antimicrobial sensitivity was tested by the disk diffusion method on Mueller Hinton Agar and performed according to National Committee for Clinical Laboratory Standards guidelines (NCCLS) [33]. The following antibacterial agents were used: penicillin G (P) (6 µg, Oxoid), cefoxitin (FOX) (30 µg, Oxoid), amoxicillin + clavulanic acid (AMC) (10 µg, Oxoid), enrofloxacin (ENR) (5 µg, Oxoid), vancomycin (VA) (30 µg, Oxoid), trimethoprim-sulfamethoxazole (SXT) (25 µg, Oxoid), clindamycin (CM) (2 µg, Oxoid), gentamicin (GM) (10 µg, Oxoid), tetracycline (TE) (30 µg, Oxoid), neomycin (N) (30 µg, Oxoid), and erythromycin (E) (15 µg, Oxoid). The results were recorded as resistant, intermediate or susceptible by the measurement of the inhibition zone diameter according to the interpretive standards of NCCLS [33]. All identified Staphylococci isolates were tested for phenotypic methicillin resistance by antibiotic disc diffusion susceptibility test with 1 µg oxacillin (OX) (Oxoid) and 30 µg cefoxitin discs. *S. aureus* ATCC 25923 strain was used as positive control. Resistance of Staphylococci isolates to three or more classes of antibiotics was considered as multidrug-resistance [34].

Genomic DNA Extraction

For nucleic acid isolation, isolates were activated on tripticase soya agar (bioMérieux, France). After overnight incubation at 37°C, single colony for each strain was resuspended on 500 µl of sterile phosphate buffer saline (PBS) (pH: 7.2). Bacterial cells were harvested by centrifugation at 3.000 × g for 10 min, the cell pellet was re-suspended in 350 µl TE buffer [10 mM tris chloride, 1 mM EDTA (pH 8.0)] with 100 µg of lysostaphin (Sigma, USA) per ml, and incubated at 37°C for 1 h. Each tube was vortexed

once every 15 min. Then, 350 µl 10% SDS with 100 µg of proteinase- K (Vivantis Technologies, Malaysia) per ml, and incubated at 37°C for 2 h. Each tube was vortexed once every 15 min. The phenol/chloroform extraction method was used for nucleic acid extraction according to Sambrook and Russel [35].The DNA precipitate was dissolved in 100 µl of TE buffer [10 mM Tris chloride-1 mM EDTA (pH 8.0)], and stored at -20°C until processing.

PCR Analysis

Simplex PCR technique was used of each gene. Properties of primers used in this study are reported in *Table 1*.

All amplification reactions were prepared in a 25 µl volume containing: 10 mM Tris/ HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 200 mM each dNTPs, 10 pmol oligonucleotide primer, 1 U Taq polymerase and 2 µl template DNA.

A pre-PCR step at 95°C for 5 min was applied. A total of 35 cycles were run at the following conditions: denaturation at 95°C for 30 sec, annealing (primer specific temperatures at *Table 1*) for 60 sec, and extension at 72°C for 60 sec. The reaction was achieved with a final extension at 72°C for 7 min. PCR products were checked using 1.5% agarose gel with 0.125 mg/l ethidium bromide. Only clear, unambiguous and reproducible bands were recorded.

Legal Permission-Ethics Committee Details

The name of institute approves the necessary ethical commission report: Laboratory of biotechnology related to animal breeding, University Saad Dahleb, Blida, Algeria.

The serial number of the approval in the material and methods section: MSRV

Statistical Analysis

The results of the phenotypic analyses of the various strains were expressed as frequencies or probabilities of observing a positive result for each performed test in a given bacterium.

Differences in frequencies of *in vitro* resistance to antimicrobials as β-lactams were determined by Pearson's chi-square test to study the possible relationship between β-lactam resistance genes and others and phenotypic resistance to antimicrobials. Value of $P<0.05$ was considered significant.

RESULTS

The antimicrobial susceptibility results of isolates of Staphylococci are summarized in *Table 2, 3,* and *4,* respectively.

The sensitivity and resistance of the isolated strains were different depending on the antibiotic tested. Regarding to the agar diffusion test, a total of 17 isolates have shown to have a methicillin resistant phenotype (i.e., resistance to oxacillin and cefoxitin). Staphylococci were resistant mostly to penicillin (95.23%), clindamycin (80.95%), vancomycine (76.19%), erythromycin (76.19%) and Amoxicillin + Clavulanic Acid (66.67%). Whereas strains were most sensitive to neomycin and gentamycin (100%), in average. The next effective antibiotics were

Table 1. *Primers and properties used in the study*
Tablo 1. *Çalışmada kullanılan primerler ve özellikleri*

Gene	Primer Name	Primer Sequences	Annealingng Temperatur (°C)	Amplicon Size (bp)	Reference
Staphylococcus spp.	Staph294-318	5′-GCCGGTGGAGTAACCTTTTAGGAGC-3′	55	106 bp	[36]
	Staph 1522-1540	5′-AGGAGGTGATCCAACCGCA-3′			
S. aureus	Sau 327	5′-GGA CGA CAT TAG ACG AAT CA-3′	64	1318 bp	[37]
	Sau 1645	5′-CGG GCA CCT ATT TTC TAT CT-3′			
Oxacillin/ Penicillin	mecA1	5′-CCTAGTAAAGCTCCGGAA-3′	54	314 bp	[38]
	mecA2	5′-CTAGTCCATTCGGTCCA-3′			
Penicillin	blaZ1	5′-ACTTCAACACCTGCTGCTTTC-3′	56	173 bp	[39]
	blaZ2	5′-TGACCACTTTTATCAGCAACC-3′			
Gentamicin	aacA-aphD 1	5′-TAA TCC AAG AGC AAT AAG GGC-3′	54	227 bp	[40]
	aacA-aphD 2	5′-GCC ACA CTA TCA TAA CCA CTA-3′			
Erythromycin	ermA 1	5′-AAG CGG TAA ACC CCT CTG A-3′	54	190 bp	[40]
	ermA 2	5′-TTC GCA AAT CCC TTC TCA AC-3′			
Erythromycin	ermC 1	5′-AAT CGT CAA TTC CTG CAT GT-3′	54	299 bp	[40]
	ermC 2	5′-TAA TCG TGG AAT ACG GGT TTG-3′			
Oxytetracyclin	tetK 1	5′-GTA GCG ACA ATA GGT AAT AGT-3′	54	360 bp	[40]
	tetK 2	5′-GTA GTG ACA ATA AAC CTC CTA-3′			
Oxytetracyclin	tetM 1	5′-AGT GGA GCG ATT ACA GAA-3′	54	158 bp	[40]
	tetM 2	5′-CAT ATG TCC TGG CGT GTC TA-3′			

Table 2. *Susceptibility to various antibiotics of staphylococci strains isolated from bovine mastitis*
Tablo 2. *Sığır mastitislerinden izole edilen Stafilokokların çeşitli antibiyotiklere karşı duyarlılıkları*

Antibiotics	Total Profile Break Points	Staphylococci (n= 21 isolates: 10 *S. aureus* and 11 CNS)					
		Sensitive		Resistance		Intermediate Sensitive	
		Number	%	Number	%	Number	%
P	≤ 28-29≥	1	04.76	20	95.23	0	0
OX	≤10-13 ≥	4	19.04	17	80.95	0	0
FOX	≤24-25≥	7	33.33	10	47.61	4	19.04
AMC	≤ 19-20≥	7	33.33	14	66.67	0	0
ENR	≤16-23≥	19	90.47	2	09.52	0	0
VA	≥15	5	23.80	16	76.19	0	0
SXT	≤10-16 ≥	17	80.95	1	04.76	3	14.28
CM	≤14-17≥	4	19.04	17	80.95	0	0
GM	≤12-15≥	21	100	0	0	0	0
TE	≤14-19 ≥	13	61.90	8	38.09	0	0
N	≤ 13-18≥	20	95.23	1	04.76	0	0
E	≤ 13-23≥	2	09.52	16	76.19	3	14.28

Table 3. *Antibiotic resistance patterns of 21 staphylococci isolates*
Tablo 3. *21 adet Stafilokok izolatının antibiyotik direnç profilleri*

Pattern	Resistance Profile 1	Resistant Isolates	
		Number	%
1	P, TE	1	04.76
2	ENR, TE	1	04.76
3	P, OX, AMC, VA, CM, TE	2	09.52
4	P, OX, FOX, AMC, VA, CM, E	7	33.34
5	P, OX, FOX, AMC, VA, CM, E	1	04.76
6	P, OX, FOX, AMC, ENR, VA, CM, TE, E	1	04.76
7	P, OX, TE	1	04.76
8	P, VA, SXT, CM, E	1	04.76
9	P, OX, VA, CM, TE, N, E	1	04.76
10	P, OX, CM, E	1	04.76
11	P, OX, FOX, AMC, VA, CM	1	04.76
12	P, OX, AMC, VA, CM, E	1	04.76
13	P, OX, AMC, VA, CM, TE, E	1	04.76
14	P	1	04.76

enrofloxacin (90.47%), Trimethoprim-Sulfamethoxazole (80.95%) and tetracycline (61.90%) according to *in vitro* tests. Antibiogram results for the isolates were classified according to pattern of resistance (types I to IX) *(Table 3)*. The most frequent pattern of resistance was type VII, which was found in 10 isolates. Antibiogram pattern IX represents resistance to nine of the drugs that are most commonly used for treatment of mastitis in Algeria.

PCR assay was made to determine whether the multidrug resistant MRS isolates from Algeria locations were genetically clustered *(Table 4)*. This table has shown in party the phenomenon of multiple resistances to three or more antibiotics, none being resistant to only one or two antibiotics. The percentage of multiple resistance strains was 87.71% (18/21).

Antimicrobial susceptibility testing reported a high resistance of Staphylococci strains to antimicrobial agents which was confirmed by PCR by the presence of *mecA* gene. Our results shows presence of *mecA* gene for all Staphylococci strains which were phenotypically resistant to cefoxitin and/or oxacillin.

In spite of the presence of some phenotypic resistance against erythromycin, no genotypic resistance genes were detected following researching *erm (C)* and *erm (A)*. The *blaZ* gene was detected in 42.85% (9/21) of isolates.

Results presented in *Fig. 1* show the presence of *mecA* gene from extracted DNA of all *S. aureus* and CNS strains tested; this result confirmed the antibiogram results for susceptibility to methicillin.

DISCUSSION

Detection of the antibiotic resistance is important for controlling and treatment of the bacterial disease. The present study was carried out to investigate the antimicrobial susceptibilities and resistance genes in staphylococci isolated from lactating cows with clinical or subclinical mastitis cases in Algeria.

The rate of penicillin resistance (100%) detected in this study is much higher than those reported in other countries such as Korea (52.9%), Switzerland (31%), Finland (32%), USA (22.1%) [41] and Brazil [42]. Strains isolated from mastitis cases were resistant to some antibiotics commonly used in treatment of cow mastitis in Algeria. Almost the same results were reported by other authors [28,29], especially on resistance to penicillin and oxacillin. The existence of antibiotic-resistant in a selective area might be due the frequent and continuing use of the same antimicrobials [18]. In fact, the antibiotics such as penicillin-G, oxacillin, streptomycin, ampicillin, amoxycillin, cloxacillin

Table 4. *Correlation between phenotypic antibiotic resistance and PCR results*
Tablo 4. *Fenotipik antibiyotik direnci ile PCR sonuçları arasındaki bağlantı*

Strains	Resistance Phenotype	Presence of Fragment						
		BlaZ	mecA	aacA-aphD	Erm (A)	Erm (C)	tetK	TetM
1	P, TE	-	+	-	-	-	-	+
2	P, AMC, VA, CM, TE, E	-	+	-	-	-	-	+
3	OX, P, FOX, AMC, VA, CM, E	-	+	-	-	-	-	+
4	OX, P, FOX, AMC, VA, CM, E	-	+	-	-	-	-	+
5	OX P, FOX, AMC, VA, CM	-	+	-	-	-	-	+
6	OX, P, FOX, AMC, ENRO, VA, CM, TE, E	+	+	-	-	-	-	+
7	OX, P, FOX, AMC, VA, CM, E	-	+	-	-	-	-	+
8	OX, P, FOX, AMC, VA, CM, E	+	+	-	-	-	-	+
9	OX, P, TE	+	+	-	-	-	-	+
10	OX, P, VA, SXT, CM, E	-	+	-	-	-	-	+
11	P, VA, CM, TE, N, E	-	+	-	-	-	-	+
12	OX, P, FOX, AMC, VA, CM, TE, E	-	+	-	-	-	-	+
13	OX, P	+	+	-	-	-	-	+
14	P, CM, E	-	+	-	-	-	-	+
15	OX, P, FOX, AMC, VA, CM	+	+	-	-	-	-	+
16	OX, P, AMC, VA, CM, E	-	+	-	-	-	-	+
17	OX, P, AMC, VA, CM, TE, E	+	+	-	-	-	-	+
18	OX, P, AMC, VA, CM, E	+	+	-	-	-	-	+
19	OX, P, FOX, AMC, VA, CM, E	+	+	-	-	-	-	+
20	OX, P, FOX, AMC, VA, CM, E	+	+	-	-	-	-	+
21	OX, ENR, TE, E	-	+	-	-	-	-	+

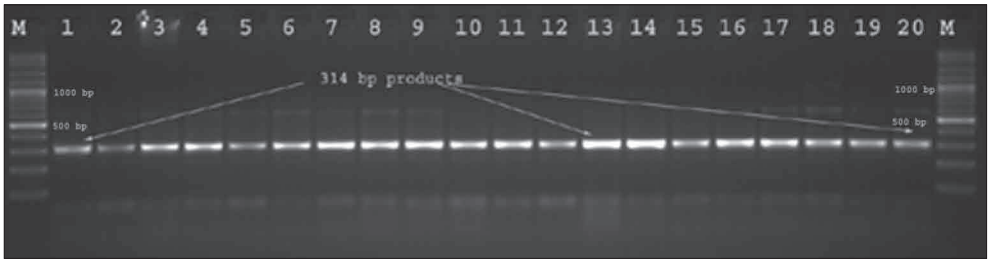

Fig 1. Agarose gel electrophoresis of *mecA* gene targeted PCR. **M:** Marker, 100 bp plus (brand). **Lines 1-35:** Amplified products of *mecA* specific PCR (Bands 314 bp)

Şekil 1. *MecA* gen hedefli PCR'nin agaroz jel elektroforezi. **M:** Marker, 100 bp plus (brand). **Kuyucuklar 1-35:** *MecA* spesifik PCR amplifiye ürünleri (314 bp)

and tetracycline are frequently used in veterinary clinics of herd selected for this study.

In the study, incidence of vancomycin-resistant isolates of *S. aureus* was observed with frequency of 76.2%. Elsewhere, none of the studies have been reported the vancomycin-resistant *S. aureus* in bovine mastitis. On the other hand, vancomycin-resistant enterococci have been reported in cattle mastitis [43].

Staphylococci examined in this study were more resistant to antibiotics than bacteria isolated earlier in Algeria [44]. But, the *in vitro* resistance to antibiotics of bacteria isolated in the same farm can change from one year to the next one [27].

The majority of authors have noted the increase in the resistance to antibiotics of staphylococci isolated from mastitis [6,19-30]. Therefore, it seems that our results are in accordance with them. The most important factor affecting the cure rates from clinical Staphylococcal mastitis was the capacity of the strain to produce β-lactamase [16]. Apart from this, Watts and Salmon [26] highlight the need to identify methicillin resistant *S. aureus* (MRSA) accurately, because these strains are resistant to all compounds currently approved for treatment of bovine mastitis. On the contrary, de Oliveira et al.[45] found that overall level of resistance was generally low to all antimicrobial agents that are currently commercially available to treat bovine mastitis.

In literature, a few studies have reported the occurrence of MRSA from bovine mastitis and proportion of resisting isolates was low [18,46,47]. In this study, 100% of isolates of *S. aureus* and CNS were methicillin-resistant. The isolates appeared to demonstrate privileged expression of *mecA* genes or production of methicillinase or appear to overproducing beta-lactamase [18,47]. The phenotypic expression of resistance could vary due to growth conditions or might be limitations in detection in microbiological methods [48]. Because of prolonged treatments with same antibiotics frequently is noticed the emergence of resistant variants of bacterial strains. In addition, it is well reported that emergence of drug resistance is the consequence of the inappropriate use of antimicrobials [49].

Analyzing the antibiograms' results of staphylococci strains it was observed that in the isolates was revealed the phenomenon of multiple resistances to three or more antibiotics, none being resistant to only one or two antibiotics. In the present study, the percentage of multiple resistance strains was 87.71% (18/21). Our finding that 18 of the isolates tested were multidrug resistant MRS isolates indicates that antibiotic resistance has emerged in dairy cattle in Algeria. In literature, multiple antimicrobial resistance is defined as resistance to three [34], four or more antimicrobials [50]. Furthermore, Waage et al.[51] reported that *S. aureus* has developed multidrug resistance with a wide variation from herd to herd. Memon et al.[52] reported that 100% of *S. aureus* isolates were multidrug resistant. While, comparatively a less percentage (52%) of isolates were also reported as multi drug resistant in Ethiopia [53]. Bardiau et al.[54] reported that all isolates were resistant to at least three antibiotics, and two-thirds of the strains (58%) were resistant to at least six antibiotics. As multiple antibiotic resistance, high resistance rates presenting was observed in study conducted by Tel et al.[55]. This is in accordance with our study. Using various antibiotics can create selection pressure, ultimately resulting in the development of antibiotic resistance [56].

Methicillin resistance was found in high percentage of *S. aureus* and CNS isolates (100%) which is greater than reported in Korea and India [18,44]. Furthermore, there was *mecA* gene in all isolates. The *S. aureus* exhibit resistance to methicillin was first reported in 1960, by the time MRSA gradually developed multiple resistances and became a source of causing serious nosocomial infections, worldwide [57].

In mastitis, most studies report a low prevalence of MRSA [46,58] but in Belgium, an unpredictably high prevalence of mastitis-associated MRSA was reported [59]. Besides to their resistance to all types of beta-lactam antibiotics, MRSA strains illustrate resistance to other antimicrobial agents, also used for treatment and prevention of mastitis [60]. Susceptibility tests showed here a wide variety of nine different resistance patterns among the isolates. In this study, all isolates except one were resistant to at least three antibiotics, and two-thirds of the isolates were resistant to at least six of the twelve antibiotics. In addition to oxacillin, all of our MRS isolates were resistant to penicillin and tetracycline. This result is in accordance with other studies [59,61]. However, these strains showed more resistance to erythromycin and clindamycin than previously reported [59,61,62]. Furthermore, this high resistance rate to some antibiotics can be explained by their frequent use in veterinary practice in Algeria. Resistance against beta-lactams and presence of *blaZ* gene in our isolates is in agreement with the result of Green and Bradley [63]. But Haveri et al.[64] found that the *blaZ* gene detected in majority of isolates and *mecA* was not found in any isolates, these findings are consistent with previous report. Detection of genes of old generation antibiotics (gentamycin and tetracycline) is also necessary. Also, a number of isolates revealed *tetK* and *tetM* genes and results are in concurrence with phenotypic observations. All isolates were found sensitive to gentamycin phenotypically and also negative to *aac-aph* gene. Following researching *aac-aph* gene from isolates which 100% phenotypically sensitive to gentamycin, no *aac-aph* gene was found. So, the *aacA-D* gene has been reported less prevalent in the mastitis isolates and results of the present study are in disagreement with a previous report [42]. Furthermore, occurrence of tetracycline-resistant genes among the bovine staphylococci isolates has been previously observed [65]. Tetracycline resistance encoding gene *tetM* was present in 100% of isolates which is higher than previously detected in china by Gao et al.[66]. The proportion of the tetracycline genes is almost similar with the previous reports [65]. Li et al.[67] reported that there is a common use of penicillin, tetracycline and erythromycin for the treatment of mastitis in China. Moreover, 76.19% of isolates were phenotypically resistant to erythromycin but all of them were negative for *ermA* and *ermC* genes. This finding is in disagreement with those from in northern China [66]. Acquisition of resistance in *S. aureus* isolates attributed to mutation in gene or due to exchange of genetic material between organisms, since resistance genes carrying mobile genetic elements of *S. aureus* have exceedingly been explored [68].

These results indicate that, these isolates are resistant at high rates to the beta-lactam antibiotics which are intensively used in the control and treatment of mastitis without any antibiogram test in Algeria. The results of present study showed some similarities with previous studies to the same antibiotics [47,69]. The presence of antibiotic-resistant genes and similar antibiotic-resistant patterns among isolates of staphylococci indicates possible diffusing or spreading of isolates between animals.

Almost of staphylococcal strains tested had an increased susceptibility to neomycin and gentamicin (100%) and all of them are resistant to penicillin (100%). The data on antimicrobial susceptibility can help determine the choice

of empirical initial treatment. Our investigations revealed high prevalence of different antibiotic-resistant genes (especially *mecA* and *tetM*) among isolates.

This study reports high prevalence of MRS isolates with having *mecA* gene. That is to say a significant observation was prevalence of methicillin-resistant isolates in the herd. These findings can be considered in designing strategic plans for treatment, prevention and control of Staphylococcal mastitis in Algeria. Occurrence of such isolates, among the mastitis cases needs attention of veterinarians and managers of herds. The findings might be helpful to control, transfer or dissemination of pathogenic strains, segregation of cows for reduction of mastitis and can be applied for treatment policies and antimicrobials strategies. The present study demonstrated that the existence of alarming level of resistance of frequently isolated mastitis agents to commonly used against antimicrobial agents in the farms in Algeria. Consequently, it is very important to implement a systematic application of an *in vitro* antibiotic susceptibility test prior to the use of antibiotics in both treatment and prevention of mastitis. The increasing occurrence of MRSA and MR-CNS should be under consideration from the point of view of antibiotic selection for mastitis treatment and prevention, especially if the possibility exists of the resistance transfer in or between bacteria. The results of this study can be used as a baseline for further investigations.

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Research Article

Effect of Different Housing Systems on Production and Blood Profile of Slow-Growing Broilers [1] [2]

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Abstract

Present study was conducted to investigate the effects of deep litter system, free-range with fixed housing system and free-range with portable housing system in wheat stubble on performance, internal organ weights, blood parameters and tonic immobility of slow-growing broilers. A total of 270 Hubbard Isa Red-JA chicks were used in experiments. The experiment lasted for 61 days. Results showed that the body weight on 21st, 28th, 35th and 42th days of birds from free-range with portable housing system was significantly lower than of those kept in deep litter and free range with fixed housing system. The viability of broilers in free-range with portable housing system was lower than broilers in free-range with fixed housing and deep litter system in the days of 14-42 and 14-61, while there were not any differences between housing systems in days of 43-61. Housing systems had no significant effect on feed conversion ratio and internal organ weight. There were no significant differences in tonic immobility durations of housing systems and some blood parameters, except hematocrit values. In conclusion, the body weight in free-range with portable housing system was significantly lower until 42nd day of the experiment, but this difference was disappeared during the rest of rearing period. It could be concluded that rural producer can be involved in broiler production after wheat harvesting.

Keywords: *Slow-growing broiler, Blood parameters, Free Range Housing system, Growth performance*

Farklı Yetiştirme Sistemlerinin Yavaş Gelişen Etlik Piliçlerde Üretim ve Kan Profiline Etkisi

Özet

Bu çalışma; altlıklı yer sistemi, buğday anızında sabit kümesli serbest yetiştirme ve buğday anızında mobil kümesli serbest yetiştirme sistemlerinin yavaş gelişen etlik piliçlerin performansına, iç organ ağırlıklarına, kan parametrelerine ve tonik immobilite sürelerine etkisini araştırmak amacıyla yapılmıştır. Çalışmada toplam 270 adet Isa Red-JA pilici kullanılmıştır. Çalışma 61 gün sonunda bitirilmiştir. Araştırma sonuçlarına göre mobil kümesli serbest yetiştirme sistemindeki piliçlerin 21. 28. 35. ve 42. gün canlı ağırlıkları istatistiki olarak diğer sistemlerden düşük bulunmuştur. Mobil kümesli serbest yetiştirme sistemindeki piliçlerin yaşama gücü 14-42 ve 14-61 günlerden diğer sistemlerden düşük çıkarken 43-61 günlerde herhangi bir istatistiki farklılık gözlenmemiştir. Yetiştirme sistemi yemden yararlanma oranı ve iç organ ağırlıklarını etkilememiştir. Yetiştirme sistemleri arasında tonik immobilite süresi ve hematokrit değer dışında kan parametreleri açısından istatistiki farklılık gözlenmemiştir. Sonuç olarak; mobil kümesli serbest yetiştirme sistemindeki etlik piliçlerin canlı ağırlıkları 42. güne kadar düşük olmuş fakat bu farklılık kalan sürede ortadan kalkmıştır. Kırsal alanda buğday hasadı sonrası anızda etlik piliç yetiştiriciliğinin yapılabileceği söylenebilir.

Anahtar sözcükler: *Yavaş gelişen etlik piliç, Kan parametreleri, Serbest yetiştirme sistemi, Büyüme performansı*

INTRODUCTION

Significant improvements have been achieved in poultry performance and yield through the developments in breeding, feeding, growing techniques and disease prevention [1,2]. This has led to reduction in chicken prices making its availability to everyone. However, sudden death syndrome and leg problems because of rapid growth on of

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respiratory and circulatory systems of broilers; and due to intensive housing systems attracted the reactions of highly sensitive publics of countries concerning about animal comfort and welfare [3]. Therefore, beside the performance of broilers, tonic immobility duration, blood parameters (heterophil/lymphocyte ratio) and internal organ weights are taken into consideration as the indicators of animal comfort and welfare [4-9]. Developments in animal rights and welfare bring important criticism about entire housing systems, including litter bed system, currently implemented in intensive poultry facilities [10,11]. Therefore, alternative housing systems taking normal behaviors and animal welfare into consideration have recently been emphasized. Free-range system is one of such alternative housing systems [10,12,13]. Free-range system allows chicken to stay on ground away from artificial atmospheres and to range at open spaces. Chickens commonly range over trashing grounds with left-over seeds. Such seed wastes cause significant losses sometimes reaching up to 20%. Wheat seed loss is around 3-4% in Turkey [14]. The demand for poultry grown rapidly and reached to slaughtering weight in a short time is decreasing in developed countries. On the other hand, organic and natural poultry housing systems have increased substantially as a result of the greater demand to natural broiler meat production [15].

There has been insufficient information on free-range with portable housing system after wheat harvest over wheat stubble lands. Accordingly, the objectives of the study reported here in were twofold: firstly, to compare the three different growing systems as of free-range with portable housing system (FRPH) in wheat stubble, free-range with fixed housing system (FRFH) and deep litter bed system (DLB) with regard to animals and environment and secondly, to examine the influence of different housing systems on performance, internal organ weight, tonic immobility duration and blood parameters of slow-growing broilers.

MATERIALS and METHODS

Experimental Birds, Feeding and Housing Systems

Animal material of the experiments was supplied from a commercial facility within the scope of a project supported by Gaziosmanpasa University Scientific Research Projects Committee (2010/75). Before the initiation of experiment, required permissions were obtained from Gaziosmanpasa University Local Ethics Committee on Experimental Animals (No: 2009-HADYEK-032) also performed in accordance with the internationally accepted principles for laboratory animal use and care. The experiment was performed between 07.07.2010 and 05.09.2010 after harvest of wheat. A total of 270 slow-growing one day-old Hubbard Isa Red-JA broiler chicks were used in experiments. A battery operated light was used for portable housing unit. Incandescent light bulb in DLB and FRFH system, and LED bulb in FRPH system was used for 24-hours per day. In the research, first 2 week; in all systems, 2 watt/m^2, for 15-61 days; in systems of DLB and FRFH, 2 watt/m^2, in system of FRFH, 2 watt/m^2 and in system of FRPH, 1 watt/m^2 light density was used. A 24 h lighting was provided in all three systems. A hang-feeder and waterer were placed into each replication of experiment. 10 cm depth of wood shavings were used as bedding material in DLB, indoor areas of FRFH and FRPH systems. In FRPH system, it is applied after 15 decar harvesting of wheat.

A free feeding program was implemented as; 1st period (23.0 CP, 3000 Kcal/kg ME) chick feed during the days 1-10; 2^{nd} period (22.0% CP, 3000 Kcal/kg ME) broiler grower feed during the days 11-35; 3^{rd} period (20.0% CP, 3100 Kcal/kg ME) broiler developer feed for the days 36-53; 4^{th} period (18.0% CP, 3100 Kcal/kg ME) broiler finishing feed for the days 53-61.

Chicks were housed in litter bed ground system for the initial two weeks. The average live weights were determined and they were separated into treatments with 30 animals in each. Average chick weights were equal in each treatment.

Experiments were carried out in DLB, FRPH and FRFH systems of poultry units at Animal Science Department of Gaziosmanpaşa University Agricultural Faculty. Three replications were performed in each housing system and each replication had 30 birds. Therefore, 90 slow-growing broilers were used in each housing systems and a total of 270 animals were used in entire treatments. A space of 5.4 m^2 was allocated to each replication of DLB system; an indoor space of 5.4 m^2 and range space of 9.5 m^2 were allocated to each replication of FRFH; a portable housing space of 2.25 m^2 and a free-range space were allocated to each replications of FRPH system. The dimensions of portable housing unit are 1.8X1.25 m (LxW), with 1.9 m front height and 1.5 m rear height. There was natural ventilation system in each housing system. In FRFH and FRPH systems, birds were allowed to range between the hours 07:00-19:00 and they were housed indoor between the hours 19:00-07:00. Place of portable housings were replaced three times in a day.

Determination of Performance and Tonic Immobility of Birds

Birds were individually weighed weekly for determination of body weight. Weekly feed consumptions were also recorded regularly until the end of experiments and feed conversion ratios corresponding to feed consumption for unit increase in BW (g feed/g BWI) were calculated. Survival rates (%) of each replication were also calculated by recording number and day of deaths.

By the end of the 61 day period, 4 birds (2 male and 2 female) with a close weight to average were randomly selected from each replication. That sums 12 birds from

each housing system and 36 from the entire experiments. Initially, tonic immobility was assessed in selected birds. The test was conducted in a rectangular container with an open end. Tonic immobility was induced by inverting the hen on its side and applying manual restrain until the hen stops struggling. Then, the hen released after 15 sec. Tonic immobility was assumed for birds immobile and not righted itself within 10 sec of release. Tonic immobility duration was recorded from the moment the hen became immobile until the hen righted itself. The birds with a tonic immobility state after 5 trials are assumed as sensitive and graded with 0. Test period was limited to 10 min and tonic immobility duration of birds immobile and not righted within the 10 min was recorded as 600 sec [16].

Slaughtering Procedures, Non-Carcass Parts, Internal Organs and Assays of Blood Parameters

The birds were slaughtered without stunning under Turkish slaughter procedure by severing the throat and major blood vessels in the neck in local processing plant. Following the slaughter and blood release, wet defeathering and manual internal removal were performed. Relative weights (g/100 g BW) of heart, liver, spleen, abdominal fat and digestion system and length of small intestine (cm) were determined [17].

Blood samples were taken into vacuum tubes from *Vena cutaneae ulnaris* of selected birds. Serum was obtained by centrifuging blood samples at 3.000 rpm for 10 min. Serum samples were preserved at -20°C and immediately transferred to laboratory for analysis [18]. *In vitro* enzymatic colorimetric method was employed in glucoses, triglyceride and cholesterol measurements. Plasma protein levels were measured by a refractometer (Atago, SPR-N, Japan). Cyano methemoglobin and microhematocrit methods were respectively used for hemoglobin and hematocrit measurements [19]. May Grünwald-Giemsa method was employed to calculate leukocyte percents from stained frothily [6].

Statistical Analyses

Experiments were carried out in completely randomized design and SPSS [20] software was used for statistical analysis of the data. The differences between groups were determined by one-way ANOVA test. Duncan multiple range test was used to compare treatment means [21].

RESULTS

Effects of housing system on performance characteristics, internal organ weights and blood parameters and tonic immobility duration of slow-growing broilers are given in *Table 1, 2,* and *3,* respectively.

The FRPH system on 21st, 28th, 35th and 42nd days of body weight was lower significantly than those other systems (P<0.05). Body weight of housing systems on initial (14th

Table 1. *Effects of housing systems on performance of slow-growing broilers*
Tablo 1. *Yetiştirme sisteminin yavaş gelişen etlik piliç performansına etkisi*

Parameter	Free-range with Portable Housing	Free-range with Fixed Housing	Deep Litter Bed System	SEM	P
Body weight (BW), g					
Initial (14th day)	281.54	281.74	281.63	1.46	NS
21st day	489.87^{b}	520.83^{a}	529.94^{a}	2.92	*
28th day	785.26^{b}	832.57^{a}	843.34^{a}	5.27	*
35th day	1116.35^{b}	1194.54^{a}	1208.61^{a}	8.09	*
42nd day	1390.12^{b}	1508.10^{a}	1498.48^{a}	10.28	*
49th day	1776.84	1841.27	1832.44	13.59	NS
56th day	2136.49	2194.13	2178.44	16.59	NS
61st day	2375.00	2471.61	2427.21	19.59	NS
Feed conversion ratio, g feed /g weight gain					
14-42 day	1.87	1.80	1.80	0.00	NS
43-61 day	2.38	2.17	2.27	0.72	NS
14-61 day	2.08	1.98	2.05	0.03	NS
Viability, %					
14-42 day	86.67^{b}	100^{a}	100^{a}	3.68	**
43-61 day	93.89	94.44	94.44	1.99	NS
14-61 day	81.11^{b}	94.44^{a}	94.44^{a}	3.81	**

SEM: *Standard Error of the Mean;* ***a-b*** *different letters in the same line indicate significant difference between means;* ******* *P<0.05;* ******** *P<0.01;* ***NS:*** *non-significant (P>0.05)*

day), 49th, 56th and 61st days were ordered respectively from higher to lower as FRFH, DLB and FRPH system, however significant (P>0.05) differences were not observed among treatments. Effects of housing systems on FCR's (14-42, 43-61 and 14-61 days) were not significant (P>0.05). While the effect of housing systems on viability (%) of 14-42nd day and 14-61st day was significant (P<0.01), effects were not significant (P>0.05) on viability of 43-61st day *(Table 1)*.

Effects of housing systems on internal organ (digestive system, heart, gizzard, spleen, abdominal fat, liver) weights and length of small intestine of slow-growing broilers were insignificant (P>0.05) *(Table 2)*.

Lower blood cholesterol levels in FRPH system and lower blood triglyceride and glucose levels in FRFH system were observed the difference between housing systems were not significant (P>0.05). The difference between housing systems with regard to total protein content of plasma were not significant (P>0.05). The difference between housing systems with regard to white corpuscle (heterophil, eosinophile, basophile, lymphocyte, monocyte, Heterophil (H)/Lymphocyte (L)) ratios were not significant (P>0.05). Effect of housing systems on hematocrit values was significant (P<0.05).Tonic immobility durations of FRFH was higher than FRPH and DLB systems *(Table 3)* but there were no significant (P>0.05) difference between these systems *(Table 3)*.

DISCUSSION

Current findings about body weight do not comply with the results of studies [22-25] indicating lower body weights for free-range systems than litter bed systems and comply with the findings of studies [15,26,27] reporting insignificant effects of housing systems on live weights. Feed conversion ratio findings of this study are contrary

Table 2. *The effect of different housing systems on some internal organ weights in slow growing broilers*
Tablo 2. *Yavaş gelişen etlik piliçlerde farklı yetiştirme sistemlerinin bazı iç organ ağırlıkları üzerine etkisi*

Parameters	Free-range with Portable Housing	Free-range with Fixed Housing	Deep Litter Bed System	SEM	P
Internal organ weights, g/100 g BW					
Digestive system	6.34	6.20	5.99	0.08	NS
Hearth	0.50	0.46	0.45	0.01	NS
Gizzard	2.04	1.91	1.81	0.07	NS
Spleen	0.14	0.15	0.14	0.01	NS
Abdominal fat	3.01	2.95	3.06	0.12	NS
Liver	1.49	1.51	1.49	0.02	NS
Length of small intestine, cm	133.50	137.17	133.42	1.95	NS

SEM: *Standard error of the mean;* ***NS:*** *non-significant (P>0.05)*

Table 3. *The effect of housing system on blood parameters and tonic immobility duration in slow-growing broiler*
Tablo 3. *Yetiştirme sisteminin yavaş gelişen etlik piliçlerde kan parametreleri ve tonik immobilite süresi üzerine etkisi*

Parameters	Free-range with Portable Housing	Free-range with Fixed Housing	Deep Litter Bed System	SEM	P
Cholesterol, mg/dL	108.85	109.65	114.46	3.11	NS
Triglyceride, mg/dL	34.42	31.50	33.74	0.89	NS
Glucose, mg/dL	207.05	203.23	217.74	4.54	NS
Plasma protein, g/dL	3.47	3.66	3.64	0.06	NS
Hemoglobin, g/dL	9.31	9.82	9.60	0.20	NS
Hematocrit, %	29.75[a]	31.33[b]	31.50[b]	0.30	*
Heterophil, %	39.70	44.55	40.75	1.14	NS
Eosinophil, %	2.80	3.27	3.63	0.21	NS
Basophil, %	2.90	1.64	2.13	0.33	NS
Lymphocyte, %	50.30	45.91	48.75	1.16	NS
Monocyte, %	4.30	3.64	4.75	0.18	NS
H/L ratio	0.81	0.99	0.87	0.05	NS
Tonic immobility duration, s	112.92	163.83	137.00	21.11	NS

SEM: *Standard Error of the Mean;* ***NS:*** *non-significant (P>0.05);* ***a-b*** *different letters in the same line indicate significant difference between means; * P<0.05*

to finding of studies [15,22,25-28] reporting significant impacts of housing systems on feed conversion ratios and indicating lower ratios for free-range systems. However, insignificant differences between housing systems were similar to finding of Sekeroglu et al.[24]. Viability rate in FRPH were lower than the other systems. Such a low viability rate was mainly due to out-season extreme temperatures and predatory animals. The rate was lower than those of earlier studies [24,26-30].

Since spleen and liver weights can easily be determined at slaughter, these weights are commonly used as an indicator for immunity levels of poultry [7]. Spleen is a lymphoid organ and a weight decrease is observed in spleen [7,8] and a weight increase is observed in liver weights under stress conditions [31]. With regard to spleen and liver weights in present study, the differences between housing systems were not found significant. Insignificant impacts of housing systems on relative weights of heart, gizzard, digestive system, spleen and liver and length of small intestine were in compliance with the findings of Sekeroglu et al.[24]. Effect of housing systems on relative abdominal fat weight was not significant and FRFH system had lower values than the other systems. Such findings comply with the findings of Sekeroglu et al.[24]. However, current findings are different from the outcomes of the researches indicating significant impacts of housing system on relative abdominal fat weight and reporting lower weights for free-range systems [22,25].

It was reported that serum glucose, cholesterol and triglyceride levels in chicken vary between 130-260 mg/dl; 125-211 mg/dl and 36-211 mg/dl, respectively [32,33]. Güneş et al.[18] claimed that different housing systems had the effect on serum glucose levels, while other reported no such effect [34]. Our findings are similar to work that of Kumar et al.[34], who concluded that glucose levels were not affected by housing systems and glucose levels in groups was within the range of values reported [32]. The results agree with those initial studies [18,24,35,36] who found no significant effect of housing system on serum cholesterol. Besides, cholesterol levels in the current study were slightly lower than that of the range value reported by Altuntaş and Fidancı [32]. Serum triglyceride levels was found to be slightly lower than that of the range value determined by Masek et al.[33]. In addition, this study found no significant changes between housing systems on serum triglyceride levels and the results was in agreement with that in the literature [37]. In contrast, Sekeroglu et al.[24] observed a significant decrease in serum triglyceride concentration compared to conventional system. There was not any significant effect of housing system on plasma protein content because total protein content of plasma is related to amount and quality of protein intake [38]. The blood hemoglobin level in chicken varies between 9.8-13.5 mg/dl [32]. In this study, hemoglobin concentration of chickens kept in FRFH system was found between the normal values, while other systems were slightly lower. However, this agrees with Olaniyi et al.[39], who indicated that housing systems have no significant differences on hemoglobin concentration. Variations in Heterophil (H)/Lymphocyte (L) ratios and leukocyte counts are considered as stress factor [1,6,31]. In general, lymphocyte, eosinophil, monocyte counts and hematocrit values decrease and basophil, heterophil and counts increase under stress conditions [1]. Heterophil (H)/Lymphocyte (L) ratio of 0.2 indicates low, 0.5 medium and 0.8 high stress levels [6]. Although there were significant differences between housing systems with regard to Heterophil (H)/Lymphocyte (L) ratio, animals were exposed to under high stress. Hematocrit values vary with the ambient temperature at which birds are reared. The exposure of chickens to high temperatures causes a decrease in blood hematocrit values [1]. Therefore decreased hematocrit values had been expected with increasing temperatures. Also the current findings of this study about tonic immobility duration were similar to finding of Campo et al.[40].

In conclusion; while broilers of FRPH system had significantly lower body weights than FRFH and DLB systems until the 42nd day, the difference between the housing systems decreased during the upcoming days. Present study may enlighten the regulations to be performed in broiler production. Further research can be carried out by taking animal welfare and customer preferences into consideration as it was in developed countries. Outcomes of present study revealed that rural producers can do broilers production after wheat harvest.

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Research Article

The Influence of Body Weight on Carcass and Carcass Part Yields, and Some Meat Quality Traits in Fast- and Slow-Growing Broiler Chickens [1]

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Abstract

This study aimed to compare the carcass and meat quality of fast- and slow-growing (FG and SG) broiler chicken genotypes in different slaughter weight as 1.5, 2.0, and 2.5 kg [light (L), medium (M), and heavy (H), resp.]. Totally 460 chicks from genotypes were raised, and 30 chicks (15 female, 15 male) from each genotype slaughtered when they reached each slaughter weight category (in total, 180 chicks). Carcass and part yields, and breast meat pHU, in addition color parameters (L*, a*, and b*) of breast skin and also meat were determined.As a result of, SGs reached the L, M, and H weight 20-24 days later. The slaughter weight increase, carcass and fat pad yields increase but wing and giblets yields decreased. In all weight categories, higher carcass and breast, but lower wing and fat pad yields were determined for FGs. H group showed higher L* and a* values than L one for skin, and FGs' skin had higher a*. Nevertheless all pHU and breast meat L* values were accepted "normal", the breast meat of SGs seems to be having lower meat quality because of slightly higher L* and lower pHU. We can conclude that, SG broilers have also some disadvantages for carcass and meat qualities, even they compared with FGs slaughtered in same slaughter weights. However, SGs' breast meat may be more attractive for consumer because of their reddish and yellowness (higher a* and b*) appearance.

Keywords: *Breast skin and meat carcass parts, Color parameters, Ultimate pH*

Hızlı ve Yavaş Gelişen Etlik Piliçlerde Canlı Ağırlığın Karkas ve Karkas Kısım Verimleri ve Bazı Et Kalite Özelliklerine Olan Etkisi

Özet

Bu çalışma farklı kesim ağırlığına [1.5, 2.0 ve 2.5 kg, sırasıyla hafif (H), orta (O) ve ağır (A)] sahip hızlı- ve yavaş-gelişen (HG ve YG) etçi piliç genotiplerinde karkas ve et kalitesini karşılaştırmayı amaçlamıştır. Genotiplerden toplam 460 civciv yetiştirilmiş ve hedeflenen ağırlıklara ulaştıklarında her bir genotipte yer alan ağırlık grubundan 30'ar piliç (15 dişi, 15 erkek) kesilmiştir (toplam 180 adet). Karkas ve parçaların oranı, göğüs eti pHU'sı, ayrıca göğüs derisi ile etinin renk parametreleri (L*, a* ve b*) belirlenmiştir. Araştırma sonunda YG'ler H, O ve A ağırlıklarına 20-24 gün daha geç ulaşmışlardır. Kesim yaşı arttıkça karkas ve karın yağı oranı artmış, ancak kanat ve sakatat oranı azalmıştır. Tüm ağırlık gruplarında HG'lerde daha yüksek karkas ve göğüs oranı ile daha düşük kanat ve karın yağı oranı saptanmıştır. A grubun derilerinde, H grubuna göre daha yüksek L* ve a* değeri ölçülmüştür ve HG'lerin derileri daha yüksek a* değeri göstermiştir. Ölçülen tüm pHU ve L* değerleri "normal" kabul edilebilir olmakla beraber; YG'lerin göğüs etleri, hafifçe yüksek L* ve düşük pHU değerleri nedeniyle, daha düşük kaliteye sahip gibi görünmektedir. YG'lerin aynı ağırlıkta kesilmiş HG'ler ile karşılaştırıldıklarında, karkas ve et kalitesi bakımlarından bazı dezavantajlara sahip olduğu sonucuna varılmıştır. Ancak, YG'lerin göğüs etlerinin daha kırmızımsı ve sarımsı (daha yüksek a* ve b*) görünümleri tüketicinin ilgisini çekebilir.

Anahtar sözcükler: *Göğüs deri ve eti, Karkas kısımları, Renk parametreleri, Son pH*

INTRODUCTION

Broiler chickens, which have been obtained as a result of genetic selections for many years, reach the slaughter weight of 2.5 kg when they are 40 days-old. Intensive feeding programs, full-controlled environment, and all day lighting have been implemented to these broilers grown in the conventional system. These broilers, which are also called fast-growing (FG), face health problems resulting from respiratory, circulatory, and skeletal system

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anomalies. These problems which are associated with rapid growth have led to serious reactions in Western public having high sensitivity to farm animal welfare issues [1-6]. The broiler sector now consists of two sections: "Conventional production" which targets mass and cheap production and "alternative production" prioritizing animal welfare and sustainability. The alternative poultry production systems have been ranked from the simplest to the complex as extensive indoor, free feeding, free range, traditional free range, and organic [7]. Each system has its own limitations [4,7,8]. The extensive indoor system has the lowest criteria in terms of rearing conditions in which the earliest slaughtering age is 56 days, and maximum stocking density is 12 broiler/m^2 or 25 kg/m^2 live weight [7,9-11].

Comparing the different broiler chicken genotypes with respect to growth, it is well known that SG broilers are disadvantaged according to standart FG birds. For example, Grashorn [12] and Aksoy et al.[2] who slaughtered FGs and SGs at same age (84th and 56th days, respectively), concluded that FG birds showed higher body weight and carcass yield (in all, $P<0.05$). According to those researches, FG birds were advantaged also in breast or breast meat yield. Grashorn [13] observed only small differences for proportions of thight yield, whereas Aksoy et al.[2] determined higher leg yield for SG ($P<0.05$). In addition, FG birds had superior feed conversion ability [2,10], but SGs had less mortality and improved bone health, which are important in an alternative system [10]. On the other hand, it should not be ignored that a substantial part of consumers is ready to pay higher prices for alternative chicken meat because of animal welfare issues and special taste [3].

The color of raw broiler chicken meat varies from pale tan to pink while many factors affect poultry meat color. Such factors can be grouped as the myoglobin content of meat, pre-slaughter factors (genetic, feeds, stress, etc), and slaughtering-chilling processes. Increased hemoglobin content of the meat results in higher redness (high a* value) and hence yields darker color (lower L* value). In addition, it is observed that darker broiler meat is associated with lower muscle ultimate pH (pHU or pH24); pHU is measured at 24 h after killing. It has been demonstrated that there is a high correlation between breast muscle ultimate pH and L* value [11,14-18]. As pH increases, the L* value decreases; a high-pH of muscles, therefore, has darker color than those of low-pH.

The meat tenderness (firmness, juiciness), taste, and aroma (smell) which are defined as "organoleptic characteristics" are closely related to the ultimate pH and also L* values. The breast meats, having pHU values between 5.7-6.1 are considered as "normal", and these meats do not reveal any quality problems. Barbut et al.[19] reported that the poultry meats with the pH value over 6.1 has been considered as "dark, firm, and dry (DFD)" and, that they are risky in terms of microbial activity and therefore have a limited shelf life, although having higher water holding capacity which is desired especially for industry because of further processing. According to Zhang and Barbut [18], the poultry meats with the pH values lower 5.7 have been accepted as "pale, soft, and exudative (PSE)". These kind of meats are less risky in terms of microbial activity but they have been known to be drier when they are cooked because of their low water holding capacity; it means that they have lower technological quality [18]. Besides, poultry meats are classified according to their L* values, because of a strong relationship between pHU and L*. For ideal broiler chicken meat quality, the L* values should be between 46 and 53, and meats with an L* value below 46 are called DFD; if L* values is higher than 53, these meats are accepted PSE [18].

In many countries, the broiler sector, either conventional or alternative, offers the carcasses at different weights. Of course, these carcasses belong to male and female birds slaughtered at different body weights. Bianchi et al.[20], who worked on FG birds, determined the differences in breast meat quality attributes in different market classes according to carcass weight (light, medium, and heavy). They concluded that light broilers produced breast meat with higher values of a* and lower pH, cooking loss, and tenderness (in all, $P<0.05$). As for that, in this study fast- and slow-growing broiler chickens, male and female mixed, were raised to three different slaughter weight categories (approximately 1.5, 2, and 2.5 kg), and their carcass and meat characteristics were determined and compared. The effect of gender factor was also examined.

MATERIAL and METHODS

The research was conducted at the facilities in the Research-Experiment Unit of the Department of Animal Science in the Faculty of Agriculture at Akdeniz University. The management and handling of the birds were performed according to the practices as required by the Akdeniz University. In the experiment, the principles of the extensive indoor production system in EU were applied [7]. Cobb 500 was used as FG genotype, whereas the SG was Hubbard ISA Red JA. Two hundered thirteen one day old chicks (male and female) from each genotype were supplied local hatchery. FG and SG chickens were weighed and equally distributed among the 14 floor pens (7 pens each genotype equally distributed within the poultry house). The chickens housed with a stocking density of 12 chicks per m^2 on the litter in floor pens (each of them 1.95x1.50 m, 2.93 m^2) located in the windowed type of concrete experimental room (11.6x7.9 m) during the experiment. Wing numbers were attached to each chicks on the first day. In order to ensure optimum temperature in the experimental unit, additional heating was provided for the first 4 weeks. The chicks were provided continuous lighting for the first day, then 22hL:2hD between the 2nd and 6th days and, thereafter, 18hL:6hD until the end of the trial [8]. The broilers were fed ad libitum a starter feed

containing 21.5% CP and 2.850 kcal/kg of ME (1-21. Days), while a grower feed containing 19.0% CP and 2.850 kcal/kg of ME was used between the 21st and slaughter days [7].

When the FG and SG broilers reached different body weights [about 1.5, 2.0 and 2.5 kg; respectively light (L), medium (M) and heavy (H)], they were slaughtered. For different categories, about 30 birds were selected randomly from each genotypes for slaughtering and we tried to take equal number birds from each genders. Total number of chickens evaluated was 180 (3 weight categories x 2 genotypes x 2 gender x 15 birds). Feed was removed for 10 h before killing. After the slaughtering and the bleeding, wet plucking and manually eviscreating was done, meanwhile the sex for each broiler was confirmation by making a sex-determination again. Following the immersion in cold water and draining, the carcasses were placed in labeled plastic bags and left in the +4ºC for along the night [21]. On the next day, firstly carcass weight was determined and then, the pH_U of muscle was measured (24 h after the slaughter) from the left breast by directly inserting the glass electrode of pH-meter (Testo-206-pH2). Then the carcasses were torn apart by experienced practitioners. The weights of breast, leg, wing, abdominal fat and edible inner organs (giblets) as liver, gizzard (empty) and hearth were determined. The basic color parameter (L*, a*, b*) of the breast skin and meat samples (3 mm thick) from the left *pectoralis major* muscle was measured by using a spectrocolorimeter (Minolta CR 200).

Data collected in this completely randomized design study were subjected to an analysis of variance [22]. A factorial arrangement for main effects (slaughter weight category, genotype, and sex) was used. The unified interactions (subgroups) of main effects were analyzed separately. The means were separated using Duncan's Multiple Range test. The level at which differences were considered significant was P<0.05.

RESULTS

The ages of reaching the targeted slaughter weights as light (L,1.5 kg), medium (M, 2 kg) and heavy (H, 2.5 kg) were determined as 41st, 53rd, 58th days for FGs and 62nd, 73rd, 82nd days for SGs. In fact, the average body weight (means of male and female) of FG birds in three categories were exactly 1479, 2183 and 2640 g respectively, while 1564, 2155 and 2513 g for SGs (data did not shown in a table). The cold carcass, breast, leg, wing, abdominal fat and edible inner organ ratios to body weight of the SG and FG broilers in different slaughter weight categories determined according to the sex are presented in *Table 1*.

The statistically significant differences between slaughter weight categories were determined in terms of carcass, wing, abdominal fat and edible inner organs ratios (P<0.05 and *Table 1*). Also, statistically significant differences were determined between the SG and the FG broilers in terms of all slaughtering characteristics (P<0.05 and *Table 1*), excuding the edible inner organ yield. On the other hand, remarkable differences were formed between male and female broilers only in terms of the breast and leg yields (P<0.05). As the slaughter weight increased the carcass yield increased, and the FG broilers had higher values than the SG broilers (P<0.05). The slaughter weight categories are the only main effect that significantly affects the edible inner organ yields (P<0.05) and as the slaughter weight increased, the edible inner organ ratios decreased *(Table 1)*. On the contrary, the carcass yield increased in parallel with the slaughter weight (P<0.05).

The means and the statistical analyzes' results of the breast meat pHU, skin and meat color parameters (L*, a*, b*) are shown in *Table 2*. When it comes to skin color, lightness was affected by only slaughter weight category factors and, light (L) birds showed lower L* value mean than medium (M) and heavy (H) counterparts (P<0.05). The merely factor which affected the redness of skin was genotype and, higher a* values deteremined for FG broilers' skin (P<0.05, and *Table 2*). In contrast with, significant differences were detected between the slaughter weight groups and the sexes in terms of the yellowness of the yellowness (b*) of skin (P<0.05); the females showed higher means especially *(Table 2)*. The slaughter weight significantly affected the pHU *(Table 2)*; the highest mean was determined in the L group carcass. The FG broilers showed higher pHU mean than the SGs. When it comes to breast meat color, the effects of slaughtering weight categories and genotype factors have been found statistically significant in terms of the brightness. Genotype had also significant effect on L* and, SGs' breast showed higher values (P<0.05). Breast meat redness significantly effected by only genotype as also for skin a*. However, the SG broilers' meats showed higher a* values (in all P<0.05) contrarily the situation observed for the skin. All three factors have led to statistically significant differences in terms of the meat yellowness (P<0.05, for all).

DISCUSSION

As expected, SGs reached to similar body weights lately. Santos et al.[23] stated that FG and SG birds reached 2.5 kg live weight at 42nd and 77th days. But, in this current research, FG and SG birds arrived to this weight lately (58 and 82 days of age). According to Fanatico et al.[4], FG, MG and SG birds gained the approximately 2.5, 2.4 and 2.1 kg body weight until 53, 67 and 81 days of age, respectively. The feeds which were used in that researches contained rather low density nutrient as our feeds, but they did not applied low period lighting (18 h/day), unlike us. The reason for this differences against our findings may be lighting application diversity.

Table 1. *Carcass and different parts' yields (%)*
Tablo 1. *Karkas ve farklı parçaların verimleri (%)*

Main Effects	Carcass[1]	Breast[2]	Leg[2]	Wing[2]	Abdominal Fat Pad[2]	Edible Inner Organs[2,3]
Category[4]						
L	72.88^{c}	26.79	30.90	13.29^{a}	1.39^{b}	4.27^{a}
M	74.18^{b}	26.86	30.88	12.53^{b}	1.73^{a}	3.79^{b}
H	75.29^{a}	27.03	30.92	12.52^{b}	1.74^{a}	3.54^{c}
Genotype[5]						
FG	74.86^{a}	29.67^{a}	30.63^{b}	11.78^{b}	1.54^{b}	3.82
SG	73.37^{b}	24.11^{b}	31.17^{a}	13.78^{a}	1.69^{a}	3.92
Sex						
♀	74.11	27.54^{a}	30.38^{b}	12.86	1.66	3.92
♂	74.12	26.24^{b}	31.42^{a}	12.70	1.57	3.82
Subgroups						
L-FG-♀	73.29^{d}	29.19^{bcd}	30.65^{bc}	12.43^{d}	1.46^{c}	4.21^{abc}
L-FG-♂	73.38^{d}	28.37^{d}	31.20^{ab}	12.12^{de}	1.37^{c}	4.11^{bcd}
L-SG-♀	73.07^{e}	25.37^{e}	30.64^{bc}	14.49^{a}	1.34^{c}	4.32^{ab}
L-SG-♂	71.79^{g}	24.22^{ef}	31.10^{ab}	14.11^{ab}	1.37^{c}	4.45^{a}
M-FG-♀	75.41^{b}	31.07^{a}	29.70^{c}	11.23^{f}	1.61^{bc}	3.78^{ef}
M-FG-♂	74.95^{b}	30.03^{abc}	31.14^{ab}	11.40^{f}	1.58^{bc}	3.86^{de}
M-SG-♀	72.66^{f}	24.01^{f}	30.75^{abc}	13.74^{bc}	1.74^{abc}	3.99^{cde}
M-SG-♂	73.69^{c}	22.34^{g}	31.95^{a}	13.75^{bc}	1.97^{ab}	3.55^{fg}
H-FG-♀	76.16^{a}	30.37^{ab}	29.80^{c}	11.85^{def}	1.72^{abc}	3.47^{fg}
H-FG-♂	75.99^{a}	29.00^{cd}	31.30^{ab}	11.65^{ef}	1.47^{c}	3.46^{g}
H-SG-♀	74.09^{c}	25.25^{e}	30.73^{abc}	13.41^{c}	2.09^{a}	3.75^{efg}
H-SG-♂	74.94^{b}	23.49^{f}	31.85^{ab}	13.15^{c}	1.66^{bc}	3.48^{fg}
SEM	0.17	0.12	0.11	0.06	0.04	0.03
Main Effects		***P Values***				
Category	0.000	0.694	0.992	0.000	0.001	0.000
Genotype	0.000	0.000	0.017	0.000	0.028	0.062
Sex	0.979	0.000	0.000	0.189	0.232	0.076

[1] Carcass weight (cold, included neck and fat pad and excluded edible organs)/body weights (after 10 h fasting) x 100; [2] Related part wights /body weights (after 10 h fasting) x 100; [3] Liver, gizzard and hearth; [4] Category for slaughter weight; ***L:*** *Light,* ***M:*** *Medium and* ***H:*** *Heavy; [5] Genotypes;* ***FG:*** *Fast-growing and* ***DG:*** *Slow-growing, Values in the same column of category or subgroups with no common superscript are differ (P<0.05)*

In this study, as the slaughter weight increased in both genotypes, the yield of carcass increased. This results are in line with conclusions of other many researches' results [24-26]. On the other hand, Grashorn [13] and Aksoy et al.[2] who salaughtered FG and SG broilers at same age (84th and 56th days, respectively) concluded that FG broilers had higher carcass performance (4 and 5%, respectively) compared to the SGs (P<0.05, P<0.05). This result is normal because of the well known relationship between live weight and carcass yield. But, Fanatico et al.[4] who raised the different broiler genotypes to market weight in the extensive indoor condition, concluded that FG broilers had reached to 2.5 kg body weight at 53th days of age showed only numerically higher carcass yield than SG birds had arrived 2.1 kg at 81st days. Again, same researchers [10] determined the higher (P<0.05) carcass yield for FG broilers were slaughtered at 63rd days of age with 3.4 kg body weight than SGs slaughtered at 91st days with 2.3 kg body weight. In fact, in this two research FG and SG birds were not closely body weights.

The question to be answered that whether the carcass yield of SG and FG broilers slaugtered at same body weight is different. In this research, we tried to slaughter two genotypes on closely body weights, because of finding the answer of this question. When we examined the subgroups means for carcass yiled in detail, it is obvious that FG birds showed higher yields than SG birds in each weight categories and sex groups. We can concluded that, they are slaughtered even very similar body weight, FG broilers again showed higher carcass yield.

We can say that FG males and females did not differ in their carcass yields and this results agrees with the some studies [4,12,27]. In spite of this, SG males showed lower carcass yield in light weight group but higher yields in medium and heavy groups (in all, P<0.05). SG broilers reached to 2 and 2.5 kg body weight at 73 and 82nd days of age, therefore it is thought that the reason of SG females' lower carcass yields than male had been caused from their newly developing reproduction organs.

Many researchers [24,28-30] stated that older chickens which have higher body weight, had more breast and thigh parts. But we found the slaughter groups similar, with regard to breast and leg yields. On the other hand, this parts of yields were affected genotype (P<0.05 and P>0.05) and FG showed higher breast yield, whereas SG had higher leg yield. Fanatico et al.[4] reported that the FG broilers grown in extensive indoor system had higher breast proportion (23.2% for FG and 17.8% for SG), while the SG broilers had a higher thigh proportion (31.1% *vs.* 33.6%). Also Aksoy et al.[2] determined that breast yield were higher in FGs, however SGs showed higher means for legs percent. In addition, the results of this study were the females had higher percentages of the breast than males, and males had greater leg yield than females (P<0.05). These findings agree with the works of Young et al.[29] and Fanatico et al.[9].

The wing and giblet yields decreased with increasing slaughter weights. This findings agree with the data deal

Table 2. *Skin color and breast meat quality parameters*
Tablo 2. *Deri renk ve göğüs et kalite parametreleri*

Main Effects	Skin[1]			Breast Meat			
	L*	a*	b*	pH_u	L*	a*	b*
Category[2]							
L	60.66^{b}	1.97	5.43^{b}	6.07^{a}	48.63^{b}	1.52	4.69^{a}
M	63.34^{a}	1.58	6.03^{ab}	5.84^{b}	50.27^{a}	1.33	4.93^{a}
H	62.24^{a}	1.8	6.50^{a}	5.85^{b}	49.44^{ab}	1.25	3.96^{b}
Genotype[3]							
FG	61.6	2.35^{a}	6.13	5.96^{a}	48.92^{b}	1.01^{b}	3.84^{b}
SG	62.56	1.22^{b}	5.84	5.89^{b}	49.97^{a}	1.72^{a}	5.22^{a}
Sex							
♀	62	1.88	6.42^{a}	5.91	49.74	1.37	4.85^{a}
♂	62.16	1.69	5.56^{b}	5.93	49.15	1.36	4.20^{b}
Subgroups							
L-FG-♀	60.67^{d}	2.39^{b}	6.38^{c}	6.08^{a}	49.24^{c}	1.28^{d}	4.62^{c}
L-FG-♂	59.99^{e}	2.86^{a}	6.62^{c}	6.11^{a}	48.16^{d}	1.20^{d}	3.66^{e}
L-SG-♀	60.70^{d}	1.20^{e}	4.30^{d}	6.05^{a}	49.29^{c}	1.75^{b}	5.66^{b}
L-SG-♂	61.27^{c}	1.42^{d}	4.42^{d}	6.05^{a}	47.81^{d}	1.86^{b}	4.84^{c}
M-FG-♀	62.84^{c}	2.11^{c}	6.87^{b}	5.85^{c}	49.66^{c}	0.77^{e}	4.18^{d}
M-FG-♂	62.79^{c}	1.45^{d}	6.16^{c}	5.93^{b}	47.63^{d}	0.90^{e}	3.45^{e}
M-SG-♀	64.08^{a}	1.56^{d}	5.84^{c}	5.83^{c}	51.40^{b}	2.11^{a}	5.99^{a}
M-SG-♂	63.64^{b}	1.18^{e}	5.26^{c}	5.75^{d}	52.38^{a}	1.53^{c}	6.11^{a}
H-FG-♀	61.14^{c}	3.02^{a}	6.29^{c}	5.87^{c}	49.37^{c}	0.77^{e}	3.65^{e}
H-FG-♂	62.17^{c}	2.24^{c}	4.47^{d}	5.89^{c}	49.44^{c}	1.14^{c}	3.47^{e}
H-SG-♀	62.56^{c}	0.98^{f}	8.84^{a}	5.79^{d}	49.48^{c}	1.53^{c}	5.03^{c}
H-SG-♂	63.09^{c}	0.96^{f}	6.41^{c}	5.85^{c}	49.47^{c}	1.55^{c}	3.70^{e}
SEM	0.26	0.1	0.21	0.01	0.23	0.06	0.12
Main Effects							
Category	0.000	0.281	0.021	0.000	0.024	0.194	0.000
Genotype	0.071	0.001	0.492	0.000	0.031	0.000	0.000
Sex	0.762	0.343	0.043	0.311	0.216	0.995	0.009

[1] Parameters were determined from breast skin, [2] Category for slaughter weight; ***L:*** *Light,* ***M:*** *Medium and H: Heavy; [3] Genotypes;* ***FG:*** *Fast-growing and* ***SG:*** *Slow-growing, Values in the same column of category or subgroups with no common superscript are differ (P<0.05)*

with Poltowicz and Doktor [26] who also found that the giblet proportions of the SG broilers slaughtered at the age of 56, 70 and 84th days has steadly decreased (6.06%, 5.27% and 4.46%, respectively, P<0.05). Probably these decreasing caused by the increasing of neck and back yields. It was observed that the SG had higher wing values than the FG as parallel to data from Fanatico et al.[4], they concluded that the wings yield was 14.5% for SG and 12.8% for FG. In addition, Aksoy et al.[2] also determined higher wing yield (P<0.01) for SG according to FG slaughterd at same age (56th day), as 13.3 *vs*. 11.9. Males and females did not differ for wing yields. Also, Fanatico et al.[4] found that there is no difference between sexes.

The light slaughter weight group (L) showed lower fat pad yield than M and H groups (P<0.05). The fact that the fat pad yields rise with increasing age and body weight is acceptable normal. Despite the effect of genotype factor was found significantly (P<0.05) for fat pad yields, when judging by carefully into subgroups' means, it was clear that they were not highly remarkable differences. Thus, very different results were concluded in this regard. Santos et al.[31] who slaughtered different genotypes at 2.5 kg body weight, concluded that as parallel to us, reported that FG broilers showed lower abdominal fat yield than SG. As regards to researcher that slaughtered different genotype on the same age, according to Aksoy et al.[2] SG birds showed higher fat yields but Lewis et al.[32] and Castellini et al.[33] confirmed the opposite result. On the other hand, Grashorn [12] found similar fat pad yield for FG and SG birds. It is well-known that genetic studies have been conducted to decrease the abdominal fat ratio for many years; therefore, the lower fat pad yield for the FG broilers is an expected situation. In H group, SG-females showed higher (P<0.05) mean for fat fad yield but in other subgroups sexes were found similar.

Because the broilers produced in alternative systems are usually sold as whole-carcass, the packaging of the product is their natural skin. According to our results obtained herein, it can be said that L* and b* means increased depending on the slaughter weight. The medium and heavy group showed brighter and more yellow skin (P<0.05), it is thought that insufficient subcutaneous fat deposition caused to lower L* and b* values in the lightest body weight groups skin. Karaoğlu et al.[34] slaughtered FG birds at 35 and 42 days of age and they measured the carcass color from the back, breast and leg parts' surface after 24 h storing at +4°C. They concluded that older birds showed lower L* (63.48 *vs*. 65.89) but higher a* (3.34 *vs*. 1.88) and b* (10.76 *vs*. 9.09) values (for all, P<0.05). Only our result for skin yellowness is similar to their findings about skin pigmentation. Karaoğlu et al.[34] observed higher a* and b* values according to our data. But Huezo et al.[15] determined that L*, a*and b* values as 61.4, 1.5 and 1.0 for broiler carcass were removed from a commercial slaughter house after 24 h storage. The results obtained herein deal with skin color showed that a* values were lower at SG according to FG (P<0.05). On the other hand, females had higher b* values than males (P<0.05), this differences may be related that the females tend to be more fat deposition.

In fact, all pH_U means determined by us were within 5.75-6.11 range which is accepted "normal" [18,35]. The lower pH_U value was determined in L groups' breast meat (P<0.05). This result has been found consistent with the finding of Santos et al.[31]. Whereas, there are also some studies reporting opposite results [20,26,36]. Also, Bianchi et al.[20] who investigated the influence of the different slaughter weight on breast meat quality traits in only standard FG broilers, found that light (1.2 kg) carcass group showed the significantly (P<0.05) lower pH_U (5.92), than medium (1.8 kg) and heavy (2.4 kg) groups' pH_U (5.99 and 5.98, respectively).

It is well known that, there is high correlation between breast muscle ultimate pH and meat brightness (L*), as pH increased the L* value decreased, therefore a high-pH of muscles have darker color than those of low-pH [34,37]. Firstly, we able to say that all L* values were determined herein are in the normal range as between 46 and 53 [18,35]. Besides, the highest breast meat L* values were found for M group as 50.27, and the lowest value were determined for L group as 48.63 (P<0.05), as H group showed intermedier L* values. The fact that high-pH of muscle deal with L group have darker color (lower L*) is parallel to well known correlation between pH and lightness. Also, lower pH and higher L* values were determined for SG breast meat than FG (P<0.05, P<0.05). Based on this, we concluded that SGs' breast meat seem to be having lower quality, because lower pH_U and higher L* means that lower water holding capacity. Fanatico et al.[9] who determined the L* of FG and SG broilers growed indoor condition as 48.2 and 49.4 (P>0.05), concluded that SG birds showed higher cooking loss than FG (P<0.05) and, this higher loss is an indicator of the lower water holding capacity. On the other hand, Debut et al.[38] suggested that SG broilers could be disadvantage for meat quality due to more struggle during shackling than FG birds.

The rednes (a*) value of broiler chicken breast meat ranges between -0.96 and 4.5 and yellowness (b*) were in the range of 6.7-13.5 [39-43]. According to Gordon and Charles [3], older birds have redder (higher a*) meat due to a higher content of myoglobin. But, in this current research, the highest a* values were determined in light group which was slaughtered at the earliest ages (P>0.05). On the other hand, Bianchi et al.[20] concluded that the breast meat from light birds was redder than medium and heavy birds' breast meat (2.14 *vs*. 1.52 and 1.59, P<0.05). Many researchers [44,45] agree that the tendency of breast meat to show a lower redness when lightness increases but our findigs were not exactly confirm this conclusion. Unlike redness, yellowness (b*) of meat were affected by slaughter age and the lowest value were determined by

us for heavy group (P<0.05). According to Bianchi et al.[20], the higher b* values were determined for medium weight carcass (6.08 *vs*. 4.75 and 4.35, P<0.05).

In case of the impact of genotype factor on a* and b* values of breast meat, SG birds showed higher redness and yellowness (P<0.01, P<0.01). It was reported that the selection for meat yield results in a decrease in redness of chicken meat [43]. Fanatico et al.[9] also determined higher a* for SG birds than FG ones (P<0.05) and, they did not find remarkable difrence for yellowness among different genotypes when raised indoors. Meat redness were not affected sex factor but females showed higher (P<0.05) yellowness, in this research.

In conclusion, the results found herein indicate that as slaughter weight increased in two genotypes, carcass and fat pad yields increase but wing and giblets ratios decreased, breast and leg yields were not affected. FGs showed higher carcass and breast yields, and lower wing and giblets ratios, as similar to the results obtained when the genotypes slaughtered in the same age. While carcass yields were similar for genders but females showed higher breast and lower leg yields (P<0.05). Although all measured values were within normal limits, the increase for weight were resulted slightly lower ultimate pH and higher L* values for breast meat, and higher slaughter weight also lead to more brilliant and yellowish skin. In different weight groups, generally SGs' carcass color was found more shining and yellow, and less reddish. The breast meat of SGs seems to be having slightly lower quality because of slightly higher L* and lower $pH_{U,}$ however we need new researches including more meat quality criteria for making accurate comparing. Also, the fact that brilliant and yellowish carcass of SGs could be attractive for consumers.

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Research Article

Polymorphism of the Kap 1.1, Kap 1.3 and K33 Genes in Chios, Kivircik and Awassi [1]

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Abstract

Keratin-associated proteins are major structural components of hair and wool fibres. They also play an important role in determining the properties of the fibre. This study is designed to examine variation in the genes encoding key keratin and keratin –associated proteins in Chios, Kivircik and Awassi sheep. Variation at these loci has the potential to be developed as genetic markers deal with wool traits. Blood samples were taken into 2 ml sterilized tubes with EDTA from vena jugularis for having genomic DNA samples. Genomic DNA isolation was obtained by standard salting out method. Regions were amplified for the determination of KAP 1.1, KAP 1.3 and K33 polymorphisms in DNA samples by using PCR. Gene polymorphisms and chi-square test were used to determine whether the populations are in Hardy-Weinberg equilibrium using by POPGENE32 software. Results of the sequence analysis of the regions were evaluated by using MEGA 5. The results have shown a possibility to improve the quality of the wool traits on those local species by doing selection trials targeting on those mentioned genes.

Keywords: *KAPs, Sequencing, Turkish sheep, Wool trait*

Sakız, Kıvırcık ve İvesi Koyun Irklarında KAP 1.1, KAP 1.3 ve K33 Gen Polimorfizmlerinin Belirlenmesi

Özet

Kreatin ilişkili proteinler, yün ve kıl yapısının komponentleridir. Lifli yapının özelliklerinin belirlenmesinde önemli rol oynarlar. Yapılan bu çalışmada amaç, Sakız, Kıvırcık ve İvesi koyun ırklarında kreatin ve kreatin ilişkili proteinleri kodlayan genlerdeki varyasyonu incelemektir. Bu lokuslardaki olası varyasyonlar, yün verimi ile ilişkili genetik markör oluşturabilir. Kan örnekleri 2 ml'lik EDTA'lı tüplere vena jugularis den alınmıştır. Standart salting out yöntemiyle genomik DNA izole edilmiştir. KAP 1.1, KAP 1.3 ve K33 için bölgeleri belirlemek için PCR yöntemi kullanılmıştır. Elde edilen sonuçler POPGEN 32 istatistik programı ile değerlendirilmiştir. Sekans analizi sonuçları için MEGA 5 programı kullanılmıştır. Sonuçlar göstermiştir ki, çalışmadaki yerli ırklarda, söz konusu genlere odaklı seleksiyon uygulamalarının , yün verim ve kalitesini geliştirmek için önem oluşturabilir.

Anahtar sözcükler: *KAPs, Sekans, Yerli koyun ırkları, Yün verimi*

INTRODUCTION

Most of the proteins in hair and wool are categorized in two types; keratin intermediate filament-forming proteins (known as keratins) and keratin-associated proteins (KAPs). The studies show that there has been an increase in the number of KAP genes defined in humans and other species and progressively accounts of variation in these genes [1]. Keratin - associated proteins are a structural constituent of hair and wool fibres. They play a critical role in determining the physic-mechanical properties of hair and wool fibres [2-5].

The wool fibre consists of three main parts, the cuticle, the cortex and medulla [6]. The cortex includes 90% of the wool fibre. It also consists of filamentous microfibrils [7,8]. Keratin intermediate filaments are formed by microfibrils. KAPs have 3 groups based on amino acids compositions; the high-sulphur proteins (KAP 1.n, KAP 2.n, and KAP 3.n),

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ultra high sulphur proteins (KAP 4.n, KAP 5.n, and KAP 10.n) and the last one; high glycine tyrosine proteins (KAP 6.n, KAP7.n, KAP 8.n) [7,9,10]. KAPs are encoded by a large number of genes which are polymorphic [11]. The KAP genes between two and nine alleles have been determined [12].

Gene expression can be affected by the structure and function of the encoded proteins [13]. It may also be affected by the variation in KAP genes and as a result of that wool traits are influenced. Wool quality might be improved if we can identify the KAP genes which affect wool traits. On the other side only 16 functional genes have been identified until now so this number of sheep KAP genes, may not be sufficient to improve the wool quality. Still many genes have not been determined in human which are homologues in the sheep genome [14-16].

MATERIAL and METHODS

One hundred and thirty five Chios, Awassi and Kivircik sheep were investigated. Information from the breeder producer was considered in order to avoid family connections. Blood from sheep was collected into tubes containing EDTA. Genomic DNA was isolated by using salt-out method [17].

The primers used to amplify the KAP 1.1 locus were designed from a published gene sequence, Gen Bank nos. AY835603- AY835605 to amplify a 311 bp fragment of the KAP1.1. and F :5´-CAACCCTCCTCTCAACCCAACTCC-3', R :5´-CGCTGCTACCCACCTGGCCAT A-3´.

The KAP 1.3 locus were designed from a published sequence, Gen Bank nos.AY835589-AY835597 to amplify a 598 bp fragment of the KAP 1.3; F :5´-GGGTGGAACAA GCAGACCAAACTC-3', R :5´- TAGTTTGTTGGGACTGTACACT GGC-3´. The K33 locus were designed from a published sequence, Gen Bank nos.AY835598-AY835602 to amplify a 480 bp fragment of the K33;

F:5´-CACAACTCTGGCTTGGTGAACTTG-3',

R:5´- CTTAGCCATATCTCGGATTCCCTC-3´ [18].

Amplification consisted of; initial denaturation 95°C for 1 min, followed by 30 cycles of 95°C for 30 s, annealing at 65°C for 30 s and extension at 72°C for 30 s with a final extension of 72°C for 7 min.

The Polymerase Chain Reaction (PCR) volume of 25 µl contained; 1 U Taq DNA Polymerase (Fermentas Life Sci., Canada), 2-2.5 mL 10X PCR buffer, 1.5 mM $MgCl_2$, 50-100 ng genomic DNA, 100mM dNTP (TaKaRa Biotechnology Co., Ltd., Japan), and 10 pmol of each primer [18]. PCR products was visualised after electrophoresis on a 2% agarose gel with a long-wavelength UV transilluminator (Thermo Fisher Scientific, Germany).

Sequencing was performed by using an ABI-3100 sequencer (PE Biosystems) and the BiyDye™ terminator cycle sequencing kit after the purification of the PCR products. Forward primer was used to sequence the PCR products. Single nucleotide polymorphisms (KAP1.1) in codons 74, 111, 177, 207, 241, 262, 289 were checked directly. For KAP 1.3; codons 53, 60, 66, 67, 160, 178, 184, 232, 241, 264, 313, 337, 352, 364, 380, 486, 557, 598 and for K33; codons 127, 160, 184, 208, 223, 251, 307, 308, 340 were checked directly.

Genotype and allele frequencies of each polymorphism were calculated by using the PopGene 32 software program and also the chi-square tests (x^2) was used to check whether the populations were in Hardy-Weinberg equilibrium using PopGene32 software [19].

Statistical Analysis

Results of the sequence analysis of the regions were evaluated by using MEGA 5 (Mega Software, USA www. megasoftware.net). MEGA5 is a collection of maximum likelihood (ML) analyses for inferring evolutionary trees, selecting best-fit substitution models (nucleotide or amino acid), inferring ancestral states and sequences (along with probabilities), and estimating evolutionary rates site-by-site [20].

RESULTS

Information on product size, genotype frequencies and allele frequencies for KAP 1.1 gene, KAP 1.3 gene and K33 gene are listed in *Table 1*.

KAP 1.1: Three amplimers of different length were obtained and designated A, B and C. The length of amplimers was 341, 311 and 281 bp respectively. For KAP 1.1 gene, the obtained results indicated that A and B alleles frequencies were higher in Awassi than the others. It was found that C allele frequency was higher in Chios than the Awassi and Kivircik.

KAP 1.3: Nine amplimers of different length were obtained and designated A-I. The results noticed that the C allele was the highest in Awassi, the G allele was the highest in Kivircik. Both of the A and D alleles were not determined in all three breed.

K33: Five unique SSCP banding were observed and designated A-E. The D allele was not obtained in all three breeds. The results indicated that the A allele was the highest in Chios and the B allele, the C allele and the E allele were the highest in Kivircik.

DISCUSSION

This study reports three alleles at the KAP 1.1 locus. The length of the amplimers from KAP 1.1 alleles A, B and C were 341, 311, 281 bp respectively. The length

Table 1. *Loci, product size, genotypes and allele frequencies for polymorphisms in three candidate genes*
Table1. *Üç aday genin, polymorfizimde lokus, ürün, genotip ve allel frekans verileri*

Locus	Breed	n	Product Size	Genotype Frequency (%)									Allele Frequency (%)									(χ2)
KAP 1.1			311	AA	AB	BB	CC						A	B	C							
	Sakız	45		7	8	20	10						0.24	0.53	0.23							56.67***
	Kıvırcık	45		1	9	27	8						0.12	0.7	0.18							47.70***
	İvesi	45		10	10	24	1						0.33	0.64	0.03							100.58***
KAP 1.3			598	AA	BB	CC	DD	EE	FF	GG	HH	II	A	B	C	D	E	F	G	H	I	
	Sakız	45			8	15		3	2	6	4	7		0.18	0.34		0.06	0.05	0.13	0.09	0.15	308.00***
	Kıvırcık	45				11		3		25	3	3			0.24		0.07		0.55	0.07	0.07	207.00***
	İvesi	45				34				6	4	1			0.75				0.14	0.08	0.03	188.57***
K33			480	AA	BB	CC	DD	EE					A	B	C	D	E					
	Sakız	45		41		3		1					0.92	0.01	0.07							53.43***
	Kıvırcık	45		35		5		5					0.81	0.04	0.11		0.04					108.92***
	İvesi	45		38		3		4					0.87	0.03	0.07		0.03					112.00***

*[1] Hardy-Weinberg equilibrium, *** P≤0.001*

polymorphism of the KAP 1.1 gene determined in this investigation has previously been reported in Romney sheep and in Merino sheep [18,21]. Our results showed that A and B alleles frequencies were higher in Awassi than the others and also the C allele frequency was the highest in Chios breed.

Five alleles were identified at the K33 locus in Merino sheep which was reported by Itenge *et al*.[18]. By the way Roger *et al*.[21] determined a diallelic polymorphism at this same loci. In our study, the D allele wasn't obtained in all three breed. Addition to this, the E allele was not obtained for Chios sheep.

Itenge *et al*.[18] reported eight alleles (except for B allele) in a 598 bp KAP 1.3 amplimer from Merino sheep. The results found by Powell *et al*.[22] also supported these findings. Roger *et al*.[21] determined six alleles from Romney sheep. In this study we obtained seven alleles for Chios sheep, five alleles for Kivircik sheep and four alleles for Awassi sheep in a 598 bp KAP 1.3 amplimer. Both of the A and D alleles were not determined in all of them. Our investigation showed that the C allele was the highest in Awassi, 0.75 by the way 0.34 for Chios and 0.24 for Kivircik, the G allele was the highest in Kivircik whereas the G allele was 0.13 in Chios and 0.14 in Awassi.

As a conclusion it has been known that Kivircik sheep breed has been preferred for wool quality and Awassi sheep breed has been preferred for wool trait among the domestic sheep breed in Turkey. Their wool quality ranges from carpet to medium-wool quality [23]. It might be a relationship between these alleles and wool quality and wool product. To support this suggestion, further linkage analyses are necessary. This will allow future analysis of how these genes may affect wool traits.

The allele numbers at the KAP1.3 and the K33 locus in Chios, Awassi and Kivircik were determined lower than the Merino sheep and Romney sheep breed. It might be suggested to make further trials on those loci to possible improvement on wool quality and wool trait in those breeds.

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Research Article

In vitro Evaluation of Short-term Preserved Stallion Semen in Different Doses and Temperatures [1] [2]

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Abstract

This study was conducted to evaluate the effects of different temperatures and doses on stallion sperm survival after 24 h and 48 h short-term storage. Ejaculates from four warm-blooded stallions were used in this study. Ejaculates were collected by an open-ended artificial vagina which separates fractions of semen into sterile plastic cups. Only the second and third fractions (spermatozoa-rich fractions) were used to obtain spermatozoa for the experiment. Semen was diluted with INRA 96 extender to final concentrations of 50, 100, 200 and 400x10^{6}sp/ml. Diluted semen were stored at different temperatures (2, 4 or 8°C) for 24-48 h. After 24 h and 48 h cooled storage, spermatozoon motility, abnormal spermatozoon ratio and membrane integrity were evaluated. According to results the highest motility values (52.5% and 55%) were determined at 50 and 100 x10^{6}sp/ml concentration at 4°C after 24 h cooled storage. After 48 h of storage, the highest motility was obtained from semen stored at 8°C. Abnormal spermatozoon and membrane integrity values were not significantly different after cooled storage. Consequently, it was determined that semen with 50 and 100 million spermatozoa/ml concentration stored at 4°C for 24 h and semen with 50, 100, 200, 400 million spermatozoa/ml concentration stored at 8°C for 48 h gave better results with respect to motility than the others.

Keywords: *Stallion semen, Doses, Temperature, Short-term preservation, In vitro evaluation of semen*

Farklı Doz ve Sıcaklıklarda Kısa Süreli Saklanan Aygır Spermasının *in vitro* Değerlendirilmesi

Özet

Bu araştırma, farklı doz ve sıcaklıkların (24-48 saat) aygır spermatozoonlarının yaşam kabiliyetleri üzerine etkisini ortaya koymak amaçlanmıştır. Çalışmada dört sıcakkanlı aygırdan alınan ejekülatlar kullanılmıştır. Ejekülatların alınması için açık uçlu suni vagina kullanılmış ve bu yolla sperma fraksiyonlar halinde cam kaplara alınmıştır. Deney süresince sadece 2. ve 3. fraksiyonlar (spermatozoondan zengin fraksiyonlar) çalışmada kullanılmıştır. Sperma INRA 96 sulandırıcısı ile final yoğunluk 50, 100, 200, 400x10^6 spermatozoa/ml (sp/ml) olacak şekilde sulandırılmıştır. Sulandırılan sperma 2°C, 4°C ve 8°C lik sıcaklıklarda 24-48 saat süreler ile saklanmıştır. 24-48 saatlik saklama sonrası spermatozoon motilitesi, anormal spermatozoa ve membran bütünlüğü yönünden muayene edilmiştir. Elde edilen sonuçlara göre kısa süreli saklanan spermada 24. saat sonunda en yüksek motilite değerleri (%52.5 ve %55) 4°C'de 50 ve 100 milyon yoğunlukta elde edilmiştir, 48. saat sonunda ise en yüksek motilite değerleri 8°C'de saklanan spermadan elde edilmiştir. Anormal spermatozoa ve membran bütünlüğü değerleri farklılık göstermemiştir. Sonuç olarak, 4°C'de 50 ve 100 milyon dozlarında 24 saat saklamanın ve 8°C'de 50, 100, 200 ve 400 milyon dozlarında 48 saat saklamanın diğerlerine göre daha iyi sonuçlar verdiği tespit edilmiştir.

Anahtar sözcükler: *Aygır sperması, Doz, Isı, Kısa süreli saklama, Spermanın in vitro değerlendirilmesi*

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INTRODUCTION

Horse breeders rarely give importance to fertility as selection criteria in mating, although many stallions do not have enough features to be used for breeding. This has resulted in stallions' transferring undesirable performance qualities to their offspring in the gene pool. Therefore, significant differences were formed in semen qualities of the stallions used for breeding. A winner stallion may also be in the gene pool even though the stallion has poor semen quality or the semen is not appropriate to use with the advanced technology of semen processing techniques. This has led the scientists' dealing with stallions, to come a long way in terms of assessment of all possibilities to increase reproductive performance [1].

Stallions' semen quality analyses are essential for fertility to be maximised. There can also be changes from poor to medium degree in ejaculates of stallions. These changes are mainly caused by; season, semen collection and processing techniques, semen collection frequency and problems in spermatogenesis. Factors such as maintenance-supply, semen collection procedures and number of mares to be artificially inseminated; as well as semen quality may effect fertility. The conventional parameters in evaluation of unprocessed semen quality are; amount, concentration, total number of spermatozoa in ejaculate, motile spermatozoa rates, morphology of spermatozoa and seminal pH [2].

Although short-term stored semen has wide range of usage in livestock animals and horses, sufficient implementation and optimum fertilisation rates have not been reached yet. In studies performed, the main step is related with storage of semen with an appropriate extender and additives (antioxidants) in 4°C for 24 h [3-6]. However, quite different and conflicting results are obtained in motility, abnormal spermatozoa, membrane integrity and fertilisation rates after storage [7,8]. Seminal plasma components provide membrane stability which protects semen during cooling, freezing and thawing. Changes in spermatozoon membrane reduce fertilising ability of frozen thawed semen. Stabilising substances in seminal plasma maintain membrane integrity and thus freezability of spermatozoa. Addition of seminal plasma from stallions with high post-thaw motility to semen from stallions with low post-thaw motility improves membrane integrity and progressive motility. Seminal plasma from different stallions contain different amounts of components which may be considered as a determining factor for the ability of spermatozoa from each stallion to survive cryopreservation. Individual differences influence seminal plasma, causing diversity in cooling and freezing processes by effecting the spermatozoa membrane integrity. Cryopreserving spermatozoa induces changes in the plasma membrane. Removal of seminal plasma by centrifugation before cryopreservation causes a premature capacitation reaction when the semen is frozen. For that reason, spermatozoa are unable to undergo the acrosome reaction and complete fertilisation [3,9,10]. Different diluents (INRA 82, lactose-EDTA, skimmed milk, skimmed milk-glucose, sugar based or ionic diluents), different cryoprotectants (Glycerol, Ethylene glycol, DMSO), different packaging methods (ampoule, vial, 0.25 and 0.5 ml sequins, 4 ml macrotube, 2.5 ml flat macrotube) and storage methods (short and long-term) were used for storage of stallion semen [10-12].

This study contains reproductive biotechnologies and preservation techniques. High fertility rates are expected, by using biotechnological methods such as transporting stored semen and artificial insemination applications, in mares. Study is directed to cryopreservation biotechnologies of sperm cells. Compared to other farm animals, sufficient fertilisation rates cannot be obtained from cooled-stored or frozen-stored stallion semen [13]. Fertilisation ability could be changed by semen concentration and storage temperature [5]. Thus, to determine the most effective semen concentrations and storage temperatures, and accordingly to increase fertility; different methods and techniques should be developed. In this study, spermatological parameters of short-term stored stallion semen with different doses at different temperatures were obtained. For this reason, spermatozoa integrity and fertilisation ability can vary in accordance with spermatozoa concentration and temperature of storage. With this respect, in vitro integrity and fertilization ability of stallion semen was aimed to be evaluated.

MATERIAL and METHODS

This study was performed in horse farms in Mahmudiye-Eskişehir and the laboratory of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine Ankara University. Materials for the study were obtained from the horse farms around the area and Dörtnal Veterinary Clinic's horses and 4 European warm-blooded stallions were used. The stallions were fed twice a day with 12 h intervals. Depending on the stallions' performance and the number of copulations, an average of 5 kg of pellet feed were given to each stallion. In addition, 2 kg clover and 2 kg dry grass were given per day. Stallions were allowed to wonder around the paddock approximately 8 h per day, no additional exercises were done. The experimental procedures were approved by the Animal Experiments Local Ethics Committee of Ankara University (No: 2008-29-138).

Semen was collected daily from stallions for 3 days in order to empty the extragonadal semen reserves at the beginning of the research and during breeding season (April-May). After 2 days of sexual resting, a total of 16 ejaculates, separated into fractions, were collected by open-ended artificial vagina, from each stallion twice a week.

The spermatozoa used for short-term storage were taken from the second and third fractions (spermatozoa rich fractions). For this purpose, only the second and third fractions were collected into the collection tube. The collected semen was diluted with skimmed-milk glucose extender with a ratio of 1:1. Extended semen was centrifuged twice at room temperature at 500 G for 10 min to separate seminal plasma. With the pellet that was obtained, concentrations were determined and using the INRA 96 extender, the pellets were extended to 50, 100, 200, 400x10^6 sp/ml.

The semen that was extended with INRA 96 extender was separated into 3 equal parts to be short-term stored and they were kept at 2, 4 and 8°C for 48 h. With reference to this, 12 trial groups including different semen doses (50, 100, 200, 400x10^6) and different temperatures (2, 4 and 8°C) were formed. After the dilution process, the semen was put in equal volume closed-end glass tubes and these were stored for 48 h. Evaluations of spermatological parameters were conducted at 0., 24., and 48. h in order for the spermatozoa fertilization abilities to be presented. Before the spermatological inspections, the short-term stored semen was put into 37°C water for 5 min. After that process, spermatozoa motility, abnormal spermatozoa ratio and membrane integrity were evaluated. Spermatozoa motility was evaluated subjectively and in percentage by 3 microscope fields scanned by 2 different observers on a microscope with heating plate (10x20) [5].

Abnormal spermatozoa ratios were determined by eosin staining method. For this purpose, samples taken from the semen were put into 3% sodium citrate, which has 2% eosin in dilution. Smears were taken from each sample and examined with a light microscope (400x). In different microscopic area, at least 200 spermatozoa were counted and abnormal spermatozoa ratio were noted as percentage [5].

Hypoosmotic swelling test (HOS) was performed in order to determine membrane integrity. For this purpose, 100 mikroliters semen was added to 1 ml lactose solution and then incubated at 37°C for 30 min. After the incubation, at least 100 spermatozoa were evaluated at the microscope according to their tail curling and membrane integrity. Each experimental group (dilution ratios and temperatures) were evaluated 3 times.

The data was analysed with General Linear Model (GLM) method in order to reveal the effect of different temperatures and different semen concentrations on some of the spermatological properties. Analysis of the temperature and concentration of each spermatological parameter were carried out with one way analysis of variance (ANOVA). The degree of significance, among experimental groups, was determined as $P<0.05$. SPSS 10.0 programme which runs under Windows software was used for the analysis.

RESULTS

Semen's fertilization ability was presented by biometrical evaluation of the data. The effect of different temperatures and different spermatozoa concentrations on some spermatological parameters were presented in *Table 1*.

Accordingly, significant differences in spermatozoa motility, abnormal spermatozoa ratio and membrane integrity were determined right after dilution (0. hour); and similar results were obtained for all 4 concentrations. Similar to this, even though no significant differences were observed after 24 h of storage in all 3 different temperatures; the different temperatures displayed differences in motility within themselves and these differences were found statistically significant. Accordingly, highest motility (50.00%) was observed in semen containing 100 million spermatozoa after storage in 2°C for 24 h. However, abnormal spermatozoa and membrane integrity was not significantly different. Similarly, when the properties of semen stored at 4°C were evaluated, significant decrease of motility from 30.00% to 24.00% was determined in concentrated semen at a concentration of 200 and 400x10^6 sp/ml. Whereas, semen storage performed at 8°C for 24 h, at a concentration of 50 and 100x10^6 sp/ml has been detected to have higher motility values with respect to more concentrated semen samples. No significant differences in abnormal spermatozoa ratio and membrane integrity were observed.

When the data obtained from storage of semen for 48 h was evaluated, in all 3 temperature degrees the semen quality was found to be decreased; resulting in decrease of spermatozoa motility, increase of abnormal spermatozoa ratio and decrease of membrane integrity.

When the temperatures were considered, in contrast to what was expected after storage for 48 h, the highest motility values were obtained from semen stored at 8°C. Similarly, membrane integrity values were higher compared to the other temperatures. However, no difference in abnormal spermatozoa ratios was observed. When all temperature values were evaluated, there was no difference in spermatological properties of different concentrations of semen stored at 2°C, semen including 100 million spermatozoa was found to have the highest motility value (30%). On the other hand, even though semen stored at 8°C shows differences when compared with other temperatures, samples of different concentrations had no differences in spermatological properties. However, after 48 h of storage the highest motility and membrane integrity values were obtained with 8°C.

DISCUSSION

In this study, after storage at 4°C for 24 h the highest motility values of 52.5% and 55% were recorded. The highest

Table 1. *Effect of different storage temperatures and spermatozoa concentrations on quality of semen*
Tablo 1. *Farklı saklama sıcaklıklarının ve spermatozoon yoğunluklarının sperma kalitesi üzerine etkisi*

Time	Temperature	Concentration (x10^6 sp/ml)	Spermatozoa Motility (%)	Abnormal Spermatozoa (%)	Membrane Integrity (%)
0. hour	Room temperature after dilution (20°C)	50	67.5±0.10	46.5±2.12	51.3±8.83
		100	62.5±0.10	48.0±1.41	50.7±6.15
		200	67.5±0.35	52.5±3.53	57.9±1.27
		400	68.0±0.01	47.5±0.70	52.9±1.55
24. hour	2°C a	50	47.5±0.35 b	76.5±2.12	36.5±12.0
		100	50.0±0.07 b	75.0±1.41	31.0±8.48
		200	40.0±0.01 a	77.0±4.24	33.5±7.77
		400	40.0±0.01 a	80.5±6.36	32.5±17.6
	4°C a	50	52.5±0.10 a	73.0±0.01	27.5±9.19
		100	55.0±0.21 a	75.0±2.01	31.0±5.65
		200	30.5±0.17 b	74.0±1.65	21.0±7.07
		400	24.5±0.24 b	79.0±3.76	24.5±13.4
	8°C a	50	35.0±0.07 a	82.0±4.24	35.5±3.53
		100	40.0±0.07 a	75.5±7.77	39.3±9.47
		200	45.0±0.21 b	81.5±3.53	32.5±7.77
		400	47.5±0.03 b	77.0±1.41	34.5±0.70
48. hour	2°C a	50	30.0±0.00	91.5±2.12	11.5±4.94
		100	25.0±0.07	87.5±0.70	18.5±9.19
		200	25.0±0.00	91.0±8.48	19.5±2.12
		400	30.0±0.00	97.0±4.24	15.0±9.89
	4°C a	50	22.5±0.03 a	92.0±4.24	18.0±2.10
		100	30.0±0.14 b	84.0±1.41	13.0±3.36
		200	20.0±0.28 a	94.0±2.94	17.0±3.03
		400	22.5±0.31 a	97.0±2.02	18.0±5.20
	8°C b	50	35.0±0.07	91.0±7.07	25.0±4.44
		100	30.0±0.10	96.0±2.82	21.5±12.0
		200	40.0±0.15	97.0±1.41	24.0±8.48
		400	40.0±0.22	96.0±5.65	28.0±11.3

a, b: *The difference of group averages in the same column between different letters are significant (P<0.05)*

motility rates of the groups stored for 48 h were noted at 8°C. Among the groups, abnormal spermatozoa and membrane integrity values were not significantly different. According to results, semen stored at 4°C in 50 and 100 million doses for 24 h and semen stored at 8°C in 50, 100, 200, 400 million doses for 48 h were observed to have better results compared to the other groups.

Many methods on short-term storage of stallion semen have been tested and it was notified that especially genetic biological differences and differences depending on the method effect spermatozoa's fertilisation ability. Some parameters such as extenders, dilution ratios and storage temperature differences have been reported to effect success by different researchers [3-8,10,11]. Similar to this, the research determined that in examinations performed after dilution ratio or spermatozoa concentration does not effect spermatological properties.

Evaluations after 24 h of storage for semen revealed that high dilution rates and low concentrations gave high motility values at 2°C and 4°C, in this case fertility rates can be higher at 2°C or 4°C with concentrations of 50 and 100 million semen. In contrast it was determined that 24 h of storage in low dilution rates at 8°C have more successful results. According to these results, high fertility rates can be obtained from semen stored at 8°C with 200 million or 400 million concentrations. Evaluations after 48 h of storage indicate that semen samples at 2°C and 4°C have low motility values. Conversely, semen stored at 8°C with low dilution rates or high semen concentirations (200 and 400×10^6 sp/ml) gave high motility and membrane integrity values. It was concluded that high fertility rates could be obtained at 8°C compared to 2°C and 4°C at 48 h storage of semen.

Results suggest that there may be an interaction

between semen concentration and storage temperature. Because high concentrated samples have low motility values at low temperatures (2°C and 4°C) whereas same samples have high motility values at a higher temperature (8°C).

Stallion ejaculation consists of 5 to 8 fractions and these fractions have different compositions because they contain secretions from different accessorial glands [14,15]. It has been reported that the reduce of seminal plasma rates increase the motility in semen cryopreservation [13,16,17]. It has been found that seminal plasma proteins increase the resistance of spermatozoa [18]. Similarly, Iwamoto et al.[19] detected that seminal plasma protects semen more efficiently against cold shock. Besides, structural differences in seminal plasma, which consists of individual differences between stallions, increase resistance of spermatozoa [18,20]. Likewise, Aurich et al.[10] stated that addition of seminal plasma from stallions with high post-thaw motility to semen from stallions with low post-thaw motility significantly improved membrane integrity and progressive motility. Varner et al.[17] reported that seminal plasma obtained from spermatozoon-rich fraction reaped a benefit more than whole ejaculates. A similar result was obtained by Katila et al.[11] although different ejaculations' effect on spermatozoon have not been revealed yet. Akçay et al.[9] indicated that last fraction (gel fraction) was essentially important for spermatozoa and also stated that, sperm plasma membranes suffer no obvious damage as the highest membrane integrity values were obtained in skim milk glucose extender. The results in the study suggest that sperm from different stallions differ in their sensitivity to the absence of seminal plasma in skim milk extender. The results also indicate that skim milk glucose extender provides protection for sperm membrane integrity but does not effectively protect motility. Conversely Moore et al.[3] reported that, seminal plasma did not affect sperm motility, viability or acrosomal integrity.

In this study, it was observed that stallion semen samples stored at 4°C for 24 h, containing the last fraction of seminal plasma, have better spermatozoa parameters. The seminal plasma proteins increase spermatozoa resistance and protect the spermatozoa against cold shock.

As a result, after the examination of samples immediately after dilution (0 h), no significant difference was observed. Additionally when analyzing the 24 h data, only temperatures among themselves, showed statistically differences in motility ($P<0.05$). After the evaluation of 48 h stored semen samples, decrease in semen quality in all three temperatures, and accordingly, reduction in semen motility, increase in the rate of abnormal spermatozoa and membrane integrity have been observed. However, after 48 h storage, some interesting results were obtained; when temperatures were compared, highest values of motility and membrane integrity were seen at 8°C. If semen is to be kept for 48 h, 8°C should be preffered in order to increase fertility rates.

In this context, the current research will be an initial step for the next studies on short-term storage of semen in horses. However, this research is based on *in vitro* examinations. Considerably actual results may be taken after *in vivo* evaluations. Thus, studies must be repeated in different ways for a successful storage protocol. Beside increasing the number of animals, storage of different breeds' stallion semen is important for future studies. More reliable results can be obtained, by having fertility rates of artificial insemination done with fresh or short-term stored semen and comparison of horse breeds and horses individually. If the semen is to be kept for a longer time, higher temperatures (i.e. 8°C) should be preferred. There are no differences among temperature rates when semen is stored for 24 h, even though it can be observed that higher temperatures (i.e. 8°C) give better results when storage period lengthens.

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Research Article

Effect of Met-Anandamide on Prevention of Hyperactivation, Cryo-Capacitation and Acrosome Reaction in Ram Semen Cryopreservation

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Abstract

Cryopreserved spermatozoon has some defects on membrane causing hyper activation, cryo-capacitation and timeless (early) acrosome reaction that lead to low fertility. While low fertility is generally accepted as a consequence of cryopreservation of sperm cells, a lot of studies are being carried out on cryopreservation techniques to improve spermatozoon membrane integrity and also viability. Some studies point out that using materials such as Cannabinoids can be effective in achieving this aim. The main effects of endocannabinoids on spermatozoa are the inhibition of motility, capacitation and acrosome reaction. The aim of this study was to determine the effect of soluted Met-anandamide in extender on post thawing sperm motility, capacitation and acrosome reaction in ram semen cryopreservation. For this aim, ten ejaculated collected from five mature Bakhtiari rams were diluted with extender containing Met-anandamide, equilibrated and then cryopreserved in liquid nitrogen vapour (approximately −125 to −130°C). Cryopreserved semen samples were preserved in liquid nitrogen until evaluation. Post thawing sperm analyses revealed that the Cannabinoids agonists affected the motility of ram sperm after thawing ($P<0.05$), resulting in an increase in the AEA (arachidonoylethanolamide) concentration (10, 100 and 1000 nM) which significantly reduced sperm motility. subsequent freezing and thawing. On the other hand, the degrading enzyme of endocannabinoid FAAH may have had a reduction effect on sperm motility and increased capacitation and early acrosome reaction ($P<0.05$). In conclusion, endocannabinoid system may be used to improve ram sperm freezing.

Keywords: *Ram, Sperm, Cryopreservation, Met-anandamide, FAAH, Capacitation*

Met-Anandamidin Koç Spermasının Dondurulmasında Hiperaktivasyon, Kryo-Kapasitasyon ve Akrozom Reaksiyonunun Önlenmesi Üzerine Etkisi

Özet

Dondurulmuş spermatazoon membranında oluşan bazı hasarlar hiperaktivasyona, kryo-kapasitasyona ve zamansız (erken) akrozom reaksiyonuna neden olarak düşük fertiliteye yol açabilmektedir. Genel olarak; düşük fertilitenin sperm hücrelerinin dondurulmasının bir sonucu olarak oluştuğu kabul edilirken, spermatazoon membran bütünlüğünü sağlama ve aynı zamanda canlılığını artırmaya yönelik dondurma teknikleri üzerinde pek çok yeni çalışma yapılmaktadır. Bu amaçla, bazı araştırmalar Cannabinoid gibi maddelerin kullanımının etkili olabileceğini göstermektedir. Endocannabinoidlerin spermatozoa üzerine asıl etkisi motiliteyi inhibe etmesi, kapasitasyon ve akrozom reaksiyonudur. Sunulan çalışmanın amacı, koç semen dondurulmasında Met-anandamid solüsyonu içeren sulandırıcıyla sulandırmanın çözünme sonrası sperm motilitesi, kapasitasyonu ve akrozom reaksiyonu üzerine etkisini araştırmaktır. Bu amaçla, 5 adet ergin Bakhtiari koçundan elde edilen 10 ejekulat Met-anandamid içeren sulandırıcı ile sulandırılarak, ekilibre edilmesinin ardından sıvı azot buharında (yaklaşık −125 ila −130°C) donduruldu. Dondurulmuş sperma örnekleri değerlendirilenceye kadar sıvı azot içerisinde muhafaza edildi. Çözdürme sonrası sperm analizleri, Cannabinoid agonistlerinin çözünme sonrası koç sperm motilitesini etkilemesi ($P<0.05$) sonucu AEA (arakidonoletanolamid) konsantrasyonundaki (10, 100 ve 1000 nM) artışın sperm motilitesini önemli derecede azalttığını gösterdi. Öte yandan, endocannabinoid FAAH enzim degredasyonu sperm motilitesini azaltıcı ve kapasitasyonu ve erken akrozom reaksiyonunu ise artırıcı etki göstermiş olabilir ($P<0.05$). Sonuç olarak, endocannabinoid sistem koç sperminin dondurulmasında daha iyi sonuç almak amacıyla kullanılabilir.

Anahtar sözcükler: *Koç, Sperm, Kryopreservasyon, Met-anandamid, FAAH, Kapasitasyon*

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INTRODUCTION

In most species, such as sheep, a lower fertility is generally determined as a consequence of cryopreservation of sperm cells and in order to improve the sperm viability in cryopreservation. However, sperm cryopreservation induces some defects such as formation of intracellular and extracellular ice crystals [1], osmotic [2-5] and chilling injury [6], cryo-capacitation and early acrosome reaction that reduced the functional life span of sperm and lead to low fertility [6,7]. Therefore, many attempts are being developed in cryopreservation techniques to improve sperm viability.

Anandamide (arachidonoylethanolamide, AEA) is a member of a group of endogenous lipids, collectively termed 'endocannabinoids'. It binds to type-1 and type-2 receptors of cannabinoid (CB1R and CB2R), thus having many functions in the central and peripheral nervous systems [8,9]. Such functions inactivation by cellular uptake through an AEA membrane transporter (AMT), completed by degradation to arachidonic acid and ethanolamine by the fatty acid amide hydrolase (FAAH) [10,11].

Some researchers believe that Cannabinoid receptor Type 1 (CB1) is effective in sperm membrane stability. They argue that endocannabinoids have an effect on CB1 receptors and then inhibit the adenylate cyclase/cAMP/PKA pathway which was earlier activated during the acrosome reaction [12-15]. They argue that the role of CB1 receptors in rapid hyper polarization of the cell membrane is also essential. However, other researchers have revealed that stimulation of vanilloid type 1 receptors (a non-selective cationic channel that belongs to the vanilloid-type transient receptor potential family), in the sperm membrane has been effective in preventing the acrosome reaction [16,17].

However, the endocannabinoids, by effect on their sperm receptors, lead to sperm remains in non-capacitated situation until they enter the female reproductive tract; concurrent with the reduction in sperm concentration endocannabinoids, and when the sperm reaches to oocyte and binds to the zona pellucida, sperm capacitation occurred [18-20]. Exactly, it prevents inappropriate capacitation and acrosome reaction, and then increases the chance of the sperm reaching to the egg. Now, due to the early capacitation problem in the process of freezing and given the need to preserve the reproductive potential of sperm after freezing and thawing, it can be effective to employ a substance that delays capacitation and acrosome reaction of sperm.

Therefore, the aim of present study is to examine the impact of different doses of Met-anandamide as a non-metabolized analog anandamide and FAAH as hydrolyzing enzyme of anandamide, on the quality of frozen Lori-Bakhtiari sheep sperm. Findings of the present research can help us to understand the beneficial or detrimental effects of the cannabinoid system on the quality of sperm after freezing and thawing. It is worth mentioning that this is the first time that Met-anandamide has been added to the freezing media for improving fertility potential of sperm.

MATERIAL and METHODS

Animals

In this study, the samples give from five 4 to 5 years rams *(Ovis aries)* that had bred for giving sperm for artificial insemination in the Lori-Bakhtiari research flock at Shooli station in Shahrekord.

Semen Extenders and Treatment Groups

The base extender used in this study (Tris base; TB) was composed of 200 mM Tris (Hydroxymethyl)-aminomethan (Merck, Darmstadt, Germany), 27.8 mM D-Glucose (Sigma; G6152), 94.7 mM Citric acid (Sigma; C1909), 100 IU/ml Penicillin (sigma) and 0.05 mg/ml streptomycin (Sigma). The osmolality and pH were adjusted to 320 mOsm and 7.2, respectively. Freezing medium was prepared by adding 25% egg yolk (egg yolk lipids were solubilized by adding 0.035% (w/v) sodium dodecyl sulfate (SDS) [21] and adding 7% Glycerol to Tris base medium. Five different extenders were designated by the addition of different concentrations of AEA (Met-anandamide) as following 10 nM; 100 nM AEA; 1000 nM AEA and FAAH (0.025 IU/ml). No addition group was considered as control .

Semen Collection and Processing

Ten ejaculates from each ram were collected by artificial vagina twice a week during the breeding season from five mature Bakhtiari rams known to have good fertility. Only normospermic ejaculates (volume >0.75 ml, sperm concentration $>3\times10^9$ sperm/mL, motility >70%, abnormal sperm <10%) were used during this study. To eliminate individual differences, semen samples from the five rams were pooled. Each pooled sample was divided into five equal aliquots and diluted with extenders containing different AEA concentrations or FAAH. The diluted semen was gradually cooled to 4°C for 2 to 3 h. The cooled semen was loaded into 0.5-mL French straws (Biovet, L'Agile France), semen samples loaded to straws were exposed to liquid nitrogen vapor for 10 min (3 cm above LN2 level, approximately -125 to -130°C), plunged into liquid nitrogen (LN2), and stored in LN2 until thawed and used for the evaluation of sperm parameters.

Evaluation of Sperm after Freezing-Thawing

- *Sperm motility:* Sperm motility parameters were measured by CASA Hamilton, with the following settings: Image collection speed, 20 frames per second; analysis time per frame, less than 15 seconds; sperm velocity that can be analyzed, 0-180 µm/s; Number of vision fields that were selected, 10/samples; magnifying power of micro-

scope (object lens), 40X measurements were done in sperm miter chambers 10 µm depth. The sperm motility was measured as fast progressive motility (class A) in percentage. The studied motion parameters was as following: VSL, VCL, VAP, ALH, BCF, LIN, STR and WOB [22].

- Sperm viability: For viability, the hypo-osmotic swelling (HOS) test and eosin-nigrosin staining were used according to Revel and Mortimer methods, respectively [23,24] and then were performed in each aliquot. In HOS test, 25 mL semen was added to 200 ml hypo-osmotic solution (100 mOsm/L, 57.6 mM fructose and 19.2 mM sodium citrate). After 30 minutes of incubation at room temperature, the mixtures were homogenized and evaluated with an inverted light microscope. A total of 200 spermatozoa were assessed in four different microscopic fields. The sperm percentage with curved and swollen tails was recorded.

In eosin-nigrosin staining technique, 0.67% eosin Y and 10% nigrosin dissolved in 0.9% sodium chloride in distilled water. Approximately equal volumes of sperm sample and stain were blended. The suspension was incubated for 30 sec. at room temperature (20°C). Then, a 12 µl droplet was transferred with the pipette to a labeled microscope slide where it was smeared by sliding a cover slip in front of it. Two smears were taken from each sample. The smears were air dried and evaluated directly. At least 200 sperms were assessed for each preparation at a magnification of 400×. Spermatozoa that were unstained or white were considered live, while those that indicated red or pink coloration in the head region were classified as dead.

- Capacitation Status: Acrosome reaction was assessed with chlortetracycline (CTC) staining as described by Perez et al.[25] with little modification. A CTC working solution (750 mM) was freshly prepared in a buffer containing 20 mM Tris, 130 mM NaCl, and 5 mM D, L-cysteine at a pH of 7.8. Five microliters of semen were mixed with 20 mL CTC working solution. After 20 seconds, the reaction was stopped by the adding 5 mL 1% (v/v) glutaraldehyde to 1 M Tris-HCl, pH 7.8. Smears were prepared on a clean microscope slide and examined under an epifluorescent microscope (Olympus). Stained spermatozoa shows three types of patterns: 1) uniform fluorescence head, or uncapacitated sperm (Noncapacitated pattern); 2) post-acrosomal region without fluorescence, capacitated sperm (Capacitated pattern); and 3) Fluorescence-free head or a thin fluorescence bond on the equatorial segment (sperm that underwent an acrosome reaction [acrosome reacted pattern]). In this study 200 sperm were assessed in each smear.

Statistical Analysis

Each treatment was replicated ten times. For each, three straws were thawed and pooled for sperm parameters evaluation. To evaluate differences between groups, we used analysis of variance (ANOVA) for comparisons of means after evaluate the normality of our data by Kolmogorov-Smirnov test. When statistical differences were revealed with the ANOVA test, the mean of the treatments was compared using Duncan's multiple range test (DMRT), and a confidence level of $P<0.05$ was regarded to be significant. Statistical evaluations were conducted using the Statistical Package for Social Studies software (Version 20; SPSS, Chicago, IL). Information on CTC staining was analyzed using the GENMOD procedure of SAS (Version 9.0, SAS Institute Inc., USA) to fit a generalized linear model with a logit link function.

RESULTS

The findings are shown separately and interpreted comprehensively below.

Sperm Motility

CASA evaluation revealed that the presence of AEA in freezing medium affected motility of rams sperm after thawing. Increases in AEA concentration reduced sperm motility significantly by using high concentration of AEA compared to the low concentrations of AEA and control group. In addition, FAAH had a positive impact on increasing the sperm motility compared to the control group and all levels of AEA ($P<0.05$) *(Table 1)*. These effects take place through the change in the number of sperm with fast progressive motility.

Table 1*. Effect of different concentration of Met-anandamide and FAAH in freezing medium on motility (mean±SE) of frozen-thawed ram sperm (evaluated by CASA)*

Tablo 1*. Dondurulmuş-çözdürülmüş koç sperminin motilitesi (ortalama±SE) üzerine farklı Met-anandamid ve FAAH konsantrasyonunun etkisi (CASA ile değerlendirme)*

Groups	Concentration	Motile Sperm	Progression (%)			
			Fast Progressive	Slow Progressive	Non- Progressive	Non- Motile
Anandamide	10 nM	50.37±5.69^{a}	19.76±6.58^{ab}	23.35±1.34	6.65±0.69	50.23±2.94^{ab}
	100 nM	38.79±3.05^{ab}	12.5±2.51^{a}	18.46±1.16	7.22±0.71	61.81±3.24^{a}
	1000 nM	31.19±3.18^{b}	6.37±0.95^{a}	16.57±1.96	7.65±1.01	69.41±3.12^{a}
FAAH	0.025 IU/ml	65.67±2.37^{c}	26.26±1.33^{b}	30.67±3.69	9.15±0.58	33.93±2.37^{b}
Control		43.74±4.01^{a}	19.49±4.7^{ab}	17.15±0.76	6.5±0.83	56.86±4.31^{ab}

^{a,b,c} Numbers with different superscripts in the same column differ significantly ($P<0.05$)

In some motility, parameters of sperm which are associated with velocity including VCL, VSL, VAP and BCF, significant differences were found between AEA groups and control group; and groups containing high concentrations of AEA (100nM and 1000nM) and FAAH group were significantly different as well (P<0.05); as in the presence of FAAH, sperm moves faster and beats cross frequency (BCF) and the mean angle degree (MAD) increased. These parameters decrease in the presence of high concentrations of AEA. High concentration of AEA significantly reduces the percentage of sperms with linearity (LIN) and straightness (STR) moving and also reduces sperm wobble (P<0.05) *(Table 2)*.

Sperm Viability

Eosin-Nigrosin and HOS tests indicated that AEA did not affect viability of sperm (P>0.05) *(Fig. 1)*; while FAAH had an impact on vitality of ram sperm significantly (P<0.05) compared to 1000nM AEA group.

Acrosomal Status

The findings are shown in *Fig. 2*. They revealed that Met-anandamide in 1.000 mg/ml concentration can protect ram sperm against induced cryo-capacitaction and acrosome reaction significantly (P<0.05) subsequent freezing and thawing and can maintain the sperm viability by delaying capacitation and acrosome reaction. Our findings suggest that FAAH stimulates acrosome reaction in spermatozoa significantly (P<0.05) *(Fig. 2)*.

DISCUSSION

Poor sperm motility after cryopreservation is associated with a significant reduction in fertility, pointing to the importance of preserving motility in cryopreserved sperm. Despite the availability of intricate assisted reproductive

Table 2. *Effect of different concentration of Met-anandamide and FAAH in freezing medium on motility pattern (mean±SE) of frozen-thawed ram sperm (evaluated by CASA)*

Tablo 2. *Dondurulmuş-çözdürülmüş koç sperminin motilite modelleri (ortalama±SE) üzerine farklı Met-anandamid ve FAAH konsantrasyonunun etkisi (CASA ile değerlendirme)*

Groups	Concentration	Sperm Motility Pattern								
		VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	MAD (°)	ALH (µm)	BCF (Hz)	LIN (%)	WOB (%)	STR (%)
Anandamide	10 nM	30.28±2.62^{ab}	12.68±2.09^{ab}	19.33±2.05^{ab}	6.85±1.25^{a}	1.94±0.08^{ac}	0.16±0.05^{ab}	37.26±2.33^{a}	54.73±1.80^{a}	56.83±2.09^{ab}
	100 nM	25.94±2.21^{a}	9.52±1.45^{a}	15.43±1.52^{a}	6.40±1.18^{a}	1.70±0.08^{ab}	0.15±0.04^{a}	28.79±1.54^{b}	46.83±1.24^{bc}	46.69±1.86^{cd}
	1000 nM	21.57±0.89^{a}	7.47±0.56^{a}	12.79±0.74^{a}	3.49±0.29^{a}	1.45±0.07^{b}	0.07±0.01^{a}	27.16±1.16^{b}	44.89±0.86^{c}	45.25±1.16^{c}
FAAH	0.025 IU/ml	38.23±3.44^{b}	17.80±1.91^{b}	23.35±2.08^{b}	12.52±1.70^{b}	2.26±0.10^{c}	0.33±0.05^{b}	39.12±2.17^{a}	55.35±1.83^{a}	61.34±1.80^{a}
Control		30.28±2.99^{ab}	12.12±2.42^{ab}	19.73±2.52^{ab}	7.78±1.17^{ab}	1.92±0.11^{ab}	0.21±0.05^{ab}	33.66±1.98^{ab}	51.48±1.95^{ab}	53.79±2.01^{bd}

^{a,b,c,d} *Numbers with different superscripts in the same column differ significantly (P<0.05)*

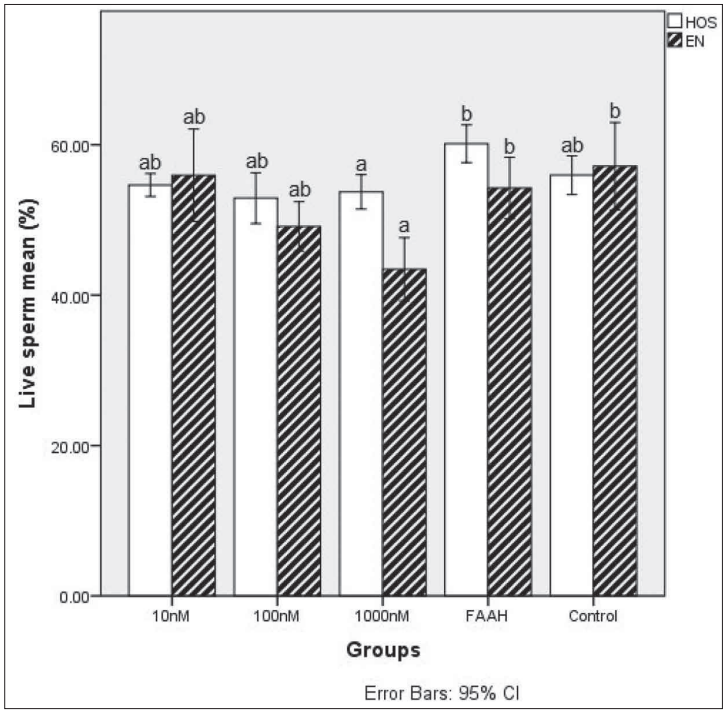
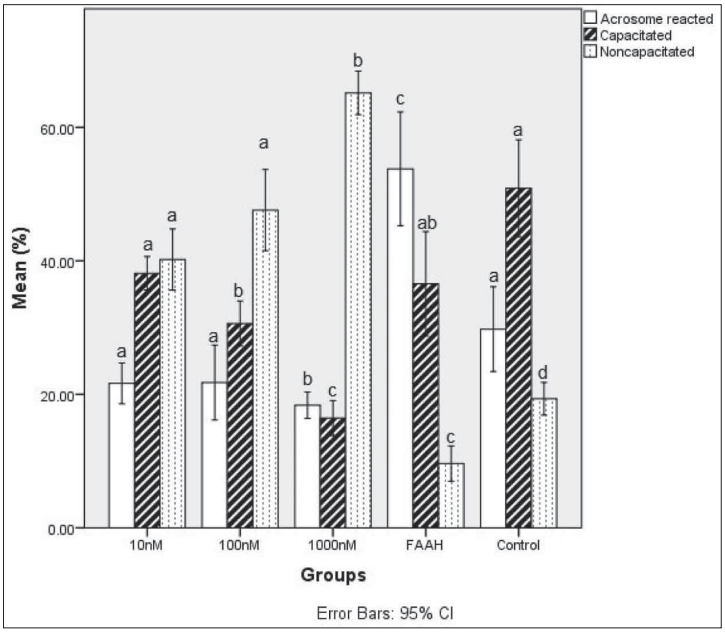


Fig 1. Comparison of sperm vitality of frozen-thawed ram sperm between treatments and control groups using Eosin-Nigrosin test and hypoosmotic swelling test (HOS test)
^{a,b} Columns with the same pattern and different superscripts differ significantly (P<0.05)

Şekil 1. Dondurulmuş-çözdürülmüş koç sperminin sperm canlılığının Eosin-Nigrosin testi ve hypoosmotic swelling testi (HOS test) kullanılarak karşılaştırılması
^{a,b} Aynı model ve farklı harf taşıyan sütunlar arasındaki fark istatistiksel olarak önemlidir (P<0.05)

Fig 2. Effect of different concentration of Met-anandamide and FAAH in freezing medium on acrosome reaction and capacitation status of frozen-thawed ram sperm (evaluated by chlortetracycline test)
[a,b,c,d] Columns with the same pattern and different superscripts differ significantly (P<0.05)

Şekil 2. Dondurulmuş-çözdürülmüş koç sperminin akrozom reaksiyonu ve kapasitasyon durumu üzerine dondurma medyumundaki farklı Met-anandamid ve FAAH konsantrasyonunun etkisi (chlortetracycline testi ile değerlendirme)
[a,b,c,d] Aynı model ve farklı harf taşıyan sütunlar arasındaki fark istatistiksel olarak önemlidir (P<0.05)



techniques, the cryosurvival and pregnancy rates with frozen semen are lower than those with fresh semen [26-28]. In this study, we observed a detrimental impact of cryopreservation on overall sperm quality. Regardless of whether the spermatozoa was treated with endocannabinoids before freezing, significant decreases in the percentage of motile spermatozoa, motion characteristics, and in the percentage of viable spermatozoa with intact acrosomes were observed.

It was also revealed through CASA evaluation that presence of AEA in freezing medium influenced motility of ram sperm after thawing. Here increases in AEA concentration reduced sperm motility significantly by using high concentration of AEA compared to the low concentrations of AEA and control group. These impacts take place through the change in the number of sperm with rapid progressive motility. AEA reduced some motility parameters of sperm that are associated with velocity like VCL, VSL, VAP and BCF and in the presence of high concentrations of AEA sperm moves slower and beats cross frequency (BCF) and the mean angle degree (MAD) were reduced. High concentration of AEA significantly reduces the percentage of sperms with linearity (LIN) and straightness (STR) moving and also reduces sperm wobble. In addition to increasing the number of motile spermatozoa, AEA can improve sperm motion characteristics in preselected subpopulations of spermatozoa, which is consistent with other studies which found that AEA reduces motility of human sperm by mitochondrial activity reduction. Athanasiou et al.[29] have revealed that cannabinoids inhibit mitochondrial membrane potential, oxygen consumption and ATP production by preventing the mitochondrial respiratory chain. Sperm motility, along with capacitation and acrosome reaction are all energy consuming processes. The sperm kinetic activity is dependent on many factors such as integrity of all flagellar structural proteins, oxidation of energetic substrates and ATP production, transformation of chemical energy into mechanical movement, the activity of all enzymes involved in the flagellar beating [19,30]. The decline in mitochondrial activity would be expected to reduce energy supply, thus affecting different sperm functions as well as motility but also capacitation and acrosome reaction. If endocannabinoids inhibit energy metabolism in sperm, then it could be possible that the inhibition of all sperm functions by endocannabinoids could origin from the same mechanism of action, i.e. reduction of the energetic reserves on which all these processes rely on [19].

To this respect, Rossato et al.[19] have shown that AEA induced a dose-dependent decrease of the sperm mitochondrial activity that paralleled the reduction of sperm motility without affecting viability and Whan et al.[20] have shown that Delta-9 tetrahydrocannabinol reduce human sperm motility. These studies are consistent with our results and our findings are consistent with these studies.

Considering our observations, such findings support the hypothesis that cryopreservation somehow precociously triggers the signal transduction pathway leading to capacitation, and that cryo-capacitation is partly responsible for the reduced fertility of thawed semen. Physiological capacitation is now accepted to be regulated by intracellular signaling pathways that results in the tyrosine phosphorylation of various sperm proteins [12-15]. The signaling pathways are not completely clear, although protein kinase A and protein tyrosine kinases appear to be involved. In the present study, the reduction of sperm capacitation of frozen-thawed ram sperm at the exposure of AEA in freezing medium confirms the above information. The results in *Fig. 2* reveal that Met-anandamide in 1000

mg/ml concentration can protect ram sperm against induced cryo-capacitaction and acrosome reaction significantly subsequent freezing and thawing and can maintain sperm viability by delaying the capacitation and acrosome reaction.

In this study AEA inhibited ram sperm acrosome reaction which is consistent with other studies which suggest AEA inhibits capacitation-induced acrosome reaction [19]. Based on the inhibitory effects of endocannabinoids on sperm capacitation, available substantially homogeneous data indicate that CB1 activation reduces sperm ability to undergo acrosome reaction both in vertebrate and invertebrates [19,20,31-33]. More importantly, endocannabinoids do not affect fertility of eggs; this shows that these effects are directed just to sperm [31,32]. The mechanisms activated by endocannabinoids determining the prevention of the sperm acrosome reaction are still unclear. It is well-known that the regulation of ion fluxes through the ion channels of sperm plasma membrane are essential for the acrosome reaction, and the most essential regulator appears to be calcium (Ca^{2+}) signaling [34,35]. In this respect, Rossato et al.[19] have revealed that the endocannabinoid AEA do not change the intracellular Ca^{2+}concentrations in human sperm, thus possibly ruling out any interfering effect of endocannabinoids on Ca^{2+} signaling. It is also known that CB1 is a receptor of G-protein coupled that has been revealed to inhibit adenylate cyclase activity [36,37]. Adenylate cyclase/cAMP/protein kinase A pathway plays an important role in the sperm capacitation and acrosome reaction: intracellular cAMP levels rise during acrosome reaction, cell permanent cAMP analogues induce the acrosome reaction, pharmacological inhibitors of cAMP-dependent PKA reduce this exocytotic event supporting a role for the adenylate cyclase/cAMP/PKA system in the signaling pathway leading to acrosome reaction of sperm and egg fertilization [12-15]. The inhibitory effects of endocannabinoids on this fundamental signaling pathway as recently described in mammalian sperm [33] may explain the negative actions of these agents activating the CB1 on sperm capacitation and acrosome reaction.

Furthermore, in inhibition of capacitation and acrosome reaction by endocannabinoids, many hypotheses may explain the inhibitory effects of endocannabinoids on mitochondrial function: endocannabinoids may interfere with mitochondrial electron transport [38,39], mitochondrial activity via depletion of NADH as previously shown [40], mitochondrial permeability transition pore complex as recently suggested [40]. The possible interference of endocannabinoids with glucose uptake and utilization for ATP production as previously suggested by experimental studies utilizing testicular extracts [41], seems not plausible since in many different cell types endocannabinoids have been shown to increase glucose uptake instead of decreasing it as we have recently demonstrated in human adipocytes [42] confirming recent observations in rat 3T3-L1 adipocytes [43]. Thus, an increase of AEA could be completing an important duty via improving mechanisms involved in the obtaining of the sperm fertilizing capacity.

Findings of this study indicated that FAAH as hydrolyzing enzyme of anandamide had a positive effect on increasing sperm motility and motility parameters associated with velocity such as VCL, VSL, VAP and BCF. Findings also suggested that in the presence of FAAH, sperm moves faster and beats cross frequency (BCF) and the mean angle degree (MAD) was increased. Sperm capacitation and acrosome reaction are also stimulated with FAAH. Endocannabinoids can be metabolized inside the cell by multiple pathways: AEA is a substrate mainly for fatty acid amide hydrolase (FAAH), that breaks the amide bond and releases arachidonic acid (AA) and ethanolamine [44]. FAAH is bound to intracellular membranes [45]. Obstruction AEA degradation by URB97 (an inhibitor of FAAH) caused sperm capacitation and the pre-incubation of sperm with CB1 or TRPV1 antagonists inverted this result [46]. It is proposed that cellular uptake of AEA can be controlled with FAAH by maintaining and/or creating an inward concentration gradient that drives the facilitated diffusion of AEA through anandamide membrane Transporter (AMT). Although FAAH is not the only factor controlling AEA transport, its pivotal function in degradation of AEA may explain why it is modulated in some pathophysiological conditions.

In conclusion, the importance of the endocannabinoid system makes it an attractive target for improvement of mammals' sperm freezing methods and subsequent *in vitro* embryo production, artificial insemination and pharmacological interventions to control male fertility.

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Research Article

Determination of Biofilm Production, Genotype and Antibiotic Resistance Profiles of *Enterococcus feacium* Isolates Originated from Dog, Cat and Human

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Abstract

The aim of the study was to determine the biofilm production, genotypes, antibiotics resistance patterns and antibiotypes of 82 E*nterococcus faecium* strains isolated from dog, cat and human. Of examined strains biofilm production detected totally 72 (87.8%) in 35 (97.2%) dog, 22 (78.6%) cat and 15 (83.3%) human isolates. Genotyping of isolates was performed by RAPD-PCR and 16, 3 and 4 different profiles were detected in dog, cat and human isolates, respectively. In total of 98.8% with a maximum resistance to nalidixic acid and 4.9% with the lowest resistance to vancomycin was found. None of vancomycin resistance 4 isolates, vancomycin resistance genes (*vanA, vanB, vanC1/C2* or *vanD*) has been detected. Antibiotyping of isolates was performed with UPGMA and 5 groups of dog, 10 groups of cat and 7 groups of human isolates were determined. The results from this study indicate that healthy dogs and cats are a source of Antibiotic resistant enterococci and may act as a reservoir of resistance that can be transferred from pets to people. Also our results demonstrated that the phenotype and genotype patterns found among enterococci strains from dogs, cats and humans were heterogeneous.

Keywords: *Antibiotic resistance, Antibiotype, Biofilm, E. faecium, Genotype*

Köpek, Kedi ve İnsan Orijinli *Enterococcus feacium* İzolatlarının Biofilm Üretimi, Genotip ve Antibiyotik Direnç Profillerinin Belirlenmesi

Özet

Bu çalışmada köpek, kedi ve insan orijinli 82 *Enterococcus faecium* izolatının biyofilm üretme özellikleri ile genotip ve antibiyotik direnç profilleri araştırıldı. İncelenen izolatların köpek 35 (%97.2), kedi 22 (%78.6) ve insan 15 (%83.3) olmak üzere toplam 72 (%87.8)'sinde biyofilm oluşumu saptandı. RAPD-PCR ile yapılan genotiplendirilmede; köpek, kedi ve insan izolatlarında sırasıyla 16, 3 ve 4 farklı profil belirlendi. Toplamda en yüksek dirençlilik %98.8 ile nalidiksik aside en düşük dirençlilik ise %4.9 ile vankomisine bulundu. Vankomisine dirençli 4 izolatın hiçbirinde vankomisin dirençlilik geni (*vanA, vanB, vanC1/2* veya *vanD*) saptanamadı. UPGMA ile yapılan antibiyotiplendirmede köpek izolatları 5, kedi izolatları 10 ve insan izolatları da 7 grup altında toplandı. Çalışma sonuçları sağlıklı köpek ve kedilerin antibiyotiklere dirençli enterokoklar için kaynak oluşturabileceğini ve insanlara direncin aktarılmasında rezervuar olarak rol oynayabileceğini göstermiştir. Ayrıca, elde edilen sonuçlarla köpek, kedi ve insanlar orijinli enterokoklarda bulunan fenotipik ve genotipik patternlerin heterojen olduğu belirlenmiştir.

Anahtar sözcükler: *Antibiyotik direnci, Antibiyotip, Biyofilm, E. faecium, Genotip*

INTRODUCTION

Enterococci are a dominant bacterial group in the intestinal flora of human and animals. They are increasingly associated with nosocomial infections. The natural ability of enterococci to acquire, accumulate, and share extra chromosomal elements encoding virulence traits or antibiotic resistance genes. Acquired resistance to various antimicrobial agents and available antibiotics currently limits the therapeutic options [1]. Biofilm is a structured community of microorganisms encapsulated within a self-developed polymeric matrix and adherent to various biotic and abiotic surfaces irreversibly. Cells in biofilms are highly resistant to antibiotics and phagocytosis, and removal

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of the medical device is frequently the only appropriate cure, which may not always be possible. Because of the importance of biofilm formation to enterococcal infection, isolating the factors involved has been of great interest [2]. Most enterococci have inherent resistance to various antibiotics such as cephalosporins and semi synthetic penicillinase resistant penicillins, aminoglycosides and clindamycin [3]. Studies have recently focused on enterococcal infections in veterinary medicine in parallel with coming out animal factor in transmission of vancomycin resistant enterococci (VRE) to humans [4,5]. To differentiate the *Enterococcus* strains, various typing methods categorized into phenotypic and genotypic methods have been used in epidemiological studies in the past two decades. Antibiotyping of enterococci isolates by several methods were performed based on their different antibiotic resistance profiles [6]. Genotyping of *Enterococcus* species can be made by several methods such as random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) [3,7]. Antibiotic-resistant enterococci were grouped by RAPD-PCR and a scattered distribution was noted, indicating that resistance was not related to a particular clone [1]. The demonstration of diversity in the RAPD patterns on the species level will be essential for understanding the molecular ecology of enterococci in the intestine of animals and humans [8]. The aims of this study were to investigate the biofilm production, genotyping, antibiotic resistance patterns and antibiotyping of *E. faecium* strains isolated from dog, cat and human. The present study also performed for detecting the relationship between biofilm production and antibiotic resistance.

MATERIAL and METHODS

Bacterial Isolates

A total of 82 *E. faecium* isolates, including 36 dogs, 28 cats and 18 human origins, were used in study. All isolates were phenotypically identified to the species level using conventional methods and were confirmed by PCR [1].

Biofilm Formation

Congo red agar was used to detect biofilm production. Black colonies on Congo red agar were evaluated as biofilm production positive, pink or colorless colonies were evaluated as biofilm production negative [9].

RAPD-PCR Amplification

RAPD-PCR analysis was done using the primer ERIC2 (5'-AAG TAA GTG ACT GGG GTG AGC G-3') as described previously [10,11]. The similarities and numbers of the bands between RAPD patterns were determined based on the Dice similarity coefficient. To create a dendrogram that graphed genetic relatedness between *E. faecium* isolates with the cut-off value of 70%, "Unweighted Pair Group Method with Arithmetic Averages (UPGMA)" was employed using CHEF-DR® III, Quantity One® Software (Bio-Rad Laboratories, Hercules, CA).

Antibiotic Susceptibility Test

All isolates were tested against 12 different antibiotics using disc diffusion method. A susceptibility test result of each antibiotic was evaluated according to CLSI interpretive standards [12].

Detection of van Genes

The genes responsible for resistance to vancomycin (*vanA*, *vanB*, *vanC1/2* and *vanD*) were investigated by PCR as described previously [4].

Antibiotyping of Isolates

The antibiotic susceptible/resistance results were recorded as susceptible, intermediate susceptible or resistant. The schematic diagram including these results have been drawn and the similarities of patterns were determined based on the Dice similarity coefficient using CHEF-DR® III, Quantity One® Software (Bio-Rad Laboratories, Hercules, CA) with a cut of value of 70% [13].

RESULTS

Capacity of Biofilm Production

E. faecium isolates from dog, cat and human feces had a high capacity for biofilm production, with 97.2%, 78.6% and 83.3% of isolates, respectively. Dog isolates had higher capacity for biofilm production compared with cat and human isolates *(Table 1)*.

RAPD-PCR and Genotyping

In genotyping by RAPD-PCR 16, 3 and 4 different profiles were determined in dog, cat and human isolates, respectively *(Fig. 1, 2* and *3)*. Analysis of RAPD-PCR patterns in dog isolates revealed the presence of 16 RAPD types (A-P) based on 70 % similarities. When considered genotypic proximity of dog isolates was observed hetero-

Table 1. *Distribution of biofilm formation in E. faecium isolates by origins*
Tablo 1. *E. faecium izolatlarında biyofilm oluşumunun orijinlerine göre dağılımı*

Origin	Biofilm Formation			
	Positive		Negative	
	n	(%)	n	(%)
Dog (n=36)	35	97.2	1	12.8
Cat (n=28)	22	78.6	6	21.4
Human (n=18)	15	83.3	3	16.7
Total (n=82)	72	87.8	10	12.2

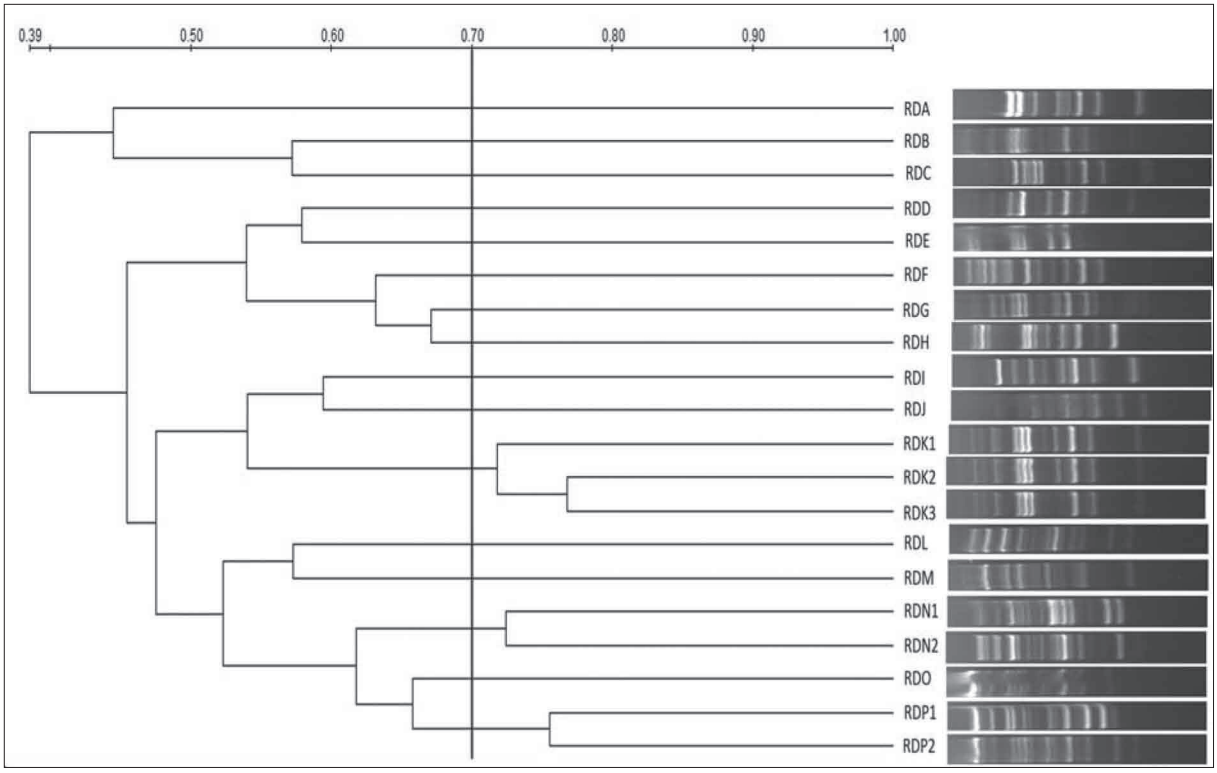

Fig 1. RAPD patterns of *E. faecium* isolated from dog and dendrogram obtained by UPGMA
Şekil 1. Köpek orijinli *E. faecium* izolatlarının RAPD patternleri ve UPGMA ile sağlanan dendrogram

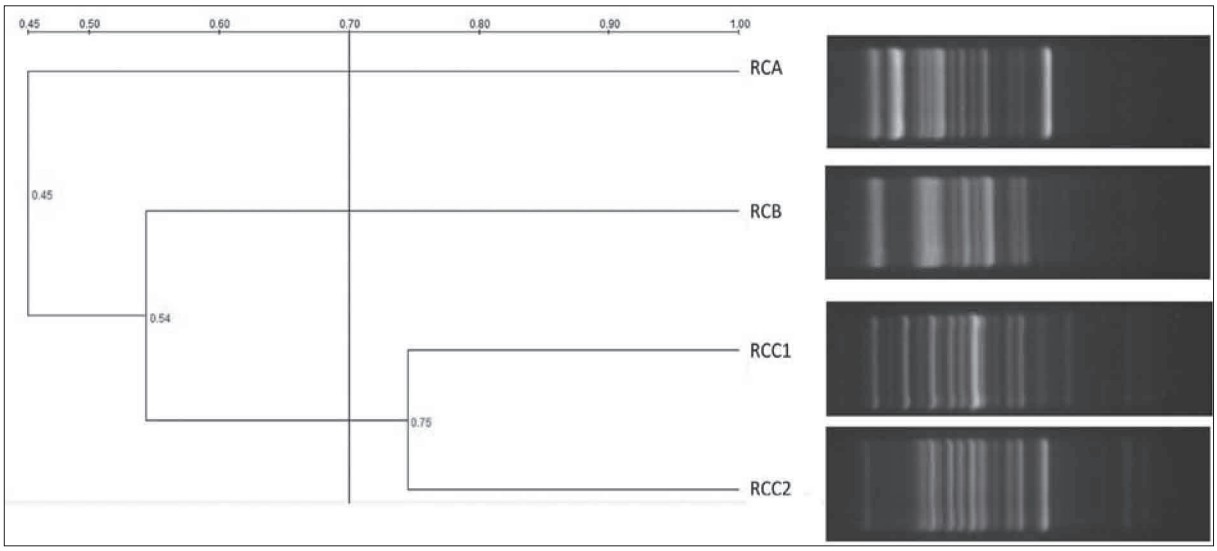

Fig 2. RAPD patterns of *E. faecium* isolated from cat and dendrogram obtained by UPGMA
Şekil 2. Kedi orijinli *E. faecium* izolatlarının RAPD patternleri ve UPGMA ile sağlanan dendrogram

geneity among groups. Isolates were represented in 7 major types: type A (n=2), type D (n=2), type E (n=2), type H (n=7), type K (n=9), type N (n=4) and type P (n=2), and others separate groups (B, C, F,G, I, J, L, M and O). On the other hand, cat isolates were collected in 3 groups (A-C) based on 70% similarities. When considered genotypic proximity of cat isolates was observed homogeneity among groups. Isolates were presented in 3 major types: type A (n=9), type B (n=12) and type C (n=7). Likewise, human isolates were classified into 4 groups (AD) based on 70% similarities. Isolates were presented in 3 major types: type B (n=7), type C (n=6) and type D (n=4), and type A (n=1) separate.

Antibiotic Susceptibility and Phenotype

Antibiotic resistance (R) / susceptibility (S) patterns

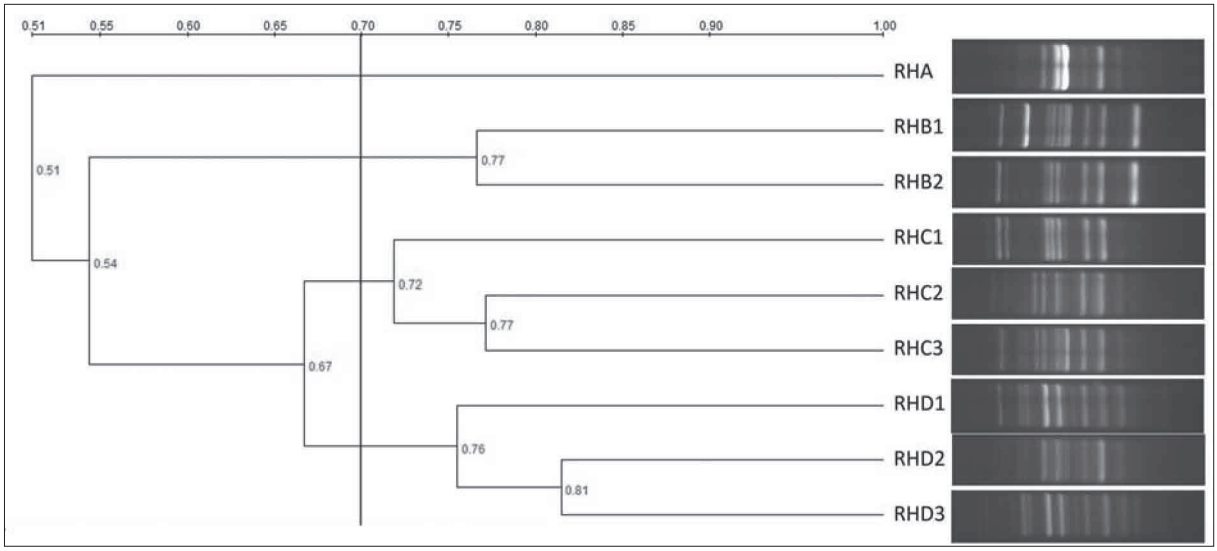

Fig 3. RAPD patterns of *E. faecium* isolated from human and dendrogram obtained by UPGMA
Şekil 3. İnsan orijinli *E. faecium* izolatlarının RAPD patternleri ve UPGMA ile sağlanan dendrogram

Table 2. *Antibiotic resistance/susceptibility patterns of E. faecium isolates*
Tablo 2. *E. faecium izolatlarının antibiyotik direnç/duyarlılık patternleri*

Antibiotics	Dog (n=36)		Cat (n=28)		Human (n=18)		Total (n=82)	
	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)
Ampicillin	0 (0)	36 (100)	8 (28.6)	20 (71.4)	1 (5.6)	17 (94.4)	9 (11)	73 (89)
Penicillin G	2 (5.6)	34 (94.4)	11 (39.3)	17 (60.7)	4 (22.2)	14 (77.8)	17 (20.7)	65 (79.3)
Vancomycin	0 (0)	36 (100)	3 (10.7)	25 (89.3)	1 (5.6)	17 (94.4)	4 (4.9)	78 (95.1)
Bacitracin	13 (36.1)	23 (63.9)	15 (53.6)	13 (46.4)	14 (77.8)	4 (22.2)	42 (51.2)	40 (48.8)
Oxytetracyclin	6 (16.7)	30 (83.3)	14 (50)	14 (50)	4 (22.2)	14 (77.8)	24 (29.3)	58 (70.7)
Kanamicin	36 (100)	0(0)	27 (96.4)	1 (3.6)	17 (94.4)	1 (5.6)	80 (97.6)	2 (2.4)
Erythromycin	11 (30.6)	25 (69.4)	15 (53.6)	13 (46.4)	9 (50)	9 (50)	35 (42.7)	47 (57.3)
Amoxicillin	0 (0)	36 (100)	5 (17.9)	23 (82.1)	4 (22.2)	14 (77.8)	9 (11)	73 (89)
Norfloxacin	7 (19.4)	29 (80.6)	3 (10.7)	25 (89.3)	5 (27.8)	13 (72.2)	15 (18.3)	67 (81.7)
Nalidixic acid	36 (100)	0 (0)	27 (96.4)	1 (3.6)	18 (100)	0 (0)	81 (98.8)	1 (1.2)
Cefalotin	22 (61.1)	14 (38.9)	18 (64.3)	10 (35.7)	11 (61.1)	7 (38.9)	51 (62.2)	31 (37.8)
Ciprofloxacin	22 (61.1)	14 (38.9)	6 (21.4)	22 (78.6)	3 (16.7)	15 (83.3)	21 (25.6)	61 (74.4)

of 82 *E. faecium* isolates are presented in *Table 2*. None of vancomycin resistance 4 isolates, vancomycin resistance genes *(vanA*, *vanB*, *vanC1/C2* or *vanD)* has been detected. Multiple antibiotic resistance phenotypes of isolates are presented in *Table 3*. Multi-drug resistance (MDR) was observed to as few as two and as many as twelve antibiotics regardless of class. Our findings showed that cat isolates resistance to multiple antibiotics greater frequency than dog and human isolates.

We also determined that biofilm forming strains showed resistance to antibiotics more frequently than not biofilm forming strains, when examined the relationship biofilm formation and antibiotic resistance in isolates *(Table 4)*.

Antibiotyping

Antibiotyping of isolates performed by UPGMA and were collected in 5 groups of dog, 10 groups of cat and 7 groups of human isolates, respectively *(Fig. 4, 5* and *6)*. According to these results; 36 dog *E. faecalis* isolates were collected in 5 main groups (A-E) based on 70% similarities. Groups were showed as ADA (n=2); ADB1-B7 (n=7); ADC (n=1); ADD (n=1) and ADE1-E17 (n=25). In like manner, 10 main groups (A-J) from 28 cat isolates were generated to 70% similarity rate. Groups were showed as ACA (n=1); ACB1-B2 (n=2); ACC1-C2 (n=2); ACD1-D8 (n=8); ACE (n=1); ACF (n=1); ACG1-G2 (n=2); ACH1-H2 (n=2); ACI (n=1) and ACJ1-J7 (n=8). On the other hand, 18 human isolates were collected in 7 main groups (A-G) to 70% similarity

Table 3. *Multidrug resistance patterns in E. faecium isolates by origin*
Tablo 3. *E. faecium izolatlarında çoklu ilaç direnç patternleri*

Num. of Antibiotics	Antibiotic Resistance Phenotype	Number of Isolates with Phenotype		
		Dog	Cat	Human
12	AMP-P-VAN-B-OTET-KAN-ERY-AMX-NOR-NAL-CEF-CIP	-	1	-
10	AMP-P-VAN-B-OTET-KAN-ERY-AMX-NAL-CEF	-	1	-
9	P-B-OTET-KAN-ERY-NOR-NAL-CEF-CIP	1	-	-
8	AMP-P-B-OTET-KAN-ERY-NAL-CEF	-	1	-
	AMP-B- OTET-KAN-ERY-AMX-NAL-CEF	-	1	-
	P-B-OTET-KAN-ERY-NOR-NAL-CIP	-	1	-
7	AMP-P-VAN-B-OTET-KAN-NAL	-	-	1
	AMP-P-OTET-KAN-ERY-NAL-CEF	-	1	-
	AMP-OTET-KAN-ERY-AMX-NAL-CEF	-	1	-
	P-B-OTET-KAN-ERY-NAL-CEF	-	2	-
	P-OTET-KAN-ERY-NAL-CEF-CIP	-	1	-
	B-OTET-KAN-ERY-NOR-NAL-CEF	1	-	-
	B-KAN-ERY-AMX-NOR-NAL-CEF	-	-	1
	B-KAN-AMX-NOR-NAL-CEF-CIP	-	-	1
6	AMP-P-KAN-NAL-CEF-CIP	-	1	-
	P-B-OTET-KAN-ERY-NAL	-	-	1
	P-B-KAN-ERY-NAL-CEF	-	-	2
	VAN-B-OTET-KAN-ERY-NAL	-	1	-
	B-OTET-KAN-ERY-NAL-CEF	-	1	-
	B-OTET-KAN-NOR-NAL-CEF	2	-	-
	B-KAN-NOR-NAL-CEF-CIP	1	-	-
	KAN-AMX-NOR-NAL-CEF-CIP	-	-	1
5	P-OTET-KAN-NAL-CEF	2	-	-
	B-KAN-ERY-NAL-CEF	1	-	1
	B-KAN-AMX-NAL-CEF	-	-	1
	B-KAN-NOR-NAL-CEF	1	-	-
	OTET-KAN-ERY-NAL-CEF	-	-	1
	OTET-KAN-NOR-NAL-CIP	-	-	1
4	P-KAN-NAL-CEF		1	
	B-KAN-ERY-NAL	1	-	2
	B-KAN-NOR-NAL	1	-	1
	B-KAN-NAL-CEF	4	1	1
	B-ERY-NAL-CEF		1	-
	KAN-ERY-NAL-CEF	4	-	1
	KAN-NAL-CEF-CIP	-	1	-
3	AMP-KAN-NAL	-	1	-
	B-KAN-NAL	1	2	1
	KAN-ERY-NAL	5	1	-
	KAN-NAL-CEF	4	-	-
	OTET-KAN-NAL	-	2	-
2	KAN-NAL	8	1	-
	KAN-CEF	-	1	-

AMP: *Ampicillin (30 mg),* ***P:*** *Penicillin G (10 mg),* ***VAN:*** *Vancomycin (30 mg),* ***B:*** *Bacitracin (10 mg),* ***OTET:*** *Oxytetracyclin (30 mg),* ***KAN:*** *Kanamycin (5 mg),* ***ERY:*** *Erythromycin (15 mg),* ***AMX:*** *Amoxicillin (25 mg),* ***NOR:*** *Norfloxacin (30 mg),* ***NAL:*** *Nalidixic acid (30 mg),* ***CEF:*** *Cefalotin (30 mg),* ***CIP:*** *Ciprofloxacin (5 mg)*

Table 4. *Relationship biofilm formation and antibiotic resistance in E. faecium isolates*
Tablo 4. *E. faecium izolatlarında biyofilm oluşturma ve antibiyotik direnç ilişkisi*

Antibiotics	Dog (n=36)		Cat (n=28)		Human (n=18)		Total (n=82)	
	Biofilm Production (n)		Biofilm Production (n)		Biofilm Production (n)		Biofilm Production (n)	
	+ (35/%)	- (1/%)	+ (22/%)	- (6/%)	+ (15/%)	- (3/%)	+ (72/%)	- (10/%)
Ampicillin	0/0	0/0	6/27.3	2/33.3	0/0	1/33.3	6/8.3	3/30
Penicillin G	2/5.7	0/0	8/36.4	3/50	2/13.3	2/66.7	12/16.7	5/50
Vancomycin	0/0	0/0	2/9.1	1/16.7	0/0	1/33.3	2/2.8	2/20
Bacitracin	13/37.1	0/0	12/54.5	3/50	11/73.3	3/100	36/50	6/60
Oxytetracyclin	6/17.1	0/0	10/45.5	4/66.7	2/13.3	2/66.7	18/25	6/60
Kanamicin	35/100	1/100	21/95.5	6/100	15/100	2/66.7	71/98.6	9/90
Erythromycin	11/31.4	0/0	11/50	3/50	8/53.3	1/33.3	30/41.7	4/40
Amoxicillin	0/0	0/0	3/13.6	2/33.3	4/26.7	0/0	7/9.7	2/20
Norfloxacin	7/20	0/0	2/9.1	1/16.7	4/26.7	1/33.3	13/18.1	2/20
Nalidixic acid	35/100	1/100	22/100	5/83.3	15/100	3/100	72/100	9/90
Cefalotin	22/62.9	0/0	13/59.1	5/83.3	11/73.3	0/0	46/63.9	5/50
Ciprofloxacin	2/5.7	0/0	4/18.2	2/33.3	3/20	0/0	9/12.5	2/20

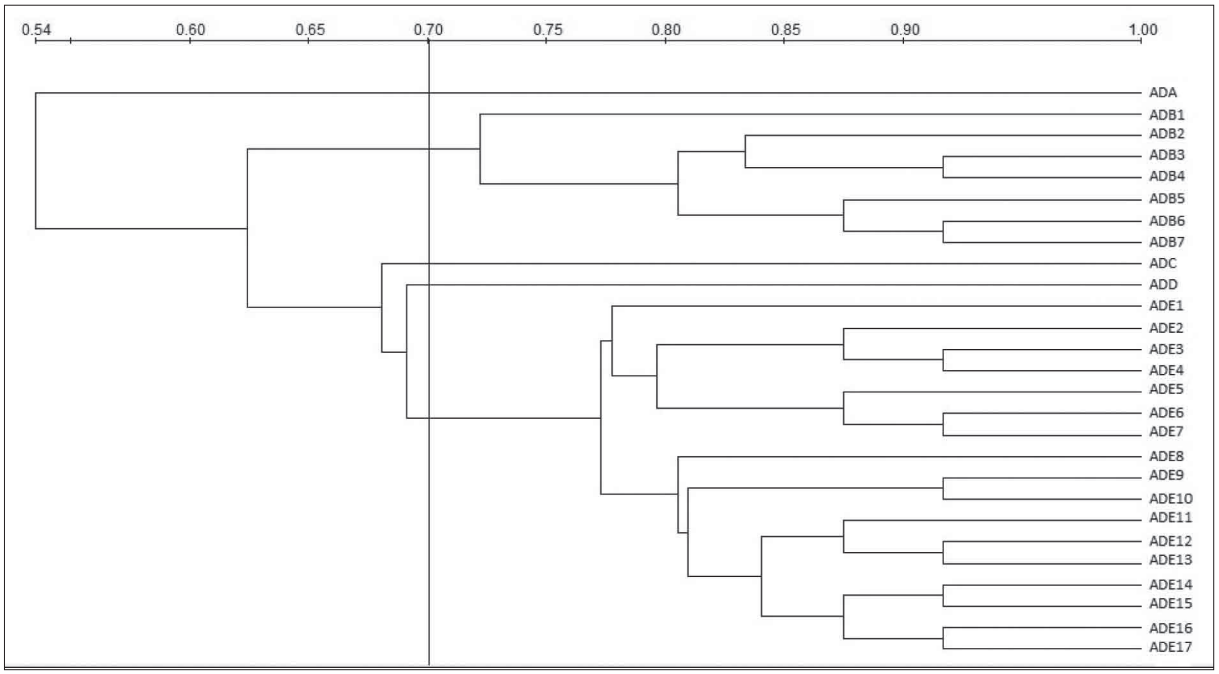

Fig 4. Antibiotype patterns of *E. faecium* isolated from dog and dendrogram obtained by UPGMA
Şekil 4. Köpek orijinli *E. faecium* izolatlarının antibiyotip patternleri ve UPGMA dendrogram

rate. Groups were showed as AHA (n=1); AHB (n=1); AHC (n=1); AHD1-D2 (n=2); AHE1-E2 (n=2); AHF1-F4 (n=4) and AHG1-G7 (n=7).

DISCUSSION

Enterococci are opportunistic pathogens and form part of the normal gastrointestinal flora in humans and animals. Over the last two decades, nosocomial infections caused by enterococci have emerged and their incidence has increased [14].

Biofilm production has been reported in some enterococcal infections. The major clinical infections have been caused by *E. faecium* capable of producing biofilms. Enterococci with biofilms are more highly resistant to antibiotics than planktonically growing enterococci, thus the potential impact of biofilm formation could be significant [7]. The prevalence of biofilm production reported

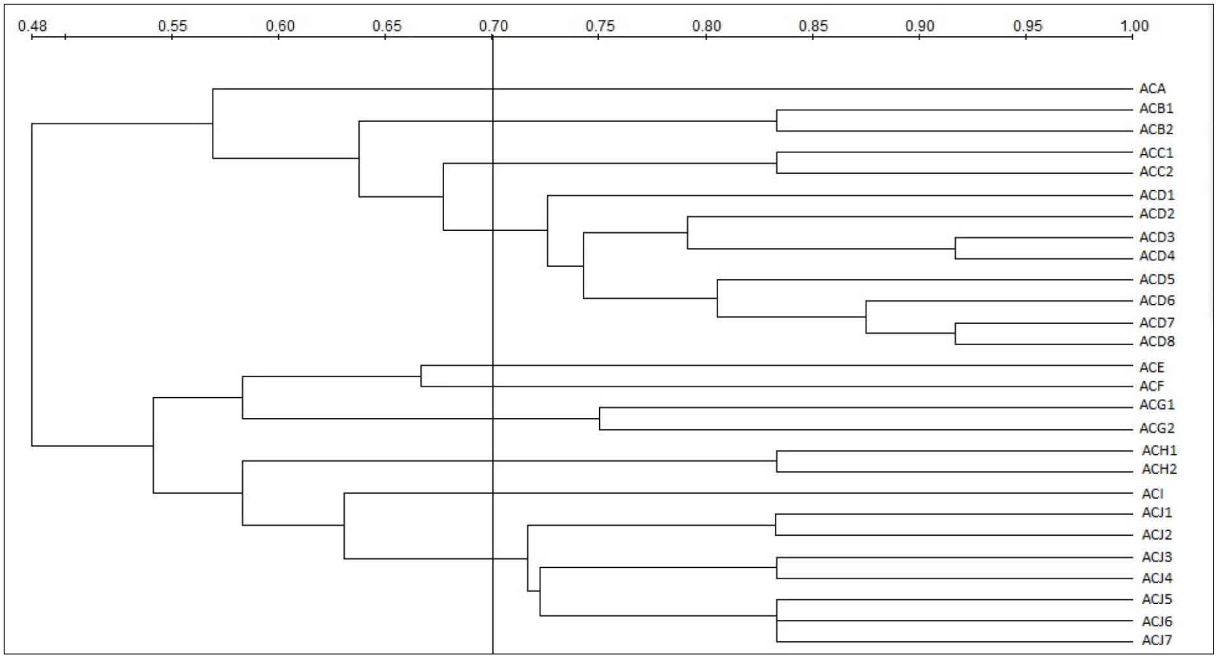

Fig 5. Antibiotype patterns of *E. faecium* isolated from cat and dendrogram obtained by UPGMA
Şekil 5. Kedi orijinli *E. faecium* izolatlarının antibiyotip patternleri ve UPGMA dendrogram

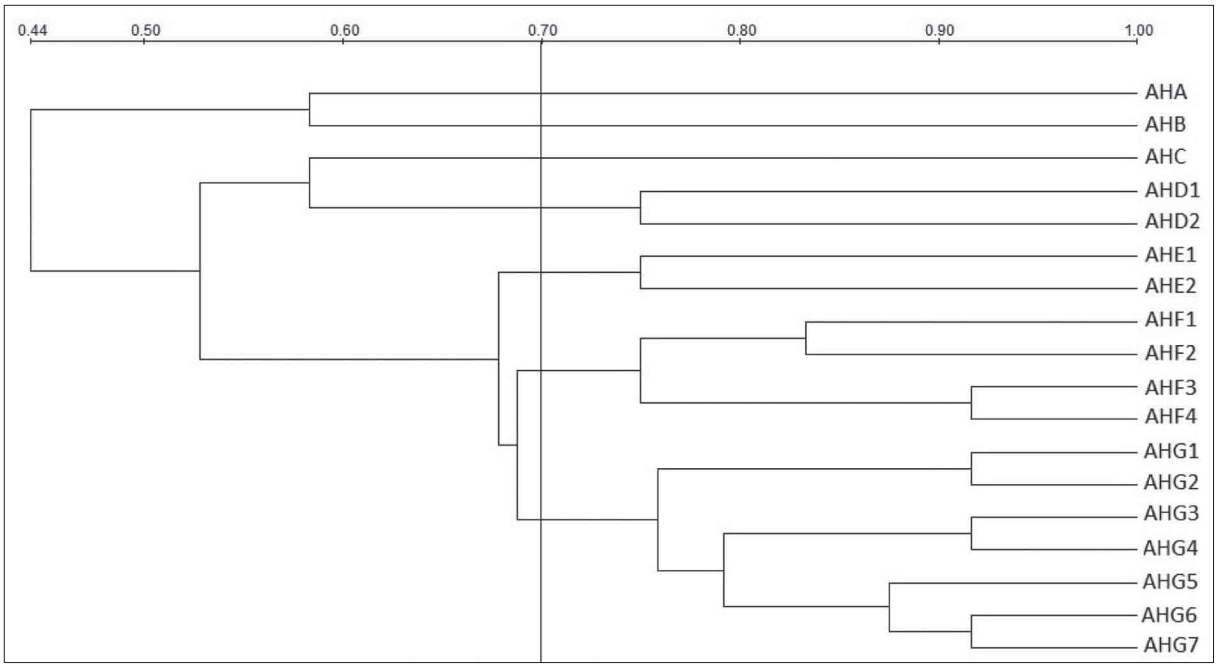

Fig 6. Antibiotype patterns of *E. faecium* isolated from human and dendrogram obtained by UPGMA
Şekil 6. İnsan orijinli *E. faecium* izolatlarının antibiyotip patternleri ve UPGMA dendrogram

previously for commensal isolates has been variable [3,9,15]. For instance, in a study from Italy 48% of *E. faecium* isolates from infected patients were able to form biofilm [16], while study from Greece reported biofilm production among 64.9% of *E. faecium* human and 34.4% of animal isolates [15]. Other investigators have reported similar results [9]. In this study, biofilm production was detected 35 (97.2%) of 36 dog, 22 (78.6%) of 28 cat and 15 (83.3%) of 18 human isolates. These results indicated that there may be more than one factor determining the production of biofilms in enterococci. In this study, we compared directly the biofilm formation between dog, cat and human *E. faecium* isolates and found that the dog isolates exhibited a significantly higher capacity for biofilm formation than isolates from cat and human isolates. Similar results reported previously [10,15].

The genotypic diversity of enterococcal isolates obtained from different origins was assessed using RAPD-PCR fingerprinting [10,11,17]. The RAPD-PCR analysis classified five profiles were discerned for *E. faecium* [11]. In a study RAPD-PCR analysis, *E. faecium* isolates (n=23) were grouped in four RAPD-types (clusters 1,4,6 and 7) at a similarity level of ca. 70% [10]. Similar results reported by Ben Omar et al.[17]. Getachew et al.[7] reported that VRE species showed diverse RAPD profiles with some clustering of strains based on the individual's background. In the other hand, in a study performed cats reported cats showed that clonal matches based on PFGE clearly demonstrate cross contamination between the resident cats and the hospital environment [3]. In this study RAPD-PCR profiles in dog isolates showed 16 types, of which 7 were predominant. When considered genotypic proximity of dog isolates was observed heterogeneity among groups. This suggests that isolates were polyclonally disseminated in our setting. On the basis of RAPD-PCR, 3 main groups could be distinguished in cat and 4 in human isolates. These findings imply that enterococci are genetically and phenotypically diverse. Our results are in good agreement with previous reports in which RAPD was found to allow rapid identification of unknown isolates [8,11,17].

Antibiotic resistances have been reported to better define the links between animals and humans [6,18,19]. Cats and dogs have played an important role in the human community [20,21], which allows them to have a good relationship with humans and contribute to their welfare; however, this relationship also poses serious risks of transmission of infectious agents to human [14]. Some enterococci are inherently resistant to some penicillins; and in the past few years, they have also shown increased resistance to vancomycin, cephalosporins, and amino-glycosides in nosocomial infections [3]. Vancomycin is, in some cases, the only antibiotic still effective in the treatment of nosocomial enterococcal infection in humans and is often considered the last treatment available in serious MDR infections [4].

Clinical cases involving VRE in companion animals are rare. Beside the several existing reports of VRE in animals [5], there are a limited number of studies dealing with the colonization of VRE in companion animals [4], even though VRE have been recorded in the intestinal tract of dogs and cats [6]. Simjee et al.[22] described the isolation of a high-level gentamicin-resistant (HLGR) and vancomycin-resistant *E. faecium* (VREfm) from a canine urinary tract infection in the USA, while Manson et al.[23] isolated a gentamicin-sensitive VREfm from a canine in New Zealand. Similar results cited by recently [5]. In contrast, no resistance to vancomycin was found several studies on enterococci from dogs and cats [3,14,21,24]. In our study only 3 cat and one human isolates were found to be resistant to vancomycin by phenotypically. However, all isolates were negative for *van* genes as recently reported [1].

In present study almost all isolates were found to be resistant to kanamycin and nalidixic acid. Cefalotin, bacitracin and erythromycin resistances were observed most frequently as compared to the other antibiotics. Similar results have been reported by other researchers [3,24]. As these antibiotics are habitually employed for treatment of a variety of infections in dogs and cats, their use could be the cause of a selective pressure for the resistance phenotype. Tetracycline, ciprofloxacin, penicillin and norfloxacin resistance was also common among isolates exhibiting MDR. These drags are used in dogs and cats for treatment of a variety of infections including urinary tract infections, periodontitis, upper respiratory tract infections and conjunctivitis [21].

The present study showed that cat isolates resistance to multiple antibiotics greater frequency than dog and human isolates as previously reported [18]. Fortunately, our isolates remain highly susceptible to ampicillin and amoxicillin as similar by Ossiprandi et al.[24]. Our findings contribute to the refinement of future therapeutic decisions in the management infections by enterococci of animals.

Antibiotyping of enterococci isolates by several methods were performed based on their different anti-biotic resistance profiles [1,3,6,10]. Antibiotic-resistant *E. faecium* isolates were grouped by RAPD-PCR and a scattered distribution was noted, indicating that resistance was not related to a particular clone as cited previously [1,23]. The spread of virulence/resistance traits in isolates of species and different RAPD-types suggest the pathogenic potential of species [10]. In a recently study, evaluated genetic similarities of the enterococcus isolates using the RAPD-PCR analysis and fingerprinting revealed no clonal lineage among tested isolates [1]. In the present study antibiotyping of isolates performed by UPGMA and were collected in 5, 10 and 7 groups of dog, cat and human isolates, respectively. These findings differ from the study by Jackson et al.[21] where they found that healthy domestic cats harbored MDR enterococcal strains of diverse clonal origin.

Previous reports demonstrated that there is a relationship between biofilm production and antibiotic resistance [9,10,15]. On the other hand, it has been proved that the very high concentrations of ampicillin, vancomycin and linezolid required inhibiting enterococcal biofilms in vitro and may explain why monotherapy with these agents frequently fails to eradicate biofilm infections. In the present study we determined that biofilm forming strains showed resistance to antibiotics more frequently than not biofilm forming strains, when examined the relationship biofilm formation and antibiotic resistance in isolates as cited previously [10].

In conclusions, we compared directly the biofilm formation between dog, cat and human *E. faecium* isolates and found that the dog isolates exhibited higher capacity

for biofilm formation than from cat and human isolates. The results demonstrated that the RAPD-PCR patterns found among enterococci strains from dog, cat and human were heterogeneous and considerably diverse. The demonstration of diversity in the RAPD patterns on the species level will be essential for understanding the molecular ecology of enterococci in the intestine of animals and humans. Further studies on the molecular typing and clinical significance of these isolates are needed. The results from this study indicate that healthy dogs and cats are a source of antibiotic resistant enterococci and may act as a reservoir of antibiotic resistance that can be transferred from pets to people. This risk is highlighted by Antibiotic resistance by use of the same antibiotics used to treat infections in humans and pets. Furthermore, the enterococcal isolates were MDR exhibiting resistance to as many as twelve antibiotics. Additional studies will address the presence of antibiotic resistance genes harbored by resistant isolates.

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Research Article

Effect of Pre-Emptive Dexketoprofen Trometamol on Acute Cortisol, Inflammatory Response and Oxidative Stress to Hot-Iron Disbudding in Calves [1]

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Abstract

The aim of this study was to evaluate the effect of dexketoprofen trometamol (DEX) on acute cortisol, inflammatory response and oxidative stress following hot-iron disbudding in calves. Twelve male Holstein Friesian dairy calves, weighing between 52 and 82 kg and 6-8 weeks of age were used for disbudding procedure. While calves (n = 6) in DEX group were treated with DEX (IV, 2 mg/kg) 30 min before disbudding, calves (n = 6) in control group were received only 0.9% saline (IV, 2 mg/kg). All calves were disbudded by an electrically heated hot-iron dehorner under local anaesthesia. The heart and respiratory rate were recorded before and after disbudding. Blood samples were collected at 30 min before and 15, 30 and 60 min after disbudding for analysis of serum cortisol, tumour necrosis factor alpha, interleukin-1 beta, interleukin-6, total antioxidant activity, malondialdehyde, glutathione and nitric oxide. In both groups, cortisol concentration was immediately increased after disbudding but dropped to lower level than baseline at 60 min after disbudding in DEX group ($P<0.05$). It was concluded that combination of a lidocaine and DEX may decrease or prevent acute cortisol, inflammatory response and oxidative stress during disbudding procedure.

Keywords: *Acute phase response, Calves, Cortisol, Dexketoprofen, Disbudding*

Boynuz Köreltme İşlemi Uygulanan Buzağılarda Pre-emptif Uygulanan Dexketoprofen Trometamol'un Akut Kortizol, İnflamatuar Yanıt ve Oksidatif Stres Üzerine Olan Etkisi

Özet

Bu çalışmanın amacı, koterle boynuz köreltme işlemi uygulanan buzağılarda, pre-emptif dexketoprofen trometamol'un (DEX) akut kortizol, inflamatuar yanıt ve oksidatif stres üzerine olan etkisinin değerlendirilmesidir. Boynuz köreltme işlemi için ağırlıkları 52-82 kg arasında, 6-8 haftalık yaşında 12 erkek Holştayn Frizyan ırkı buzağı kullanıldı. DEX grubunda bulunan buzağılara (n=6) boynuz köreltme işleminden 30 dk önce DEX (İV, 2 mg/kg) uygulanırken, kontrol grubuna (n=6) serum fizyolojik (İV, 2 mg/kg) uygulandı. Bütün buzağıların boynuzları lokal anestezi altında elektrokoterle köreltildi. Kalp frekansı ve solunum sayısı boynuz köreltme işleminden önce ve sonra kaydedildi. Serum kortizol, tümör nekrozis faktör alfa, interlöykin-1 beta, interlöykin-6, total antioksidan aktivite, malondialdehit, glutatyon ve nitrik oksit düzeylerinin belirlenebilmesi için boynuz köreltme işleminden 30 dk önce ve 15, 30, 60 dk sonra kan örnekleri toplandı. Her iki grupta boynuz köreltme işleminden hemen sonra kortizol konsantrasyonlarının yükseldiği ancak DEX grubunda boynuz köreltme işlemi sonrası 60. dk'da başlangıç değerlerin altına indiği gözlendi ($p<0.05$). Sonuç olarak, boynuz köreltme işlemi sırasında lokal anestezik ve DEX kombinasyonunun akut kortizol, inflamatuar yanıt ve oksitadif stresi azalttığı veya önlediği belirlendi.

Anahtar sözcükler: *Akut faz yanıt, Buzağı, Kortizol, Dexketoprofen, Boynuz köreltme*

INTRODUCTION

Disbudding is a routine but also painful procedure performed in young calves in dairy farms [1,2]. Two methods are frequently used such as caustic paste and hot-iron [2,3]. The physiological, behavioural and production responses before, during and after the procedure with or without local anaesthesia or systemic analgesia are the main factors for the evaluation of the presence of pain-related distress caused by various techniques of dehorning and

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disbudding [3,4]. Restraining or holding of horned animals may be associated with both human and animal safety issues. Therefore, horn buds of young calves normally removed to decrease the risk of injuries to farm workers or other animals [5].

Nonsteroidal anti-inflammatory drugs (NSAIDs) prevent inflammation by inhibiting cyclooxygenase (COX) enzyme which involved in the synthesis of prostaglandins. COX-1 and COX-2 are two primary forms of COX. Prostaglandins associated with COX-1 isoform mainly regulate maintenance of the gastrointestinal tract, renal function and other homeostatic processes, whereas COX-2 isoform are commonly related to pain and inflammation associated with tissue injuries [6]. Ketoprofen is a NSAID, inhibits COX-1 and COX-2 enzymes reversibly and effectively reduces inflammation-related pain responses to dehorning [7]. Dexketoprofen is the water soluble salt of the S-isomer of racemic NSAID ketoprofen. Dexketoprofen has some advantages as compared to ketoprofen, since it has faster onset of action, increasing potency and decreasing potential for gastrointestinal side effects [8]. It is hypothesized that dexketoprofen trometamol (DEX) may have more adequate pain-relieving effect during painful procedures such as disbudding in calves. Therefore, this study was designed to evaluate the effect of DEX and local anaesthetic lidocaine on acute cortisol, inflammatory response and oxidative stress following hot-iron disbudding in calves.

MATERIAL and METHODS

Twelve male Holstein Friesian dairy calves, housed in the same farm, weighing between 52 and 82 kg and 6-8 weeks of age were used for disbudding procedure. The study was approved by the Animal Ethics Committee, Afyon Kocatepe University (registration number 295-13). The day before each trial, calves were kept in individual pens, bedded with straw. Calves were randomly allocated into two groups. "DEX" group (n = 6) was treated by intravenous injection of DEX (Arveles, UFSA, Turkey) at a dose of 2 mg/kg body weight 30 min before disbudding. Whereas 0.9% saline (2 mg/kg) was administered 30 min before procedure in "Control" group (n = 6).

Twenty minutes before disbudding, local anaesthesia was achieved by subcutaneous injection of 6 mL of 2% lidocaine hydrochloride with 0.01 mg/mL epinephrine (Adokain, Sanovel, Turkey) to each cornual nerve, just ventral to the lateral edge of the frontal bone midway between the base of the horn and the lateral cantus. Local anaesthesia was confirmed by pricking the horn-base with a needle. For disbudding procedure, an electrically heated hot-iron dehorner was used. Before procedure, iron was preheated for at least 10 min to a temperature of approximately 600°C. Disbudding was then carried out with the electro-cautery by applied over each horn bud for 30-60 sec. All cases were disbudded by the same person and at the same time of the day.

The heart rate (HR) was measured by auscultation and the respiratory rate (RR) by counting thoracic excursions before and after disbudding procedure.

Blood samples (8 ml) were collected by jugular vein-puncture into glass tube containing gel at 30 min before disbudding and at 15, 30 and 60 min after disbudding for analysis of serum cortisol, tumour necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), total antioxidant activity (AOA), malondialdehyde (MDA), glutathione (GSH), and nitric oxide (NO). Blood samples were collected by a same person during the study and the procedure was completed in 15 sec. Samples were kept on ice then centrifuged, serum was removed and stored at -20°C until analysis.

An ELISA method was used to determine cortisol concentrations (Bovine Cortisol ELISA Kit, Hangzhou Eastbiopharm, China), TNF-α (Bovine TNF-α ELISA Kit, Hangzhou Eastbiopharm, China), IL-1β (Bovine IL-1β ELISA Kit, Hangzhou Eastbiopharm, China) and IL-6 (Bovine IL-6 ELISA Kit, Hangzhou Eastbiopharm, China) in serum samples.

NO level was quantified indirectly by measuring nitrites (NO_2) and nitrates (NO_3), using the Griess method [9]. Plasma lipid peroxidation [10], serum GSH [11] and the total AOA [12] levels were measured spectrophotometrically by the previously described methods.

Statistical Analysis

The comparison of parameters measured at same sampling times was evaluated by Mann-Whitney U test between groups. Data of measurement obtained at -30, 15, 30 and 60 min for each parameters were analysed using the analysis of variance (ANOVA) followed by Tukey test (SPSS 16.0) within groups. All data were presented as mean±standard error (SEM). Data were considered to be significantly different at $P<0.05$.

RESULTS

It was observed that HR was increased at 15 min after disbudding and dropped to baseline levels at 30 and 60 min after disbudding in control groups *(Table 1)*. This alteration in HR was noted at 15 and 60 min after disbudding within control group was statistically significant ($P<0.05$). However, the time-dependent changes of HR did not show any significant within DEX group ($P>0.05$). The HR values detected at the same sampling time periods between control and DEX groups did not show any significant changes ($P>0.05$). The RR reached its peak value at 15 min after disbudding, but it was dropped to baseline value at 30 and 60 min after disbudding in control group. The RR detected at 15 and 30 min after disbudding was statistically significant when compared to baseline value in control

group (P<0.05). The RR in DEX group was significantly increased at 15 min after disbudding (P<0.05). There was significant difference in RR at 15, 30 and 60 min after disbudding between control and DEX groups (P<0.05).

The concentrations of cortisol in control group did not show any significant difference in time-dependent manner (P>0.05) *(Table 2)*. In DEX group, the cortisol concentration was immediately increased at 15 min after disbudding but decreased to lower level than baseline at 60 min after disbudding. However, the decreased cortisol concentrations detected at 60 min after disbudding were significant, when compared to all measured time intervals (P<0.05). There was no significant difference in serum cortisol concentrations between control and DEX groups at any sampling time periods (P>0.05).

Serum TNF-α, IL-1β and IL-6 levels in control and DEX groups were presented in *Table 2*. TNF-α concentration was significantly increased at 15 min after disbudding with respect to baseline value (30 min before disbudding) in control group (P<0.05). The concentrations of TNF-α were not significant within DEX group (P>0.05). The concentrations of IL-1β were not significant within control group, whereas IL-1β concentrations in DEX group significantly decreased at 15, 30 and 60 min after disbudding, as compared to concentration detected at baseline value (P<0.05). The concentrations of IL-6 at 15, 30 and 60 min after disbudding were not significant, when compared to baseline value within both control and DEX groups (P>0.05). In addition, there was no significant difference in TNF-α, IL-1β and IL-6 concentrations between control and DEX groups at any sampling time periods (P>0.05).

Serum oxidant and antioxidant status were presented in *Table 3*. The concentrations of NO and MDA within control and DEX groups did not reveal any significant difference (P>0.05). Similarly, the alteration of concentrations of GSH observed in time-dependent manner was not significant statistically in control group (P>0.05). However, GSH concentrations significantly increased at 15 min after disbudding in DEX group (P<0.05). The concentrations of AOA detected at 15 min after disbudding within control group significantly increased (P<0.05), whereas the alteration of AOA concentrations did not show any significant difference within DEX group (P>0.05). Furthermore, the concentrations of NO, GSH, MDA and AOP did not present any significant difference between control and DEX groups at all measured time intervals (P>0.05).

Table 1. *Heart rate and respiratory rate in control (CO) and dexketoprofen trometamol (DEX) groups (Mean±SEM)*

Tablo 1. *Dexketoprofen trometamol ve kontrol gruplarında kalp frekansı ve solunum sayıları (Ort±SEM)*

Time (Min)	Group (n=6)	Heart Rate (Pulse/min)	Respiratory Rate (Beat/min)
-30	CO DEX	97.3±6.7^{b} 119.3±4.1	30.3±1.4^{b} 25.3±1.0^{b}
15	CO DEX	137.3±3.5^{a} 127.0±8.5	62.0±2.3^{a} 32.6±2.7^{a}
30	CO DEX	113.3±3.7^{b} 110.3±5.4	57.0±4.1^{a} 26.3±2.1^{b}
60	CO DEX	108.6±5.2^{c} 94.6±5.7	36.0±2.2^{b} 21.3±1.0^{b}

*Small $^{(abc)}$ letters in the same column indicate significant differences within the group (P<0.05); * Indicate significant differences between the groups (P<0.05)*

DISCUSSION

Evaluation of changes in cortisol concentrations widely used as an indicator of acute distress responses to a wide range of painful husbandry practices such as castration, disbudding or dehorning procedures [4,7,13]. When calves are disbudded by cautery after a cornual blockade with lidocaine, there is a small transient increase in cortisol concentrations which returns to baseline concentrations at 60 min after disbudding [14]. Similarly, the cortisol concentrations of calves disbudded by hot-iron after a cornual nerve blockade with lidocaine instantly increase without any significant difference but return to the

Table 2. *Serum TNF - α, IL - 1β, IL - 6 and cortisol concentrations of calves disbudded with hot - iron in control (CO) and dexketoprofen trometamol (DEX) groups (Mean±SEM)*

Tablo 2. *Dexketoprofen trometamol ve kontrol gruplarında boynuz köreltme uygulanan buzağıların serum TNF - α, IL - 1β, IL - 6 and kortizol konsantrasyonları (Ort±SEM)*

Time (Min)	Group (n=6)	TNF-α (ng/L)	IL - Iβ (pg/mL)	IL - 6 (ng/L)	Cortisol (nmol/L)
-30	CO DEX	187±6.4^{b} 150±6.7	116.3±28 103. 1±11.1^{a}	148.4±12.9 136.1±12.5	38.2±11.7 51.2±3.1^{a}
15	CO DEX	225.8±4.1^{a} 169±9.9	116.2±19.8 69.6±9.5^{b}	166.3±30.8 118±14.5	47.1±11.4 63.4±6.6^{a}
30	CO DEX	172.8±9.9^{b} 154.1±18.1	146.5±37.1 63.6±12.1^{b}	166.6 ±41.3 174.3±12.1	59.3±5.6 50.4±3.8^{a}
60	CO DEX	191.3±10.1^{b} 198.5±18.9	148.4±31.8 62.6±8.5^{b}	168.9±13.1 161.8±22.1	39.1±6.1 34.1±6.3^{b}

CO: *calves disbudded after regional lidocaine application,* ***DEX:*** *calves disbudded after treatment with dexketoprofen trometamol and regional lidocaine*
^{ab}Superscript letters in the same column indicate significant differences within the groups (P<0.05)

Table 3. *Serum oxidant and antioxidant levels of calves disbudded with hot - iron in control (CO) and dexketoprofen trometamol (DEX) groups (Mean±SEM)*
Tablo 3. *Dexketoprofen trometamol ve kontrol gruplarında boynuz köreltme uygulanan buzağıların serum oksidan ve antioksidan düzeyleri (Ort±SEM)*

Time (Min)	Group (n=6)	NO (µmol/L)	GSH (nmol/mL)	MDA (nmol/mL)	AOA (mmol/L)
-30	CO	14.5±1.6	15.9±0.7	7.9±0.5	6.2±0.6[b]
	DEX	15.5±2.5	14.4±0.3[b]	7.6±0.9	7.4±0.4
15	CO	15.3±2.1	15.1±0.4	7.7±0.3	7.9±0.3[a]
	DEX	14.2±1.5	17.7±1.9[a]	6.7±0.5	6.9±0.7
30	CO	13.6±2.3	15.1±0.5	8.5±1.4	7.1±0.3[ab]
	DEX	13.4±1.9	16.2±0.1[ab]	7.5±1.1	6.9±0.2
60	CO	14.4±2.4	14.7±0.8	6.3±0.4	7.2±0.1[ab]
	DEX	14.6±1.1	15.9±0.2[ab]	8.3±1.5	6.3±0.6

CO: *calves disbudded after regional lidocaine application,* ***DEX:*** *calves disbudded after treatment with dexketoprofen trometamol and regional lidocaine;* [ab]*Superscript letters in the same column indicate significant differences within the groups (P<0.05)*

initial levels approximately one hour after disbudding [1,2]. The observation of the temporary increment of cortisol concentration immediately after disbudding and the decreasing cortisol concentration at 30 min after disbudding and similar concentrations as initial cortisol levels detected at one hour after disbudding in control group as in results of the present study supports the observations of other reports [1,2].

There are several studies indicating the use of local anaesthetics and NSAIDs to prevent or decrease of distress after disbudding [2,7,13,15,16]. However, there is no report indicating the effect of DEX on distress. The use of ketoprofen has been shown to significantly reduce cortisol response to the scoop dehorning of calves, when it is combined with a local anaesthetic lidocaine [7]. Moreover, it has been stated that the cortisol response of disbudded calves given the NSAID ketoprofen and local anaesthesia is less than the response of calves given local anaesthetic alone in hot-iron disbudded calves [2,13]. Stilwell *et al.*[2] reported that the cortisol concentration detected at one hour after disbudding the calves following the treatment with regional lidocaine and carprofen had less concentration than initial cortisol level. In addition, the authors assessed no significant difference in decreasing cortisol concentration at first hour between the calves disbudded only regional lidocaine and the calves disbudded by regional lidocaine and carprofen. In the present study, a decreasing cortisol concentration at 30 min after disbudding following a transient increasing cortisol level was observed. It was also found that the cortisol level which was detected at one hour after disbudding was lower than initial cortisol concentration in DEX group. This non-significant decrement in DEX group might be due to pain-relieving effect by the inhibition of prostaglandin synthesis via COX inhibition in addition to that of local anaesthesia alone following DEX injection before hot-iron disbudding.

The stressful events such as castration and disbudding raise the HR in cattle [15,17] and therefore can possibly be used as a measure of relative stress [17]. Schartzkopf-Genswein *et al.*[17] reported that HR increased at 15 min after dehorning and started to decrease at 30 min after dehorning in calves as compared to initial HR. However, the HR increased during five min following cautery disbudding and returned the baseline values at 15 min after cautery disbudding with and without local anaesthetic [16]. Besides, Heinrich *et al.*[15] reported lower HR and RR following administration of meloxicam with local anaesthesia in comparison to calves those given only local anaesthesia in disbudding procedure. When compared to control group, DEX group had lower heart and respiratory rates after disbudding, as well as lower cortisol concentrations during 60 min after disbudding. Hence, our data may support the hypothesis that DEX reduces distress caused by the hot-iron disbudding.

The acute phase response is a complex systemic early defence system that is activated by trauma, infection, stress, neoplasia and inflammation [18]. There are three pro-inflammatory cytokines; TNF-α, IL-1β, and IL-6, are regarded to be the chief stimulators of the systemic reaction to inflammation and these cytokines are major mediators of acute phase response. Inflammation, infection or tissue injury triggers cytokine release by defence-oriented cells, thereby inducing acute phase protein synthesis. It is widely accepted that physical and psychological stress increase plasma IL-6 and acute phase proteins levels in humans and experimental animals. There is also evidence in cattle that physical stress can induce acute phase proteins [19]. Ballou *et al.*[20] reported that giving local anaesthetic and a systemic analgesic prevented the decrease in supernatant concentrations of TNF-α among surgical castration or dehorning procedure in calves. Leukocytosis and neutrophilia were previously reported following hot-iron dehorning in calves [21]. In our study, it was determined that TNF-α concentrations increased at 15 min after disbudding in both groups however, this increment was higher in control group than those in DEX group (P<0.05). Furthermore, it was seen that IL-1β and IL - 6 concentrations in control group increased after disbudding but decreased in DEX group as compared to initial levels. In addition, IL-6 concentrations in DEX group

decreased after disbudding but subsequently increased at 30 and 60 min after disbudding. It is suggested that no increasing TNF-α concentration and decreasing IL-1β and IL-6 concentrations after disbudding in DEX group may be related to either inhibition of pro-inflammatory cytokine synthesis of glucocorticoids [22] or anti-inflammatory effect of DEX itself.

The trauma activates the hypothalamic-pituitary-adrenal (HPA) axis which is the main response for stress [23]. The cytokines, NO and prostaglandins are the molecules secreting from adrenal cortex induced by pituitary gland for adaption to stress [24]. Furthermore, surgical trauma may lead to oxidative stress by increasing oxidation and lipid peroxidation or activation of inflammatory response in tissue [25]. Lipid peroxidation and the plasma MDA concentrations have been stated to directly correlate with the damage severity [26]. In this study, it was observed that the concentrations of MDA which was known as a marker of lipid peroxidation, decreased at 15 min after disbudding in both groups and raised at 30 min after disbudding without any significant difference. Moreover, it was determined that GSH concentration in DEX group increased at 15 min after disbudding ($P<0.05$), while control group did not show any significant difference at 15, 30 and 60 min after disbudding ($P>0.05$). NO concentration increased at 15 min after disbudding in control group ($P>0.05$), while NO level decreased at 15, 30 and 60 min after disbudding in DEX group without any significant difference. It has been indicated that there is a positive relationship between COX enzymes and NO action. NO is elevated by COX enzymes which are considered highly significant receptor targets for the activation of NO [27,28]. Hence, it is suggested that COX inhibition by DEX may cause in decreasing NO concentrations in DEX group.

In conclusion, the present study re-stated that hot-iron disbudding was a painful procedure in calves. Combination of DEX and local anaesthesia showed lower cortisol response than local anaesthetic alone in hot-iron disbudded calves. The combination of a local anaesthetic agent with DEX may decrease or prevent the acute inflammatory response and oxidative stress during disbudding procedure.

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Research Article

Evaluation of Certain Oxidative Stress Parameters in Heifers that were Administered Short Term PRID

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Abstract

The purpose of this study was to investigate the effect of short term administration of PRID on the levels of serum progesterone, nitric oxide (NO), malondialdehyde (MDA), total antioxidant capacity (TAC) and total oxidant capacity (TOC) in heifers. This purpose, 200 heifers were synchronized with the Cosynch-72 protocol in which progesterone was administered for 5 days. Blood was taken from the heifers 10 days prior to starting synchronization (day -10), the day the PRID was attached (day 0), the day the PRID was removed (day 5) and on the day of insemination (day 8). Blood taken on day -10 and day 0 were accepted as control values for the study. Serum progesterone, NO, MDA, TAC and TOC were measured on days 5 and 8. The difference in serum NO and MDA levels was found to be statistically significant on day 5 of the study ($P<0.001$). It was determined that the procedure caused a drop in the TAC level, while the TOC level was not affected. In conclusion, it was determined that short-term administration of PRID in heifers caused an increase in serum NO and MDA levels, a decrease in TAC level and had no effect on the TOC level.

Keywords: *Heifer, Nitric oxide, Oxidative Stress, PRID*

Kısa Süreli PRID Uygulanan Düvelerde Bazı Oksidatif Stres Parametrelerinin Değerlendirilmesi

Özet

Bu çalışmada, düvelere kısa süreli PRID uygulamasının; serum progesteron, nitrik oksit (NO), malondialdehit (MDA), total antioksidant (TAK) ve oksidant kapasitesi (TOK) düzeyleri üzerine etkisinin araştırılması amaçlanmıştır. Bu amaçla, 200 adet düve 5 gün progesteron uygulanan Cosynch-72 protokolüyle senkronize edildi. Düvelerden senkronizasyona başlamadan 10 gün önce (-10. gün), PRID takılma günü (0. gün), PRID çıkarılma günü (5. gün) ve tohumlama günü (8. gün) kan alındı. Çalışmada -10 ve 0. gün alınan kanlar kontrol olarak değerlendirildi. Beş ve 8. günlerde serum progesteron, NO, MDA, TAK ve TOK değerleri araştırıldı. Çalışmada 5. gün serum NO ve MDA düzeyleri diğer günlerden istatistiksel olarak farklı olduğu tespit edildi ($P<0,001$). Uygulamanın TAK düzeyinde düşmeye neden olduğu, TOK değerini ise etkilemediği belirlendi. Sonuç olarak, düvelere kısa süreli PRID uygulamasının serum NO ve MDA düzeylerinde artışa neden olduğu, TAK düzeyinde düşmeye ve TOK değerini ise etkilemediği belirlendi.

Anahtar sözcükler: *Düve, Nitrik oksit, Oksidatif stres, PRID*

INTRODUCTION

Oxidative stress is a new and pertinent issue in the field of veterinary medicine. Reactive oxygen metabolites (ROM) are known to be the free radicals of an organism. The body consists of about 1-2% ROM, which play a role in many body activities [1]. These metabolites are found in low concentrations and play a role in functions such as protein phosphorylation, cell maturation, apoptosis, oocyte maturation, steroidogenesis, cell immunity, ovulation, implantation, blastocyst formation, luteolysis, acrosome reactions, fertilization and luteal maintenance during pregnancy [1-4]. However, when ROM is produced in large amounts in an organism, it causes damage to lipids, protein and DNA in the cell, resulting in the loss of cell functions. Disturbance of the balance between ROM and the anti-oxidant systems that eliminate these substances (such as superoxide dismutase and glutathione peroxidase) causes oxidative stress (OS) [1,2,5].

A high level of free radicals reacts with fatty acids of the cell membrane and creates peroxidation products.

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In this process, the fatty acid on the cell membrane loses a hydrogen molecule, which increases the level of malondialdehyde (MDA) in the environment. Measuring the MDA level provides information about lipid peroxidation [6,7]. Peroxynitrites develop after free radicals cause damage to the fatty acids on the cell membrane. Nitric oxide (NO) plays a role in many of the body's physiological functions and is produced by nitric oxide synthesis. NO is mostly produced by macrophage, neutrophil and mast cells [2,8,9].

Studies performed on cattle have reported that OS factors increase when the animals become sick [9-11]. Other studies have shown that the level of OS factors increases in cases of retentio secundinarum, mastitis, subclinical mastitis, udder edema, anestrus, repeat breeders, follicular cysts and metabolic diseases [1,7,12-15].

Many different synchronization protocols are used to control reproduction in cattle. Synchronization methods include the use of progesterone-releasing intravaginal devices (PRID or CIDR), ear implants and feed additives [16-18]. It is known that the removal of progesterone-releasing intravaginal devices irritates the tissue, causing vaginitis, which in turn causes the animal to undergo stress [19-21]. For example, Aksu et al.[22] conducted a study on cows in which the use of CIDR in the synchronization protocol resulted in higher MDA levels than in those cows for which the devices were not used. Other studies conducted on cattle found that TAC and TOC levels were higher during the period of the cycle which had high progesterone levels than they were during the estrus period [3,23]. It has also been reported that estrogen and progesterone can affect OS parameters in the body [2].

The purpose of this study was to determine the effect of short-term administration of PRID on the levels of serum progesterone, NO, MDA, TAC and TOC in heifers.

MATERIAL and METHODS

This study was conducted after obtaining approval from the Kafkas University Animal Experiments Local Ethics Committee (KAÜ HADYEK - Submission: 2014/04-Decision no: 040). The study material consisted of 200 clinically healthy Holstein heifers age 14-16 months and weighing 350-420 kg.

The heifers were synchronized with the method described by Colazo and Ambrose [24], using the Cosynch-72 protocol with short-term progesterone (5 days) administered via PRID. For all the heifers, blood was taken from the *Vena coccygea* into evacuated 8.5 ml gel tubes without an anticoagulant (BD Vakutainer®, Tıpkimsan, Turkey) 10 days prior to the synchronization, on the day the PRID was attached (day 0), on the day the PRID was removed (day 5) and on the day of artificial insemination (day 8). The samples were centrifuged at 4.000 RPM for 10 min, after which they were stored at -18°C until the analysis was performed.

Serum nitric oxide concentration was measured according to the method reported by Miranda et al.[25] and serum MDA levels were measured according to the method reported by Yoshioka et al.[26].

A colorimetric assay (PowerWave XS, Biotek, Instruments, USA) was performed on serum total antioxidant and oxidant capacity using a commercial kit (Rel Assay Diagnostic®, Turkey).

Progesterone levels in blood taken from the heifers was measured with an Enzyme-Linked Immunosorbent Assay (ELISA) reader (Epoch®, Biotek, USA) using ELISA commercial kits (EIA 1561®, DRG International, Germany).

Statistical evaluation of the results was performed using SPSS® (SPSS 20, IL, USA). One-Way ANOVA was used to evaluate statistical differences between the groups. The results were analyzed as X±SE. $P<0.05$ was considered to be statistically significant.

RESULTS

On the day of the synchronization protocol in which the PRID was removed (day 5), it was noted that vaginitis had developed in all heifers and when the device was removed, there was a purulent discharge from between the vulva lips.

Analysis showed that the serum NO level varied depending on the time of administration *(Fig. 1)*. Although no statistically significant difference was found between samples taken 10 days prior to administering synchronization and on day 0, a significant difference was found between all of the days in which the procedure was administered ($P<0.001$).

No statistically significant difference was found in the MDA levels between the control samples (days -10 and 0) and the serum samples taken during the days in which synchronization was administered. A statistically significant difference was found between all days in which the procedure was administered (*Fig. 2*; $P<0.001$).

Analysis of the changes in total antioxidant capacity during the administration of the procedure revealed that the level dropped up until day 5 after the PRID was administered and increased from day 5 through day 8, with a statistically significant difference between these levels and the other days (*Fig. 3*; $P<0.001$). No statistically significant difference was found between the groups after measuring total oxidant capacity (*Fig. 4*; $P>0.05$).

Measurements of serum progesterone levels performed during the synchronization were found to be similar to those conducted on days -10 and 0 ($P>0.05$), although

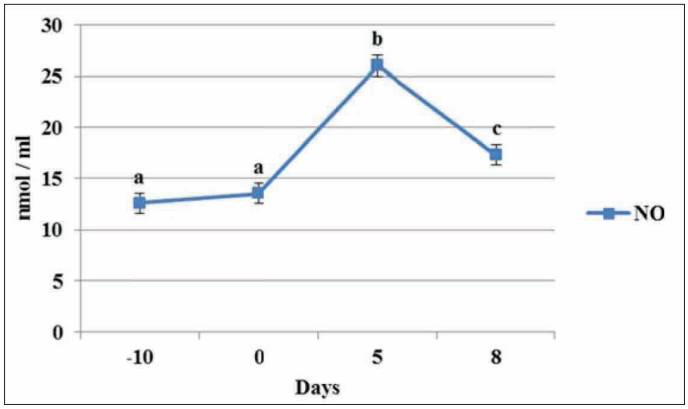
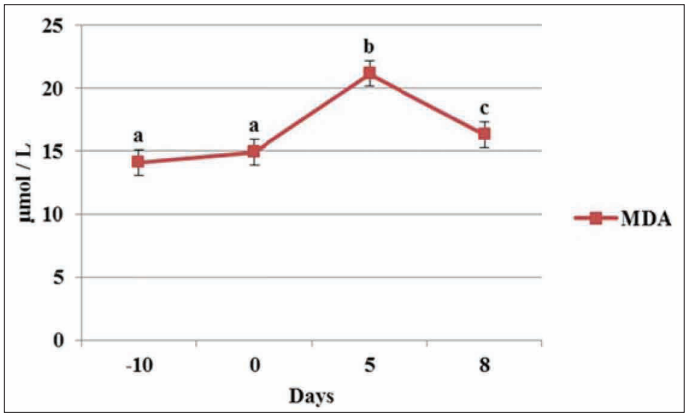


Fig 1. Serum NO levels according to the days of synchronization (P<0.001)

Şekil 1. Senkronizasyon günlerine göre serum NO düzeyleri (P<0.001)

Fig 2. Serum MDA levels according to the days of synchronization (P<0.001)

Şekil 2. Senkronizasyon günlerine göre serum MDA düzeyleri (P<0.001)



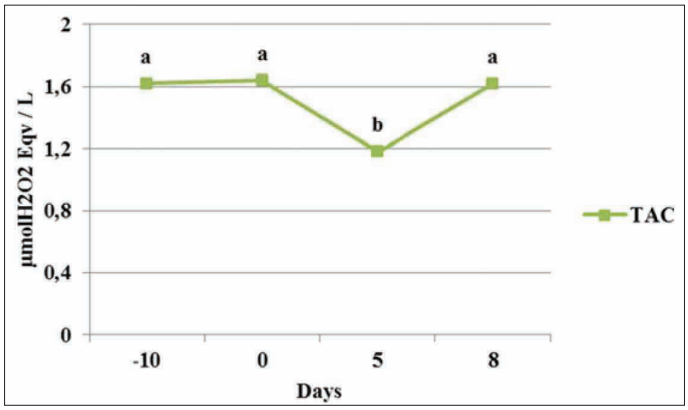
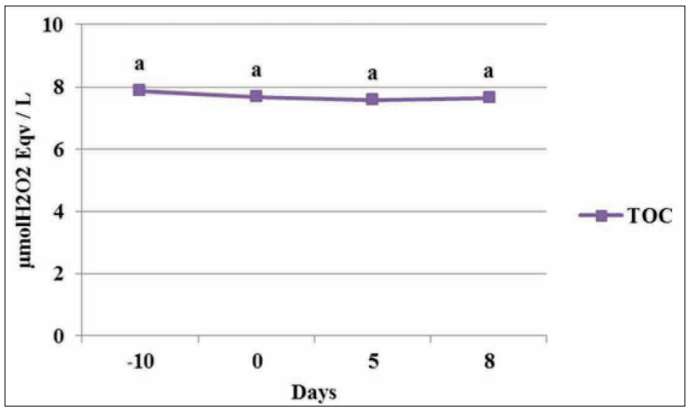


Fig 3. Serum TAC levels according to the days of synchronization (P<0.001)

Şekil 3. Senkronizasyon günlerine göre serum TAK düzeyleri (P<0.001)

Fig 4. Serum TOC levels according to the days of synchronization (P>0.05)

Şekil 4. Senkronizasyon günlerine göre serum TOK düzeyleri (P>0.05)



the highest level was found to be on day 5 and the lowest level was on day 8 *(Fig. 5)*. Serum NO, MDA, TAC, TOC and progesterone levels have been shown in *Table 1* according to the days of synchronization.

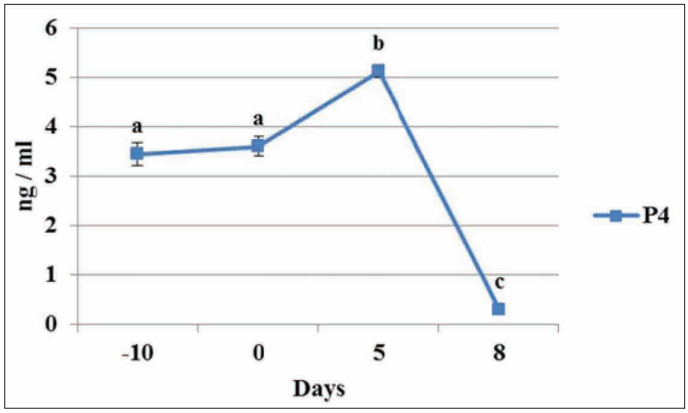


Fig 5. Serum P4 (progesterone) levels according to the days of synchronization (P<0.001)

Şekil 5. Senkronizasyon günlerine göre serum P4 (progesteron) değerleri (P<0.001)

Table 1. *Changes in NO, MDA, TAC, TOC and progesterone according to days*
Tablo 1. *Günlere göre NO, MDA, TAK, TOK ve progesteron değişimleri*

Days	NO (nmol/ml)	MDA (µmol/l)	TAC (mmol Trolox Eqv/L)	TOC (µmolH_2O_2 Eqv/L)	Progesterone (ng/ml)
-10	12.59± 020^a	14.10±0.21^a	1.62±0.01^a	7.89±0.08	3.44±0.23^a
0	13.52±0.23^a	14.93±0.25^a	1.64±0.01^a	7.68±0.10	3.60±0.19^a
5	26.02±0.33^b	21.16±0.28^b	1.18±0.02^b	7.59±0.11	5.12±0.13^b
8	17.30±0.25^c	16.31±0.27^c	1.62±0.01^a	7.65±0.09	0.30±0.02^c
P	P<0.001	P<0.001	P<0.001	P=0.172	P<0.001

DISCUSSION

A number of different progesterone-based synchronization protocols have been developed for cattle. Some studies have reported that progesterone-release intravaginal devices frequently cause vaginitis and cause the animal stress [20,21]. The present study also studied the degree to which progesterone releasing devices affect OS factors in animals.

Studies in the field of veterinary medicine have shown that OS factors increase when certain diseases occur [1]. In a study conducted by Ergönül and Kontaş Aşkar [8], the authors had the goal of identifying MDA levels in cattle with Anaplasmosis and in healthy cattle (control group). At the conclusion of the study, they found that MDA levels averaged 15.23 µmol/L in the control group. They reported that this level was much higher in diseased animals. In our study, we found that MDA levels were 14.10 and 14.93 µmol/L in the control samples (days -10 and 0). The results of our study were similar to that of the aforementioned study. Aksu et al.[22] conducted a study in which they administered CIDR to a group of cows for the purpose of synchronization. They found that there was a change in serum MDA levels on the day the CIDR was removed and on the day of insemination. They identified a statistically significant difference in MDA levels between the first blood sample and the second sample. The authors reported that the MDA level was higher on the day the CIDR was removed than on the day of insemination and that the progesterone-release intravaginal device caused stress, resulting in an increase in the MDA levels. Similarly, our study also found that MDA levels were at the highest on the day the PRID was removed but lower on the day of insemination. MDA level is thought to increased due to the stress that occurs after PRID application.

A number of studies have been conducted to measure NO levels in order to analyze oxidative stress. A study conducted by Bozukluhan et al.[9] found that serum NO levels in healthy animals (control group) were lower than those of diseased animals. In our study, the PRID was administered to healthy animals and because the device caused stress to the animal, we found that NO levels rose after the procedure. Agarwal et al.[2] reported that a low level of nitric oxide is important for ovarian activity and implantation. Furthermore, they reported that a high level of NO has a harmful effect on motile sperm, that it can have a toxic effect on the embryo and that it can prevent implantation. In our study, we found that the NO level increased when the PRID was administered and that it decreased notably from day 5 to day 8 of the protocol. Nitric oxide rising by PRID application, it was determined that fell to normal levels at the artificial insemination day. Declining in the value of nitric oxide will not adversely affect fertility parameters and in synchronization protocols concluded that PRID application can be used in a safe manner.

Aydilek et al.[3] conducted a study in which they attempted to measure TAC and TOC levels at various points of the reproductive cycle in cows. The study identified an increase in both factors during the estrus period. On the other hand, it was determined that TAC and TOC levels were lower during the times when the progesterone level

was high. In our study, we found that the TAC level was at its lowest at the end of the administration of progesterone (the day the PRID was removed) (P<0.001), while there was no change in the TOC level (P>0.05). In the present study, it is believed that after the PRID application which forms stress causes a decrease on the levels of TAC. Talukder et al.[23] reported that some OS parameters decreased together with progesterone from the time the CIDR was removed until ovulation. In our study, we also observed a drop in the level of progesterone, MDA and NO after the PRID was removed.

In conclusion, it was determined that short-term administration of PRID in heifers caused an increase in serum progesterone, NO and MDA levels, a decrease in the TAC level and had no effect on the TOC level.

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Research Article

Besi Sığırcılığı İşletmelerindeki Farklı Barındırma Sistemlerinin Hayvan Refahı Bakımından ANI 35 L/2000 Yöntemi İle Karşılaştırılması [1]

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Özet

Bu çalışmada, farklı barınak özelliklerine sahip besi sığırı çiftliklerindeki hayvan refahı düzeylerinin Animal Needs Index (ANI) 35L yöntemi ile karşılaştırmalı olarak ortaya konulması amaçlanmaktadır. Araştırma kapsamında 42 besi sığırı çiftliği, barınak yapısı (bağlı ya da serbest sistem), ahır içi mekanizasyon ile teknoloji ve işgücü kullanımı kriterleri gözetilerek 3 alt gruba ayrılmıştır: i) bağlı sistem kullanan aile işletmeleri (n=18), ii) bağlı sistem kullanan iyileştirilmiş işletmeler (n=11) ve iii) serbest sistemdeki işletmeler (n=13). Hareket olanağı, ahır içi iklim koşulları ve sosyal iletişim kategorileri bakımından serbest sistemdeki işletmeler bağlı sistemdeki işletmelere göre daha yüksek puanlar almışlardır (P<0.001). Zemin kalitesi bakımından bağlı sistem kullanan aile işletmelerine diğer iki sisteme göre daha düşük puan verilmiştir (P<0.01). Toplam ANI puanı bakımından ise serbest sistemdeki işletmelerin her iki bağlı sistemdeki çiftliklere göre daha yüksek ortalamaya sahip oldukları belirlenmiştir. ANI değerlendirmesi sonucunda "hayvan refahı açısından uygun bulunmayan" işletme oranı, bağlı sistem kullanan aile işletmelerinde %94.44, bağlı sistem kullanan iyileştirilmiş işletmelerde ise %63.64 olarak saptanırken, serbest sistemdeki işletmelerin hiç birisi bu grupta yer almamıştır. Çalışmada, serbest sistemdeki hayvanların avluya çıkma olanağının bulunmasının hayvan refahına olumlu katkı sağladığı ve bağlı sistemdeki işletmelerde durak alanı ve servis yolunun yapısal özelliklerinin hayvan refahını olumsuz etkilediği sonucuna ulaşılmıştır.

Anahtar sözcükler: *Çiftlik düzeyinde refah değerlendirmesi, Hayvan refahı, Hayvan ihtiyaç indeksi, Hava kalitesi, Besi sığırı*

Assessment of Animal Welfare in Different Beef Cattle Housing Systems by ANI 35 L/2000 Method

Abstract

The aim of the study includes assessment of animal welfare using ANI 35L system to compare different types of beef housing systems. 42 beef farms were divided into 3 sub groups according to housing type (tie stall or loose type), condition of mechanical and technical equipment and use of labour. The sub groups of the study are: i) family type tether system (n: 18), ii) improved farms with tether systems (n: 11), iii) loose housing system (n: 13). Points given to the loose housing system found higher when they compared to tether system farms in the locomotion, social interaction and light and air categories (P<0.001). In the flooring category, family type tether systems had the lowest points when they compared to other housing systems (P<0.01). Loose housing system had higher mean about total ANI points when they compared to tether systems. As a result of ANI assessment, 94.44% of farms from family type and 63.64% of farms from improved tether systems found "not suitable for animal welfare" according to the sum of ANI points. None of the loose housing system farms take a part in this welfare category. As a result of the study, opportunity of outside exercise helps farms to improve animal welfare and structural requirements of the stall areas and service roads in the tether systems cause various welfare problems for animals.

Keywords: *On-farm welfare assessment, Animal welfare, Animal needs index, Air quality, Beef cattle*

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GİRİŞ

Özellikle 20. yüzyılın ikinci yarısında batı toplumlarındaki sosyo-ekonomik gelişime paralel olarak kişi başına düşen hayvansal gıda tüketiminde büyük bir artış gözlenmiştir. Hayvansal gıda ihtiyacını karşılamak için hayvancılık bir endüstri haline dönüşmüş ve entansif çiftliklerin sayısı hızla artmıştır. Bu süreçte barınak büyüklükleri, yapısı ve iç dizaynı aynı anda çok sayıda hayvanı barındıracak şekilde düzenlenmeye başlanmış, meraya dayalı sistemler yerine enerji ve protein oranı yüksek konsantre yemlerin ağırlıklı olarak kullanıldığı üretim sistemleri hızla yaygınlaşmıştır [1]. Fakat bakım, besleme ve barınma koşullarındaki bu değişimler, hayvanların yeni koşullara uyum sağlamakta sorun yaşamalarına sebep olmuş; hayvanların sağlıklarında, metabolizmalarında ve davranışlarında bozukluklar görülmeye başlanmıştır [2]. Entansif çiftliklerde hayvanların yüksek verime zorlanması, dar bir alana çok sayıda hayvanın konulması gibi uygulamalar ise toplumlarda giderek artan eleştirilere sebep olmuştur. Avrupa ülkelerinde tüketiciler, hayvanların çiftliklerde hangi koşullarda yetiştirildiği, nasıl nakledildikleri ve kesildikleri, modern üretim tekniklerinin hayvanlar üzerindeki etkileri gibi konulara daha fazla ilgi göstermeye başlamışlar ve dolayısı ile "hayvan refahı" kavramının önemi giderek artmıştır [1,3].

Hayvan refahı, bir hayvanın içinde yaşadığı koşullarla ne ölçüde başa çıkabildiğidir [4,5]. Eğer bir hayvan sağlıklı ise, konforlu ortamda ise, iyi besleniyorsa, güvende ise, doğal davranışlarını sergileyebiliyorsa; ağrı, korku, huzursuzluk gibi istenmeyen durumlardan dolayı acı çekmiyorsa o hayvan iyi refah düzeyindedir. İyi hayvan refahı hastalıklardan korunma ve veteriner hekim tedavisini, uygun barınma ortamı, bakım, besleme, insancıl müdahale ve kesim/öldürme'yi gerektirir [5]. Hayvan refahı konusunda duyarlılığın artması, çeşitli türlerden çiftlik hayvanları için mevcut refah düzeyinin ortaya konulması ihtiyacını doğurmuştur [6]. Sürü bazında refah değerlendirmesi amacıyla çeşitli araştırma ekiplerince hayvana dayalı ve çevresel refah ölçüm parametrelerinin kombine edildiği refah değerlendirme sistemlerinin geliştirildiği görülmektedir. Bu sistemlerin en önemlileri; Avusturya orjinli "Animal Needs Index (ANI 35L/2000)" ve bir AB projesi kapsamında geliştirilen "Welfare Quality" protokolüdür [6,7].

ANI 35L sisteminde iyi bir hayvan refahı düzeyi için duyulan gereksinimlerin mevcut bakım, besleme ve barınma koşullarında ne ölçüde karşılandığı değerlendirilmektedir. Sığır çiftliklerinde yapılacak olan ANI 35L değerlendirmesinde; ahırın serbest ya da bağlı sistemde oluşu gibi barınak özellikleri ile hayvan başına düşen alan, altlık temizleme ve havalandırma sistemleri gibi barınak içi koşullar önemli bir paya sahiptir [7]. Trakya Bölgesi'nde sığır besiciliği genellikle küçük aile işletmelerinde, kurbanlık dana üretimi amacıyla ve geleneksel yöntemlerle yürütülmektedir. Bu işletmelerin büyük bölümü bağlı sistemde olup, işçilik aile bireyleri tarafından sağlanmaktadır [8]. Son yıllarda serbest sistemde kurgulanmış, yüksek kapasiteli entegre besi işletmelerinin sayısında da belirgin bir artış olduğu gözlenmektedir. Yeni kurulan bu işletmelerde, hayvan başına daha fazla alan sağlandığı, altlık temizleme ve havalandırma işlemlerinde modern teknolojilerden daha çok yararlanıldığı görülmektedir. Diğer yandan ekonomik koşulları nispeten daha iyi olan bazı aile işletmelerinin aynı anda daha fazla sayıda hayvanı besiye alabilmek amacıyla daha büyük ahırlar inşa ettikleri, ancak geleneksel yöntemlerle ve bağlı sistemde sığır besisine de devam ettikleri görülmektedir.

Bu çalışmada, ANI 35L yöntemi ile Trakya Bölgesi'nde yaygın kullanılan bağlı sistemdeki aile tipi, bağlı sistemdeki iyileştirilmiş ve serbest sistemdeki besi sığırı çiftliklerinde hayvan refahı düzeylerinin karşılaştırmalı olarak ortaya konulması amaçlanmıştır. Çalışmada ayrıca bölgedeki sığır çiftliklerinde ANI 35L yönteminin hayvan refahı değerlendirmede kullanılabilirliği konusunda da bilgiler üretilmesi hedeflenmiştir.

MATERYAL ve METOT

Araştırma kapsamında İstanbul'un Çatalca ve Silivri, Tekirdağ Merkez ve Malkara, Kırklareli Merkez ve Lüleburgaz ilçelerinde yer alan 42 besi sığırı çiftliği ziyaret edilerek, ANI 35L yöntemi ile hayvan refahı değerlendirmesi yapılmıştır. Çalışmanın yürütüldüğü Trakya Bölgesi'nde, yılın en soğuk ayı olan Ocak ayındaki ortalama sıcaklık 2.8°C, en sıcak ay olan Temmuz ayında ortalama sıcaklık 23.9°C, yıllık ortalama sıcaklık ise 13.2°C'dir. Yıllık ortalama nispi nem %69.6'dır [9].

İncelenen çiftlikler; i) barınak özellikleri (bağlı ya da serbest), ii) altlık temizleme, yemleme ve havalandırma işlemlerinde kullanılan teknoloji, iii) işgücü kullanımı (aile içerisinden sağlanması ya da ücretli işçi çalıştırılması), kriterleri gözetilerek 3 alt gruba ayrılmıştır:

a) Bağlı Sistem Kullanan Aile İşletmeleri (n=18): Hayvanların sürekli olarak bağlı tutulduğu; yataklık alanında altlık materyali olarak saman kullanılan; ahırın günlük temizliği, hayvanların yemleme ve sulama işlemlerinin aile bireyleri tarafından gerçekleştirildiği ve bu işlemler için herhangi bir mekanizasyon sistemine sahip olmayan; havalandırmanın pencere ve/veya bacalarla sağlanmaya çalışıldığı; çoğunlukla ailenin kendi konakladığı binanın hemen yanında yer alan işletmeler *"bağlı sistem kullanan aile işletmeleri"* olarak gruplandırılmıştır. Bu ahırların ortalama zemin alanları 82.8±8.68 m^2, besideki hayvan sayısı ise 16.9±1.70 olarak belirlenmiştir.

b) Bağlı Sistem Kullanan İyileştirilmiş İşletmeler (n=11): Hayvanların bakımı ve ahırın temizliği amacı ile aile dışı bireylerin de çalıştırıldığı, ahır içi koşullarda çeşitli iyileştirmelerin (yemleme, sulama veya havalandırma işlem-

lerinde mekanizasyona başvurulması gibi) yapıldığı sabit bağlamalı sistemdeki aile işletmeleri "*bağlı sistem kullanan iyileştirilmiş işletmeler*" olarak gruplandırılmıştır. Bu ahırların ortalama zemin alanları 208.6±32.90 m^2, besideki hayvan sayısı ise 25.7±4.25 olarak belirlenmiştir.

c) Serbest Sistemdeki İşletmeler (n=13): Hayvanların bakımı ve ahır temizliğinin ücretli işçiler tarafından yapıldığı, yemleme, sulama ve havalandırma işlemleri için mekanizasyon kullanılan, kayıtların düzenli olarak tutulduğu, serbest gezinmeli sisteme sahip işletmeler *"serbest sistemdeki işletmeler"* olarak gruplandırılmıştır. Bu ahırların ortalama zemin alanları 1011.9±264.39 m^2, besideki hayvan sayısı ise 96.2±20.94 olarak belirlenmiştir.

Araştırma kapsamında 3 farklı yapıdaki besi işletmesinde Bartussek ve ark.'nın [7] bildirimine uygun olarak ANI sistemine göre refah değerlendirmesi 4 araştırmacı tarafından yapılmıştır. ANI sisteminde i) hareket olanağı, ii) sosyal iletişim, iii) zemin kalitesi, iv) ahır içi iklim koşulları (aydınlatma düzeyi, havalandırma ve gürültü), v) bakım kategorileri altında toplam 30 kriter için puanlama yapılmıştır. Her bir değerlendirme kategorisi altında incelenen kriterler ve bu kriterler bakımından besi işletmelerinin alabilecekleri minimum ve maksimum puanlar *Tablo 1*'de verilmiştir.

ANI uygulama kılavuzunun [7] 5. bölümü olan "bakım" kategorisinde yer alan "hayvan sağlığı" kriterinin değerlendirilmesinde; infeksiyöz ve paraziter hastalıkların şiddeti ve insidensleri ile genel hijyen ve beslenme durumları, döl verimi ve yaşama gücü parametrelerinin ele alınacağı belirtilmektedir. Bununla birlikte işletmede herhangi bir sağlık kaydı tutulmamışsa, sadece verilecek puanın 0.5'i geçemeyeceği bildirilmiş olup, puanlamanın nasıl yapılacağına ilişkin ayrıntılı bir açıklamaya yer verilmemiştir. Bu belirsizliği gidermek ve puanlamada bir standart oluşturabilmek amacıyla Earley ve ark.'nın [10] ANI sistemini revize ederek geliştirdikleri Animal Welfare Index (AWI) yönteminden yararlanılmıştır. AWI yönteminde "hayvan sağlığı" kriterine -0.5 ile 1.0 arasında bir puan öngörülmektedir. AWI değerlendirmesinde hayvan sağlığı kriterinin puanlanması, hafif hastalıkların ve semptomların (solunum sistemi hastalıkları, ishal, iç ve dış parazitler) ve şiddetli hastalıkların (BVD, BRD, tüberküloz, paratüberküloz ve leptospiroz) görülme sıklığına göre yapılmaktadır. Bu yönteme göre hafif ve şiddetli hastalıkların hiçbirisinin görülmediği çiftliklere 1 puan; en fazla iki hafif hastalığın görüldüğü çiftliklere 0.5 puan; iki şiddetli hastalık ya da ikiden fazla hafif hastalığın varlığında 0 puan; ikiden fazla ağır hastalığın ya da 4 hafif hastalığın görüldüğü çiftliklere -0.5 puan verilmektedir.

Refah değerlendirme işlemlerinin standardize edilmesini sağlamak amacıyla 2 farklı çiftlikte ön değerlendirme yapılmıştır. Belirli bir refah kategorisinin değerlendirilmesi tüm çiftliklerde aynı araştırmacı tarafından yapılmıştır.

Çiftlik ziyaretleri sırasında Testo 400 hava kalitesi ölçüm cihazı ile ahır içi sıcaklık, hava hızı, nem ve CO_2 miktarı ölçümleri yapılmıştır. Ahırlardaki olası sıcaklık stresinin varlığını belirlemek amacı ile ahır içi sıcaklık (T) ve nispi nem (RH) ölçüm değerleri kullanılarak aşağıda verilen formül ile sıcaklık - nem indeksi (THI) hesaplanmıştır [11]:

$$THI = 0.8T + [(RH/100) (T-14.3)] + 46.4$$

ANI değerlendirme işlemi tamamlandıktan sonra işletmeler aldıkları toplam ANI puanına göre 6 farklı refah kategorisine ayrılmışlardır [12]. Alınan toplam puanlara göre refah kategorileri:

<11: hayvan refahı açısından uygun değil,

11-16: hayvan refahı açısından sınırda uygun,

16.5-21: hayvan refahı açısından kısmen uygun (vasat),

21.5-24: hayvan refahı açısından büyük ölçüde uygun,

24.5-28: hayvan refahı açısından uygun,

>28: hayvan refahı açısından çok uygun, olarak belirlenmiştir.

İstatistik Analiz

Farklı barınma sistemlerindeki besi sığırı çiftliklerine ANI 35L kategorilerinde yer alan kriterler için verilen puanların istatistiki karşılaştırılması amacıyla SPSS 13.0 programında tek yönlü varyans analizi ve Duncan testi uygulanmıştır. Sadece bağlı sistemlerdeki işletmelerde değerlendirilen kriterlerin istatistiki karşılaştırılmasında bağımsız örneklem t-testi kullanılmıştır. ANI 35L değerlendirmesine göre farklı refah kategorilerinde yer alan işletmelerin oranları bakımından barınma sistemlerinin karşılaştırılması ise ki-kare testi ile yapılmıştır.

BULGULAR

ANI 35L yöntemine göre farklı barınma sistemlerindeki besi sığırı çiftliklerine verilen puanlara ait ortalamalar *Tablo 1*'de sunulmuştur. Hareket olanağı, sosyal iletişim ve ahır içi iklim koşulları kategorilerinde alınan toplam puanların serbest sistemdeki işletmelerde, bağlı işletmelere kıyasla daha yüksek olduğu görülmektedir. Zemin kalitesi bakımından bağlı sistem kullanan aile işletmelerinin diğer iki sisteme kıyasla daha düşük puan aldıkları belirlenmiştir ($P<0.01$). ANI 35L yöntemine göre alınan toplam puan incelendiğinde; bağlı sistem kullanan aile ve iyileştirilmiş işletmeler arasındaki farklılığın önemli olmadığı, ancak serbest sistemdeki işletmelerin her iki bağlı sistemdeki çiftliklere göre daha yüksek puan aldıkları görülmektedir ($P<0.001$).

Araştırma kapsamında ANI 35L yöntemi ile refah değerlendirmesi yapılan bağlı sistem kullanan aile, bağlı

Tablo 1. *ANI 35L değerlendirme kategorileri ve kriterleri için farklı barınma sistemlerindeki besi sığırı işletmelerine verilen ortalama puanlar*
Table 1. *Mean points given to each category and criteria for different housing systems according to ANI 35L evaluation*

Kategori/Kriter	Model[4]	Alınabilecek puan Min./ Maks.	Bağlı - Aile İşl.		Bağlı - İyileştirilmiş İşl.		Serbest İşl.		P Değeri
			$\bar{X}$	$S\bar{x}$	$\bar{X}$	$S\bar{x}$	$\bar{X}$	$S\bar{x}$	
Hareket Olanağı			0.94[c]	0.08	1.27[b]	0.14	9.00[a]	0.00	<0.001
Kullanılabilir yüzey alanı [1] (m^2/AWU)	S	0/3.0	-	-	-	-	3.00	0.00	-
Yatma, kalkma ve dinlenme davranışları	S	0/3.0	-	-	-	-	3.00	0.00	-
Durak boyutu ve özellikleri	B	0/1.0	0.08[b]	0.05	0.36[a]	0.07	-	-	0.002
Zincir hareketi (m)	B	0/1.0	0.86	0.05	0.91	0.09	-	-	0.632
Avlu kullanımı (Gün/yıl)	S	1.0/3.0	-	-	-	-	3.00	0.00	-
Sosyal İletişim			0.92[b]	0.08	0.91[b]	0.20	6.69[a]	0.22	<0.001
Kullanılabilir yüzey alanı	S B	0/3.0 0.5	0.50	0.00	0.50	0.00	3.00	0.00	#
Sürünün sosyal yapısı	S,B	-0.5/2.0	0.00[c]	0.00	0.27[b]	0.14	1.00[a]	0.00	<0.001
Gençlerin yönetimi	S,B	-0.5/1.0	0.42	0.08	0.14	0.17	0.38	0.12	0.220
Avlu kullanımı	S	0.5/2.5	-	-	-	-	2.31	0.19	-
Zemin Kalitesi			0.42[b]	0.29	1.45[a]	0.44	2.19[a]	0.31	0.002
Yatma alanının yumuşaklığı	S,B	-0.5/2.5	0.56	0.06	0.64	0.10	0.77	0.11	0.183
Yatma alanının temizliği	S,B	-0.5/1.0	-0.08	0.11	0.32	0.19	-0.08	0.11	0.087
Yatma alanının kayganlığı	S,B	-0.5/1.0	0.03[b]	0.09	0.23[ab]	0.16	0.42[a]	0.08	0.027
Aktivite alanı/Servis yolları	S,B	-0.5/1.0	-0.08[b]	0.07	0.27[a]	0.10	0.50[a]	0.06	<0.001
Avlu	S	-0.5/1.5	-	-	-	-	0.58	0.10	-
Ahır İçi İklim Koşulları			2.86[b]	0.22	3.36[b]	0.44	8.00[a]	0.52	<0.001
Barınaktaki gün ışığı	S,B	-0.5/2.0	0.11[b]	0.01	0.64[b]	0.27	1.46[a]	0.24	<0.001
Hava kalitesi ve hava akımı	S,B	-0.5/1.5	1.00	0.18	0.86	0.28	1.35	0.15	0.269
Durak alanındaki cereyan	S,B	-0.5/1.0	0.75	0.10	0.86	0.07	0.73	0.11	0.648
Gürültü	S,B	-0.5/1.0	1.00[a]	0.00	1.00[a]	0.00	0.90[b]	0.07	0.025
Açık alan kullanımı[2] (gün/yıl)	S	0.5/2.0	-	-	-	-	1.88	0.13	-
Açık alan kullanımı (saat/gün)	S	0.5/2.0	-	-	-	-	2.00	0.00	-
Bakım			3.22	0.15	3.09	0.28	3.69	0.33	0.228
Bölme, yemlik ve sulukların temizliği	S,B	-0.5/1.0	0.44[b]	0.04	0.50[b]	0.00	0.69[a]	0.07	0.001
Teknik ekipmanların durumu	S,B	-0.5/1.0	0.47[b]	0.03	0.59[ab]	0.06	0.73[a]	0.07	0.003
Derinin durumu	S,B	-0.5/1.0	1.03	0.06	0.82	0.12	0.73	0.12	0.078
Hayvanların temizliği	S,B	-0.5/0.5	-0.36[b]	0.07	-0.18[b]	0.10	0.19[a]	0.09	<0.001
Tırnakların durumu	S,B	-0.5/1.5	1.00[a]	0.07	0.86[ab]	0.10	0.62[b]	0.13	0.021
Teknopatiler[3]	S,B	-0.5/1.5	1.14[a]	0.07	1.00[ab]	0.10	0.81[b]	0.11	0.029
Hayvan sağlığı	S,B	-0.5/1.5	-0.50[b]	0.00	-0.50[b]	0.00	-0.08[a]	0.03	<0.001
Toplam Puan			8.36[b]	0.37	10.09[b]	0.89	29.58[a]	0.86	<0.001

[a,b,c] Aynı satırda farklı harf taşıyan ortalama değerler arasındaki farklılık önemlidir (P<0.05); # Grup içi varyanslar "0" olduğu için Varyans Analizinde "F" ve "P" değerleri hesaplanamamıştır; [1] İşletmedeki hayvanların her zaman ulaşabildikleri toplam yatma ve aktivite alanı, 1 AWU = 500 kg canlı ağırlık; [2] İşletmedeki hayvanlar tarafından günün herhangi bir saati ulaşılabilir olan çatılı ya da çatısız tüm açık alanlar; [3] Barınakta kullanılan teknoloji veya ekipmanların doğrudan veya dolaylı olarak sebep olduğu yaralanmalar; [4] Uygulandığı barınma modeli, S:Serbest, B: Bağlı

Tablo 2. *Farklı barınma sistemlerindeki çiftliklerin ANI değerlendirmesinde aldıkları toplam puanlara göre dağılımı*
Table 2. *Distribution of farms in different housing systems according to total ANI points*

Refah Kategorisi	Bağlı - Aile İşl.		Bağlı - İyileştirilmiş İşl.		Serbest İşl.		P Değeri
	n	%	n	%	n	%	
<11 puan (hayvan refahı açısından uygun değil)	17	94.44[a]	7	63.64[b]	0	0[c]	<0.001
11-16 puan (sınırda uygun)	1	5.56[b]	4	36.36[a]	0	0[c]	0.013
16.5-21 puan (kısmen uygun - vasat)	0	0	0	0	0	0	-
21.5-24 puan (büyük ölçüde uygun)	0	0	0	0	0	0	-
24.5-28 puan (uygun)	0	0[b]	0	0[b]	4	30.77[a]	0.007
>28 puan (çok uygun)	0	0[b]	0	0[b]	9	69.23[a]	<0.001

[a, b, c] Aynı satırda farklı harf taşıyan ortalama değerler arasındaki farklılık önemlidir (P<0.05)

Tablo 3. *Farklı barınma sistemlerinde barınak içi çevre koşulları*
Table 3. *Enviromental conditions of the farms in different housing systems*

Parametre	Bağlı - Aile İşl.		Bağlı - İyileştirilmiş İşl.		Serbest İşl.		P Değeri
	$\bar{X}$	$S_{\bar{x}}$	$\bar{X}$	$S_{\bar{x}}$	$\bar{X}$	$S_{\bar{x}}$	
Nem (%)	62.37[a]	1.81	62.65[a]	4.19	52.84[b]	2.70	0.030
Sıcaklık (°C)	26.06[a]	0.54	22.00[b]	1.24	17.64[c]	1.21	<0.001
THI	74.59[a]	0.79	69.02[b]	1.86	61.00[c]	1.95	<0.001
Hava hızı (m/sn)	0.36	0.09	0.32	0.08	0.63	0.12	0.085
CO_2 (ppm)	905.70	115.94	1020.00	170.73	636.75	27.71	0.093

[a, b, c] Aynı satırda farklı harf taşıyan ortalama değerler arasındaki farklılık önemlidir (P<0.05)

sistem kullanan iyileştirilmiş ve serbest sistemdeki işletmelerin aldıkları toplam puanlara göre çeşitli refah kategorilerindeki dağılımları *Tablo 2*'de yer almaktadır. ANI değerlendirmesi sonucunda 11'den az puan alarak "hayvan refahı açısından uygun bulunmayan" işletme oranı, bağlı sistem kullanan aile işletmelerinde %94.44, iyileştirilmiş işletmelerde %63.64 olarak saptanırken, serbest sistemdeki işletmelerin hiç birisi bu grupta yer almamıştır (P<0.001).

Farklı barınma sistemlerinde ahır içi çevre koşulları *Tablo 3*'te sunulmuştur. Ahır içi nem, sıcaklık ve THI ortalamaları, serbest sistemdeki işletmelerde her iki bağlı sistemdeki ahırlara kıyasla daha düşük bulunmuştur. Ayrıca, bağlı sistem kullanan aile işletmelerinde ahır içi sıcaklık ve THI'nın bağlı sistem kullanan iyileştirilmiş işletmelerden daha yüksek olduğu sonucuna ulaşılmıştır (P<0.001).

TARTIŞMA ve SONUÇ

ANI 35L yöntemi ile bir işletmede hayvanlara sağlanan bakım, besleme ve barınma koşullarının iyi refah düzeyi üretme potansiyellerini belirlemek amacıyla, hayvan refahı düzeyini etkileyebilecek 5 kategoride puanlamalar yapılmaktadır [13]. Değerlendirilen kategorilerden ilki "hareket olanağı"dır. Doğal ortamlarında hayvanların davranışları gözlenecek olursa; yeme, su içme, ruminasyon, otlama, yürüme, yatma-dinlenme gibi çok sayıda davranış özelliklerini sergilemektedirler [14]. Çiftlik ortamında ise bu davranışlardan bazılarının kısıtlanabildiği; entansifleşmeye paralel olarak genellikle gezinme ve otlama davranışlarının sınırlandırıldığı, yüksek yerleşim sıklığı ya da yataklık alanının yetersizliği gibi durumlarda da yatma ve ayağa kalkma davranışlarının zorlaştığı görülmektedir [2]. "Normal davranışları sergileyebilme özgürlüğü" hayvan refahının temel bileşenlerini ifade eden 5 özgürlük arasında yer almaktadır [15]. Çiftlik sisteminin yapısı gereği belirli davranışların sergilenmesinin sürekli olarak kısıtlanması; hayvanlarda kronik strese, homeostazis'in bozulmasına, dil yuvarlama gibi stereotipik davranışların şekillenmesine, fiziksel ve mental sağlığın bozulmasına sebep olabilmektedir. Dolayısı ile davranışsal kısıtlamalar hayvanın stresle başa çıkabilme ve içinde yaşadığı koşullara uyum sağlama yeteneğini azaltarak, hayvan refahını olumsuz etkileyebilmektedir [16,17].

Araştırmada beklenilebileceği üzere serbest sistemdeki işletmeler hareket olanağı kategorisi bakımından bağlı sistemdeki işletmelere kıyasla daha yüksek puan almışlardır (P<0.001). Araştırma kapsamında incelenen serbest sistemdeki işletmelerde hayvanların meraya hiç çıkarılmadığı belirlenmiş ve dolayısı ile bu kriter bakımından puanlama yapılamamıştır. Hareket olanağı kategorisinde değerlendirilen diğer kriterler bakımından ise serbest sistemdeki tüm işletmelere en yüksek puan olan 3 puan verilmiştir.

Araştırmada bağlı sistem kullanan iyileştirilmiş işletmelerin, bağlı sistem kullanan aile işletmelerine kıyasla daha yüksek hareket olanağı puanı aldıkları görülmektedir. Bağlı sistemdeki işletmelerin hareket olanağı kategorisi bakımından değerlendirilmesinde "durak boyutu ve özellikleri" ile "zincirin hayvanın ileri-geri ya da sağa-sola hareket etmesine ne ölçüde olanak sağladığı" kriterlerine bakılmaktadır. Zincir hareketi puanlaması bakımından bağlı sistem kullanan aile işletmeleri ile iyileştirilmiş işletmeler arasındaki farklılık önemsiz bulunurken; durak boyutu ve özellikleri bakımından bağlı sistem kullanan iyileştirilmiş işletmelerin bağlı sistem kullanan aile işletmelerine kıyasla daha yüksek puan aldıkları görülmektedir. Dolayısı ile bağlı sistem kullanan iki farklı işletme modeli arasındaki hareket olanağı bakımından gözlenen farklılığın, bu işletmelere durak boyutu ve özelliği bakımından verilen puanlardan kaynaklandığı düşünülmektedir.

Sığırlar doğal ortamlarında zamanlarının yaklaşık %50'sini yatarak geçirirler [18]. Durak boyutlarının hayvanların ağırlıkları, vücut ölçüleri, normal yatma pozisyonları, yatma ve kalkma davranışları gözetilerek planlanmış olması hayvan refahı ve konforu açısından önem taşımaktadır [19,20]. Yatan bir inek ayağa kalkarken; önce başını biraz öne ve aşağıya doğru uzatarak carpal eklemleri üzerinde doğrulur, daha sonra ileriye doğru hamle yaparak arka ayakları üzerinde durur, sonra başı ile ileriye doğru hamle yaparak ön ayaklardan biri üzerinde durur ve en son diğer ön ayağını kaldırarak dört ayağı üzerinde durmaya başlar [20-22]. Dolayısı ile durak uzunluğunun hayvan refahı açısından uygunluğu değerlendirilirken, hayvanın vücut ve baş uzunluğunun yanı sıra yatma kalkma davranışları sırasında yapacağı ileri hamleler de göz önünde bulundurulmalıdır [19]. ANI 35L yönteminde durak boyutu ve özellikleri kriterinin değerlendirilmesinde; durak uzunluğu, durak ayırma demiri yüksekliği, yemlik yüksekliği, yemlik kenarları ile durak eşiği kenarının yapısal özellikleri/keskinliği incelenmektedir. Bu araştırmada durak boyutu ve özelliği kriteri bakımından bağlı sistem kullanan iyileştirilmiş işletmelerin bağlı sistem kullanan aile işletmelerine kıyasla daha yüksek puan alması, ekonomik açıdan nispeten daha iyi koşullara sahip olan aile işletmelerinin ahırlarını iyileştirirken durak alanındaki koşulları hayvan refahı açısından daha iyi seviyeye getirdiklerine işaret etmektedir.

Araştırmada bağlı sistem kullanan besi işletmelerine "sosyal iletişim" kategorisi için verilen puanların serbest sistemdeki işletmelere oranla daha düşük olduğu görülmektedir. Sığırların sürekli olarak bağlı tutulması, hayvanların normal davranış kalıplarının değişmesine, sosyal davranışların frekansının azalmasına ve anormal davranışların oluşma riskinin artmasına sebep olabilmektedir [23]. "Sosyal iletişim" kategorisindeki puanlama kriterleri ele alınacak olursa; gençlerin yönetimi bakımından barınma sistemleri arasında önemli farklılık bulunmadığı ($P>0.05$); ancak kullanılabilir yüzey alanı, sürünün sosyal yapısı ve avlu kullanımı kriterleri bakımından serbest sistemdeki işletmelerin daha yüksek puan almaları nedeniyle "Sosyal İletişim" kategorisine verilen toplam puan bakımından barınma sistemleri arasında istatistik önemde farklılık oluştuğu sonucuna ulaşılmaktadır.

Serbest sistemdeki ahırlarda sığırların "birbiri ile sosyal iletişime geçebilme" ve "çevreyi inceleyebilme" olanaklarına sahip olması hayvan refahına olumlu katkı sağlarken; yerleşim sıklığının yüksek olduğu durumlarda hayvanlar arasındaki sosyal mücadelelerin artmasına bağlı olarak refah düzeyi olumsuz etkilenebilmektedir [24]. Bu çalışmada yer alan serbest sistemdeki 13 ahırda hayvan başına düşen ortalama zemin alanı 10.52 ± 2.45 m^2 olarak tespit edilmiş olup; kullanılabilir zemin alanı kriteri bakımından tüm serbest sistemdeki işletmelerin ANI 35L değerlendirme yöntemindeki en yüksek puanı elde ettikleri görülmüştür. Dolayısıyla, incelenen serbest sistemdeki ahırlarda yüksek yerleşim sıklığından kaynaklanan hayvan refahı problemlerinin gözlenmediği sonucuna ulaşılmıştır.

Araştırmada bağlı sistem kullanan aile işletmelerinin "zemin kalitesi" kategorisi bakımından diğer barınma sistemlerine kıyasla daha düşük puan aldıkları belirlenmiştir. Zemin kalitesi bakımından bağlı sistem kullanan iyileştirilmiş işletmeler ile bağlı sistem kullanan aile işletmeleri arasındaki farklılığın "aktivite alanı/servis yolunun zemin kalitesi" kriterinden kaynaklandığı görülmektedir. Ayrıca, serbest sistemdeki işletmelerin "yatma alanının kayganlığı" ve "aktivite alanı/servis yolunun zemin kalitesi" kriterleri bakımından bağlı sistem kullanan aile işletmelerine kıyasla daha yüksek puan aldıkları görülmektedir.

Bağlı sistem kullanan ahırlarda "aktivite alanı/servis yolunun zemin kalitesi" kriteri; durak eşiği, gübre kanalı ve servis yolu zemininin kayganlık düzeyi ile tırnaklar üzerine olası olumsuz etkilerine göre puanlanmaktadır. ANI 35L yöntemine göre, bağlı sistem kullanan aile işletmelerinin "aktivite alanı/servis yollarının zemin kalitesi" kriteri bakımından diğer işletme tiplerine göre daha düşük puan alması, bu işletmelerdeki servis yollarının teknik özelliklerinin refah açısından daha kötü olduğuna ya da bu alanların temizliğinin yetersiz yapılmasına bağlı olarak zeminin daha kaygan olduğuna işaret etmektedir. Bu araştırmada elde edilen sonuçları destekler nitelikte, bağlı ve serbest sistem kullanan işletmelerdeki yatma alanının temizliğinin incelendiği bir araştırmada [25], barınma sistemleri arasındaki farklılık önemsiz bulunmuştur. Diğer yandan Popescu ve ark.'nın [26] Romanya'da 24 bağlı sistem kullanan çiftliğin zemin kalitesi kategorisi için belirledikleri puanın (3.52), bu çalışmada "bağlı sistem kullanan aile işletmeleri" ve "bağlı sistem kullanan iyileştirilmiş" işletmelere verilen puanlardan (sırasıyla 0.42 ve 1.45) daha yüksek olduğu görülmektedir.

Ahır içi iklim koşulları kategorisi için verilen toplam puanlar dikkate alındığında, serbest sistemin, bağlı sistem kullanan ahırlara kıyasla hayvan refahı açısından daha uygun

olduğu görülmektedir. Ahır içi iklim koşulları bakımından serbest sistemin üstünlüğünün "barınaktaki gün ışığı" ve "açık alan kullanımı" kriterlerinden kaynaklandığı görülmektedir. ANI 35L değerlendirmesindeki "hava kalitesi ve hava akımı" ile "yataklık alanındaki cereyan" kriterleri bakımından ise barınma sistemleri arasındaki farklılık önemsiz bulunmuştur. Bu bulgu, farklı barınma sistemlerindeki ahır içi hava hızı ve CO_2 düzeylerinin benzer oluşu *(Tablo 3)* ile paralellik göstermektedir. Diğer yandan bağlı sistem kullanan işletmelerde ahır içindeki nem, sıcaklık ve THI düzeylerinin serbest sistemdeki işletmelere kıyasla daha yüksek olduğu sonucu elde edilmiştir.

Çevre sıcaklığı 25°C'yi aştığında, sığırlarda solunum sayısı ve terleme artarken, kalp atım sayısı azalır. Ayrıca, sığırlar yüksek çevre sıcaklığına adapte olabilmek amacıyla yem tüketimini ve buna bağlı olarak metabolik ısı üretimini de azaltırlar [11]. Sıcaklık - nem indeksinin (THI) çeşitli türlerden çiftlik hayvanlarında mikroklimaya bağlı potansiyel stresi değerlendirmek amacıyla kullanılabilecek etkin bir araç olduğu bildirilmektedir [27]. Besi sığırlarında 74 THI değeri, sıcaklık stresi için eşik değer olarak kabul edilmektedir [27]. Dolayısı ile bağlı sistem kullanan aile işletmelerindeki ahır içi sıcaklık ve THI değerlerinin, kötü hayvan refahı düzeyini işaret ettiği söylenebilir. Diğer yandan, ahır içi sıcaklık, nem ve THI düzeylerinin hayvan refahı ile yakın ilişkili olmalarına rağmen ANI 35L yönteminde sıcaklık, nem ve/veya THI parametrelerine yer verilmeyişi, bu sistemin önemli bir eksikliği olarak değerlendirilmiştir.

Araştırmada, bağlı sistem kullanan işletmelerde teknik gürültü düzeyinin serbest sistemdeki işletmelere kıyasla daha az olduğu belirlenmiştir. Bu sonuç, bağlı sistem kullanan işletmelerde havalandırma, altlık temizliği, yemleme gibi işlemler için teknoloji kullanımının minimal seviyede olması ile açıklanabilir. Bu çalışmada ANI 35L yöntemi ile bağlı sistem kullanan işletmelerde "ahır içi iklim koşulları" kriteri için belirlenen puanlar, Popescu ve ark.'nın [26] bağlı sistemdeki 24 süt sığırı çiftliği için saptadıkları puanlar (3.56±0.17) ile uyumlu bulunmuştur.

Çalışmada "bakım" kategorisi açısından barınma sistemleri arasındaki farklılığın önemsiz olduğu görülmektedir ($P>0.05$). Ancak, bu kategorinin değerlendirilmesinde kullanılan "bölme, yemlik ve sulukların temizliği", "hayvanların temizliği" ve "hayvan sağlığı" kriterleri bakımından serbest sistemdeki işletmelerin bağlı sistem kullanan işletmelere göre daha yüksek puan aldıkları sonucuna ulaşılmıştır. Buna karşılık, "tırnakların durumu" ve "teknopatiler" kriterleri bakımından serbest sistemdeki işletmelerin bağlı sistem kullanan aile işletmelerine kıyasla daha düşük puan aldıkları görülmektedir. Bağlı sistem kullanan işletmelerin "bölme, yemlik ve sulukların temizliği" kriteri bakımından daha düşük puan almaları, bu işletmelerdeki bakıcı ilgisi yetersizliğine işaret etmektedir. Hayvanların temizliği kriteri bakımından bağlı sistem kullanan işletmelerin daha düşük puan almaları ise, bağlamalı durağın yapısal özelliğine bağlı olarak hayvanların yatma, dışkılama, idrar yapma gibi tüm faaliyetlerini durak alanında gerçekleştirmeleri ile açıklanabilir. Popescu ve ark.[28] bacağın alt kısmının serbest sistemdeki ineklerde, bacağın üst kısmı ve karın bölgelerinin ise bağlı sistemdeki ineklerde daha kirli olduğunu belirlemişlerdir.

Ayak hastalıkları (özellikle topallığa neden olacak şiddette iseler) hayvan refahını olumsuz etkileyen en önemli sağlık problemleri arasında yer almaktadır [29]. Ayak hastalıklarına bağlı şekillenen ağrı, hayvanın duruşu ve hareketlerini belirgin bir şekilde etkileyebilmekte ve sonuçta hayvanın yaşadığı çevreye uyum sağlama yeteneğini azaltabilmektedir. Hayvanların daha çok hareket edebilme olanağı bulduğu serbest sistemdeki ahırlarda, ayak hastalıklarının en aza indirilebilmesi için yürüyüş alanının konforlu olması gerekir. Serbest sistemdeki ahırlarda gezinme alanının ızgaralı ya da sert zeminli oluşu, daha fazla ayak hastalığı görülmesine sebep olabilmektedir [30]. Alban ve Agger [24] serbest sistemdeki barınaklarda fekal kontaminasyonun fazla olması ile tırnak hastalıkları insidensinin artması arasında bir ilişki olabileceğini bildirmişlerdir. Bu çalışmadaki "tırnakların durumu bakımından serbest sistemdeki ahırların daha düşük puan alması" bulgusu, Norveç [30] ve ABD'de [31] yürütülen araştırmalarda elde edilen "serbest sistemdeki ineklerde topallık oranının bağlı sistem kullanan işletmelerdeki ineklere kıyasla daha yüksek olduğu" sonucu ile uyum göstermektedir.

Hayvan refahı kavramı, hayvanın tam bir fiziksel ve mental sağlığa sahip olması, çevresi ile harmoni içerisinde olması ve insanlar tarafından sağlanan yapay çevreye acı çekmeksizin uyum sağlayabilmesi olgularını içermektedir [32]. Hastalıklar, hayvanlarda fiziksel ve/veya mental acıya sebep olduğu için, hastalıkların yokluğu "yaşam kalitesi" açısından büyük öneme sahiptir [2]. Bakım besleme koşullarının iyi olduğu bağlı sistem kullanan çiftliklerde bakıcının/çiftçinin hayvanlarla teması genellikle daha yoğun olduğundan, olası hastalıklar daha çabuk fark edilebilmektedir [24]. Ayrıca serbest sistemdeki çiftliklerde bulaşıcı hastalıklar çok daha hızlı yayılabilmektedir [33]. Ancak bu çalışmada serbest sistemdeki çiftliklerin hayvan sağlığı kriteri bakımından bağlı sistem kullanan çiftliklere kıyasla daha yüksek puan aldıkları görülmektedir. Bu sonucun, serbest sistemdeki çiftliklerin sürü sağlığının korunması açısından daha profesyonel yönetilmelerine bağlı olduğu düşünülmektedir. Araştırma kapsamında refah değerlendirmesi yapılan tüm serbest sistemdeki çiftliklerde iç ve dış parazitlere, BVD, BRD, paratüberküloz, tüberküloz ve leptozpiroza karşı mücadele yapıldığı, bu durumun da serbest sistemdeki çiftliklerin "hayvan sağlığı" kriteri bakımından daha yüksek puan almalarını sağladığı sonucuna ulaşılmıştır. Romanya'da 60 süt sığırı çiftliğinde yürütülen bir çalışmada da [28], bağlı sistem kullanan ahırlardaki ishal, burun ve göz akıntısı, mastitis

ve ölüm oranının serbest sisteme kıyasla daha yüksek olduğu bildirilmiştir. Bu çalışmada "bakım" kategorisi için saptanan puanların, Popescu ve ark.'nın [26] bağlı sistemdeki 24 süt sığırı çiftliği için tespit ettikleri ortalama puanlar (3.65±0.91) ile uyumlu olduğu görülmektedir.

ANI 35L sistemi ile refah değerlendirmesi yapılırken hareket olanağı, sosyal iletişim, zemin kalitesi, ahır içi iklim koşulları ve bakım kategorilerinden alınan puanlar toplanarak işletme için ANI puanı hesaplanmakta ve bu puan üzerinden çiftlikteki hayvanların refah düzeyi hakkında karar verilmektedir. Bu çalışmada, serbest sistemdeki işletmelerin toplam ANI puanının bağlı sistem kullanan çiftliklere göre daha yüksek olduğu görülmektedir. Bağlı sistem kullanan işletmelerin aldıkları toplam puanlar, bu işletmelerin "hayvan refahı açısından uygun olmadığına (<11 puan)" veya "sınırda uygun olduğuna (11-16 puan)" işaret etmektedir. Serbest sistemdeki çiftlikler ise hayvan refahı açısından "uygun (24.5-28 puan)" veya "çok uygun (>28 puan)" bulunmuşlardır. Bu sonuçlar işletmelerin bağlı ya da serbest sisteme sahip oluşunun ANI sistemi ile yapılan değerlendirme sonucu üzerinde önemli etkiye sahip olduğuna işaret etmektedir. ANI 35L sistemine göre hayvanların sürekli bağlı tutulduğu, mera ya da avluya çıkarılmadığı bir işletmenin alabileceği en yüksek puan 25.5'tir. Diğer yandan ANI 35L sisteminde "avlu ve meraya yıl boyunca sürekli çıkabilme olanağının bulunması" durumunda 14.5 puan alınabilmektedir.

ANI 35L yönteminde toplam puan üzerinden refah düzeyi hakkında karar verilmesi, çeşitli çiftliklerin kolaylıkla karşılaştırılabilmesi avantajını sağlamaktadır. Ayrıca, ANI yöntemi ile hedeflenen refah düzeyine ulaşmak amacıyla hangi konularda iyileştirme yapılması gerektiği konusunda da bilgiler edinilebilmektedir [3,12]. ANI yönteminin en önemli dezavantajı ise, bir kategori bakımından çok yüksek puan alınması durumunda, refah açısından çok önemli olan bazı kriterlerden çok düşük puan alınsa dahi işletmenin refah düzeyinin iyi olarak değerlendirilebilmesidir [12]. Bu çalışmada, serbest sistemdeki işletmelerde hayvanların avluya çıkabilme olanağının bulunması, bu işletmelerin bağlı sistem kullanan işletmelere kıyasla 9.77 puan daha fazla almalarını sağlamıştır. ANI 35L yönteminde ağırlıklı olarak "kaynağa ve yönetim uygulamalarına dayalı parametreler" değerlendirilmekte ve hayvanın yaşadığı çevreye ait unsurların hayvan refahı için ne ölçüde uygun olduğu sorgulanmaktadır [34]. Hayvana dayalı refah parametreleri için ise en fazla 6 puan (toplam puanın %13.2'si) verilebilmektedir. Bu noktada her bir kriter için öngörülen puanların; i) bilimsel yöntemlere dayalı olarak belirlenmiş olması ve ii) o kriterin refah düzeyi üzerine oransal katkısını yansıtmasının önemi ön plana çıkmaktadır.

Hayvanlar ile çevre arasındaki etkileşimin sonuçları olan hayvana dayalı parametreler, hayvanların içinde bulundukları ortama ne düzeyde uyum sağlayabildiklerinin direkt göstergeleridir [15,35]. Dolayısı ile birçok araştırmacı refah düzeyi değerlendirmesinde hayvana dayalı parametrelere daha fazla ağırlık verilmesini önermektedir [36]. Ancak hayvana dayalı parametrelerin ölçümü daha fazla işgücü ve zaman gerektirmekte, dolayısı ile de bu parametrelerin çiftlik ortamında değerlendirilmesi her zaman mümkün olamamaktadır [37]. Bu durum, değerlendirilmesi mümkün olan hayvana dayalı parametreleri temel alan ve ayrıca refah üzerine belirgin etkisi olan çevresel parametreleri de içeren refah değerlendirme sistemlerinin geliştirilmesinin gerekliliğine işaret etmektedir. Diğer yandan, ideal bir hayvan refahı değerlendirme sistemi, hayvanların mevcut refah durumunu güvenilir bir şekilde ortaya koymanın yanı sıra sürü bazında refah düzeyini iyileştirmek amacıyla nelerin yapılması gerektiği konusunda da geri bildirim sağlamalıdır. Bu bağlamda, EFSA hayvan sağlığı ve refahı komitesi (AHAW) iyi bir refah düzeyi elde edebilmek için "kaynağa ve yönetim uygulamalarına dayalı parametreler kullanılarak zayıf refah riskinin azaltılması" ve "hayvana dayalı ölçüler kullanılarak mevcut refah düzeyinin gözlenmesi" gerektiğini bildirmektedir [15].

Araştırma materyalinin bir bölümünü oluşturan serbest sistemdeki işletmelerde, çeşitli verim ve sağlık kayıtlarının tutulduğu ve hayvana dayalı birçok refah parametresinin değerlendirilebileceği gözlenmiştir. Diğer yandan bağlı sistem kullanan işletmelerde verim ve sağlık kayıtlarının tutulmadığı, dolayısı ile incelenebilecek hayvana dayalı refah parametrelerinin sadece çiftlik ziyaretleri sırasındaki gözlemlerle elde edilebilecek veriler olduğu belirlenmiştir. Bu durum çalışmanın refah değerlendirme yöntemi belirleme sürecini etkilemiş, "Welfare Quality" protokolü gibi daha fazla hayvana dayalı parametreyi içeren bir yöntem yerine büyük ölçüde çevre koşullarının hayvan refahı açısından uygunluğunun değerlendirildiği ANI 35L yöntemi tercih edilmiştir. Türkiye'deki besi sığırı işletmelerinin büyük çoğunluğunu geleneksel yöntemlerle üretim yapan, düzenli kayıt tutulmayan küçük aile işletmeleri oluşturmaktadır [38]. Bu araştırmada farklı barınma sistemine sahip işletmelerde ANI 35L yönteminin başarı ile uygulanması; hayvana dayalı parametrelerin incelenmesinin mümkün olmadığı, geleneksel yöntemlerle üretim yapan küçük aile işletmelerinin hayvan refahı açısından değerlendirilmesi amacıyla ANI 35L yönteminin kullanımının önerilebileceğine işaret etmektedir.

Bu çalışmada elde edilen sonuçlar; serbest sistemde hayvanların avluya çıkma olanağının bulunmasının hayvan refahına olumlu katkı sağladığı, bağlı sistem kullanan işletmelerde durak alanı ve servis yolunun yapısal özelliklerinin hayvan refahını olumsuz etkilediği ve bağlı sistem kullanan aile işletmelerindeki ahır içi sıcaklık ve THI düzeylerinin sıcaklık stresi eşik değerine ulaştığına işaret etmektedir. Dolayısı ile daha iyi hayvan refahı düzeyine ulaşabilmek için serbest sistem kullanan işletmelerin yaygınlaştırılması gerektiği sonucuna ulaşılmıştır.

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Research Article

The Comparative Analysis of Phenotypic and Genotypic Properties of *Aeromonas sobria* Strains Isolated from Rainbow Trout (Oncorhynchus mykiss, Walbaum, 1972) [1]

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Abstract

Phenotypic and molecular characterization of *Aeromonas sobria (A. sobria)* isolates by antibiotyping, sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of whole cell proteins, random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) was aimed in this study. For this aim, thirty-six *A. sobria* isolates were analysed. Isolates were divided into 12 different antibiotypes and 4 proteotypes according to their antibiotic susceptibilities and SDS-PAGE patterns, respectively. Thirteen RAPD types were observed among all isolates. In conclusion, the use of double or triple combination of typing methods in this study was found to be more useful for discriminating the strains. The results obtained from this study may give information about phenotypic and genotypic variability of the *A. sobria* strains isolated from different regions of Turkey and can be helpful to control disease in fish through guiding the antibiotic therapy and giving information that will be useful to development vaccine.

Keywords: *Aeromonas sobria, Antibiotic resistance, Fish, RAPD, SDS-PAGE*

Gökkuşağı Alabalıklarından (Oncorhynchus mykiss, Walbaum, 1972) İzole Edilen *Aeromonas sobria* Suşlarının Fenotip ve Genotip Yönünden Karşılaştırmalı Analizi

Özet

Bu araştırmada *Aeromonas sobria (A. sobria)* izolatlarının antibiyotiplendirme, tüm hücre proteinlerinin sodyum dodesilsulfat-poliakrilamid jel electroforezi (SDS-PAGE) ile analizi, rastgele çoğaltılmış polimorfik DNA (RAPD) PCR ile genotiplendirme ile fenotipik ve genotipik karakterizasyonu amaçlanmıştır. Bu amaçla otuzaltı adet *A. sobria* izolatı incelenmiştir. İzolatlar antibiyotik duyarlılık sonuçlarına göre 12 farklı antibiyotip ve SDS-PAGE profiline göre 4 farklı proteotipe sahip oldukları belirlenmiştir. İzolatların 13 farklı genotipe sahip oldukları saptanmıştır. Sonuç olarak, Türkiye izolatı *A. sobria* suşlarının fenotipik ve genotipik olarak çeşitlilik gösterdiği ve çalışmada kullanılan tiplendirme metodlarının ikili veya üçlü kombinasyonlar halinde kullanılmasının suş ayrımında daha faydalı olduğu belirlenmiştir. Çalışmada elde edilen sonuçların balıklarda *A. sobria* infeksiyonlarının antibiyotik tedavi seçeneklerinin belirlenmesi konusunda faydalı olmakla birlikte, konu ile ilgili olarak yapılacak olan aşı ve teşhis kiti geliştirilmesi çalışmalarına öncülük edeceği düşünülmektedir.

Anahtar sözcükler: *Aeromonas sobria, Antibiyotik direnci, Balık, RAPD, SDS-PAGE*

INTRODUCTION

Motile Aeromonads which are *Aeromonas hydrophila, Aeromonas caviae* and *Aeromonas sobria*, all are small, Gram negative and rod-shaped bacteria are responsible for some troublesome diseases in pond fish [1]. These bacteria show wide-spread distribution in aquatic environment and several stress factors in aquatic culture systems predispose

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the fish to infections. The diseases caused by motile aeromonads are called as Motile Aeromonas Septicemia (MAS), Motile Aeromonad Infection (MAI), hemorrhagic septicemia, red pest or red sore. Whatever they are called, they are responsible for considerable economic losses [2]. Aeromonas septicemia is also fatal in reptiles, amphibians and humans. These organisms are considered to be a food and water borne pathogen causing an acute diarrheal disease human [3].

Motile Aeromonads that have multiple DNA groups (phenospecies) could not be differentiated from one another by biochemical tests readily and there are taxonomic complexities within the genus [4]. However, several reliable molecular *Aeromonas* identification methods have enabled new species such as *Aeromonas tecta* and *Aeromonas piscicola* to be discovered and also other known species associated to fish disease to be recognized such as *Aeromonas bestiarum, Aeromonas sobria, Aeromonas encheleia, Aeromonas veronii, Aeromonas eucrenophila* and *Aeromonas media* [5-8].

To characterize and type the bacterial isolates, conventional methods based on phenotypic characteristics such as biochemical properties and antimicrobial resistance, bacteriophage susceptibilities and rection to antisera together with molecular methods are offered as effective ways. Now, several genotypic methods such as polymerase chain reaction (PCR), pulsed-field gel electrophoresis (PFGE), DNA sequencing and restriction enzyme digestion with Southern blotting of chromosomal DNA and plasmids are used easily and rapidly. To determinate the genetic relatedness between bacterial strains by molecular methods are powerful and more commonly useful for bacterial typing [9]. Several PCR based protocols have been designed to identify and characterization of *Aeromonas* strains [10,11]. Moreover RAPD PCR with ERIC primer has been used for typing *Aeromonas* isolates [12,13]. Also some phenotypic and molecular methods have been used together to characterize *Aeromonas* strains and the results from these methods were evaluated comperatively [14,15].

In the current study, we aimed to characterize *A.sobria* isolates from rainbow trouts using both RAPD as a molecular method and phenotypic methods including SDS-PAGE analysis and antibiotyping.

MATERIAL and METHODS

Aeromonas sobria Strains

Total of 89 *Aeromonas* strains isolated between 2007-2011 from Rainbow trout *(Oncorhynchus mykiss)* were examined. To identify these isolates at the species level, strains were inoculated onto Tryptic Soy Agar (TSA) and incubated at 28°C. Macroscopically suspected colonies were selected. Strains were identified according to their Gram staining and biochemical characteristics based on Aerokey II group of tests for the identification of *Aeromonas* [16,17].

Identification of A. sobria Isolates by PCR

To confirm and genotype the *A. sobria* strains, DNA extractions of all isolates were performed using commercial DNA extraction kit (Invitrogen, Canada). The concentrations of extracted DNAs were measured and equalized to 10 ng/ml. To identify and confirm the strains as *A. sobria*, PCR was carried out according to Das et al.[10] with *asa*1 gene-specific primers F (5'-TAA AGG GAA ATA ATG ACG GCG-3') and R (5'-GGC TGT AGG TAT CGG TTT TCG-3'). The expected size of the PCR product was 249 bp. *A. sobria* ATCC 43979 was used as standard control strains in this study.

Genotyping of A. sobria Isolates by RAPD-PCR

ERIC-2 (5'-AAG TAA GTG ACT GGG GTG AGC G-3') primer was used to determine RAPD patterns of *A. sobria* strains. Amplification was performed according to the method reported by Onuk et al.[18] and amplification products were analysed by 1.5% agarose gel electrophoresis. DNA was visualized by staining with ethidium bromide. Dendogram of these patterns was obtained using UPGMA (Unweighted Pair Group Method with Arithmetic Averages). The genetic relatedness between the strains was determined applying the 70% similarity index.

Antibiotyping

To determine the resistance of the isolates against several antibiotics using in fish farming, Kirby-Bauer Disc Diffusion Method was used [19]. The antimicrobial agents were tested including nalidixic acid (30 µg), gentamycin (10 µg), sulfamethoxazole + trimethoprim (1.25 µg/23.75 µg), ampicillin (10 µg), oxolinic acid (2 µg), flumequine (30 µg), erythromycin (15 µg), oxytetracycline (30 µg), neomycin (10 µg), kanamycin (30 µg), florfenicol (30 µg), amoxicillin (25 µg), enrofloxacin (5 µg) (Bioanalyse, Turkey). Results were evaluated as sensitive, intermediate and resistant according to NCCLS [19]. A dendogram was created using UPGMA. The relatedness between the strains was determined applying the 70% similarity index.

Determination of SDS-PAGE Profiles

Whole cell protein profiles of *A. sobria* strains were analysed by SDS-PAGE [20,21]. Strains were propoagated onto Brain Heart Infusion Agar at 37°C for 24 h and after incubation period colones were harvested from agar plate by washing physiological saline and cells were inactivated by incubating at 60°C for 30 min. Inactivated bacterial suspension was centrifugated at 10.000 rpm for 15 min and supernatant was decanted. After pellet was washed three times it was resuspended in 0,01M Tris-HCl (pH 7.4) and optical density (OD) was adjusted to 5.0 at 470 nm spectrophotometrically. Before loading samples into gel for SDS-PAGE, samples were mixed with the sample buffer

in 2:1 (v/v) and sonicated for 5 min. Then mixture was incubated at 100°C in a water bath for providing protein denaturation. Twenty microliter samples were loaded into each well of 10% polyacrilamide gel. After electrophoresis at 200 V for 20 min, Blue Silver staining method was used for visualizing the protein bands. In electrophoresis, a molecular weight standard (205-6,6 kDa, Sigma S8445) was used for determining and calculating the molecular weights of protein bands.

Determination of Typeability, Reproducibility, Discrimination Power and Confidence Intervals of Typing Methods

To determine the typeability, a formula, T = Nt/N where Nt is the number of isolates assigned a type and N is the number of isolates tested, was used. The reproducibility was determined by using the formula: R = Nr/N, where Nr is the number of isolates assigned the same type on repeat testing and N is the number of isolates tested [22]. Discriminatory indices and confidence intervals of the typing methods were determined according to the formulas described previously [23,24].

RESULTS

Identification of A. sobria

Fourty five of eighty nine *Aeromonas* isolates from rainbow trout were identified as *A. sobria* by conventional cultural tests. Thirty six of 45 isolates were confirmed by PCR as *A. sobria*. *A. sobria* isolates including a reference *A. sobria* strain gave a specific 249 bp band. Further analyzes were carried out with using the 36 isolates which were commonly identified as *A.sobria* by conventional tests and PCR.

RAPD-PCR Profiles of A. sobria Strains

All *A. sobria* strains (n=36) were able to typed by this method. The strains showed genetic variability and given the 70% similarity indices, they were grouped into 13 RAPD types. One of them, R4 was a cluster including two subtypes (R4a and R4b) and the others were unique types *(Fig. 1)*. Most of the isolates (33.33%) were assigned to R13 type and the second predominant RAPD type, R1 included 8 (22.22%) isolates.

Antibiotyping

According to the antibiogram results, *A. sobria* strains (n=36) were grouped into 12 antibiotypes depending upon their susceptibilities to 13 different antibiotics. Dendogram of antibiotyping of these isolates is shown in *Fig. 2*. These antibiotypes (A1-A12) included four clusters (A3, A4, A10 and A12) and eight unique types. Cluster A3 and A12 were differentiated into 8 and 9 subtypes, respectively. All isolates except for one (97.2%) were resistant against oxolinic acid and most of the isolates (91.7%) were susceptible to florfenicole. More than 60% of isolates were resistant against oxytetracycline, amoxycillin and ampicilline. The predominant types, A3 and A12 included 11 and 12 isolates, respectively. While in A3 cluster, dominant pattern was susceptible to the most of antibiotics (at least seven), in A12 cluster most of the isolates were resistant against at least 5 antibiotics. The percentages of isolates that were susceptible, intermediate or resistant to 13 antibiotics were given in the *Table 1*.

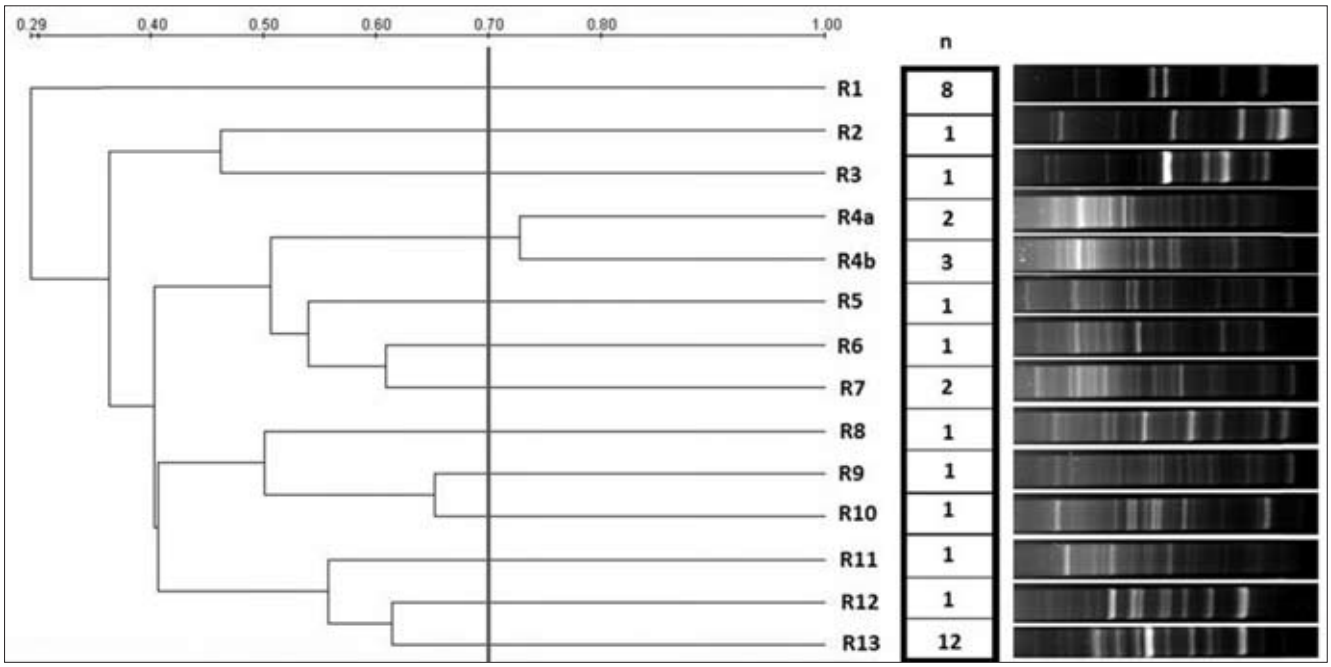

Fig 1. The phylogenetic tree of *A. sobria* strains with RAPD-PCR patterns
Şekil 1. RAPD-PCR profiline göre *A. sobria* suşlarının filogenetik ağacı

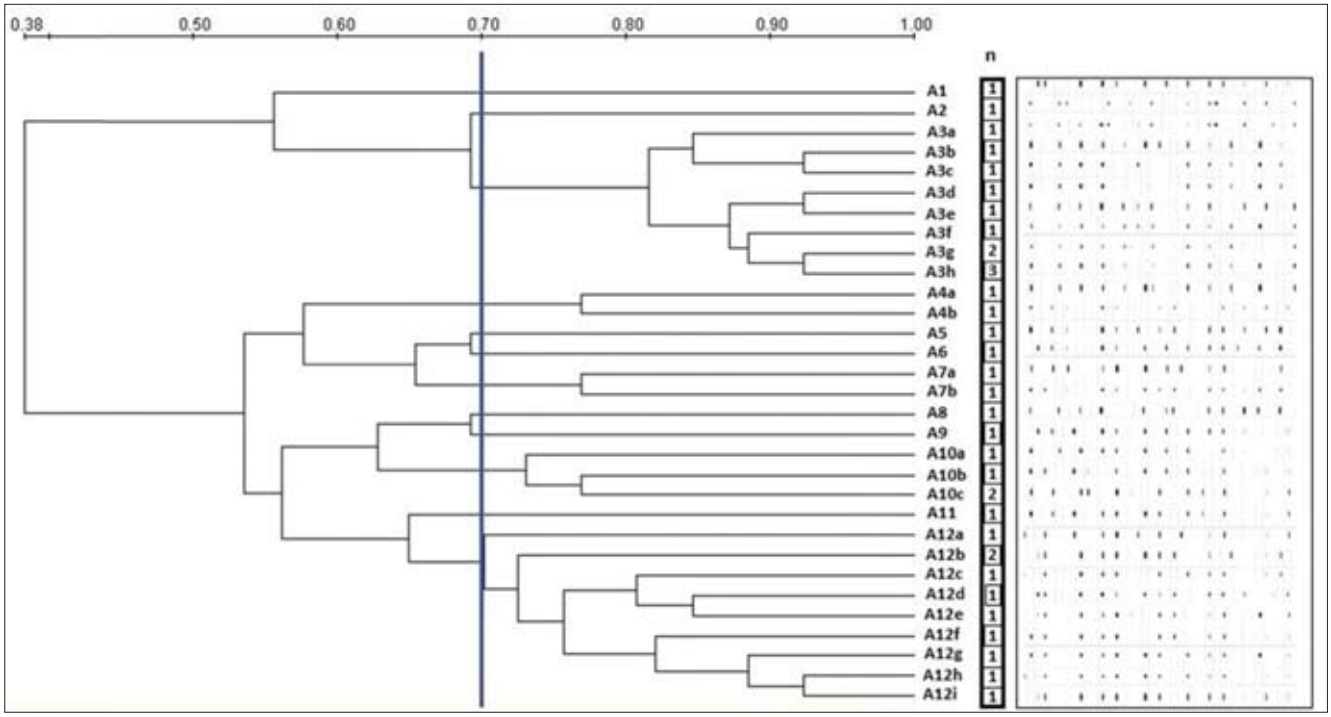

Fig 2. Antibiotyping dendogram of *A. sobria* isolates depending upon susceptibilities to 13 antibiotics
Şekil 2. *A. sobria* suşlarının 13 antibiyotiğe karşı duyarlılıklarına gore antibiyotip dendogramı

Table 1. *Antibiotic susceptibilities of A. sobria isolates*
Tablo 1. *A. sobria izolatlarının antibiyotik duyarlılıkları*

Antibiotic Discs	Resistance Profiles					
	R		I		S	
	n	%	n	%	n	%
Oxolinic acid (2 µg)	35	97.2	1	2.8	0	0
Oxytetracycline (30 µg)	25	69.5	4	11.1	7	19.4
Amoxycillin (25 µg)	23	63.8	2	5.6	11	30.6
Ampicilline (10 µg)	22	61.1	2	5.6	12	33.3
Nalidixic acid (30 µg)	17	47,2	12	33.3	7	19.5
Flumequine (30 µg)	16	44.4	14	38.9	6	16.7
Erythromycin (15 µg)	15	41.7	11	30.6	10	27.7
Neomycin (10 µg)	8	22.2	13	36.1	15	41.7
Sulphamethoxazole-trimetoprim (25 µg)	6	16.7	0	0	30	83.3
Enrofloxacin (5 µg)	6	16.7	11	30.6	19	52.7
Kanamycin (30 µg)	5	13.9	6	16.7	25	69.4
Gentamycin (10 µg)	2	5.6	8	22.2	26	72.2
Florfenicol (30 µg)	0	0	3	8.3	33	91.7

R: *Resistance* ***I:*** *Intermedier resistance.* ***S:*** *Susceptibility*

Protein Profile Analysis by SDS-PAGE

All thirty six *A. sobria* isolates were able to typed according to their whole cell protein profiles by SDS-PAGE. Strains were clustered at 70% similarity level using UPGMA method. Strains were grouped into two cluster (S2 and S4) and 2 unique types. Most of the isolates (66.66%) were assigned to S2 cluster and the similarity level between two subtypes (S2a and S2b) within this cluster was 75%. Besides the second predominant cluster including seven (19.44%) isolates included two subtypes, S4a and S4b and 87% of similarity was shown between these subtypes. A dendogram illustrating SDS-PAGE protein patterns of *A. sobria* isolates is shown in *Fig. 3*.

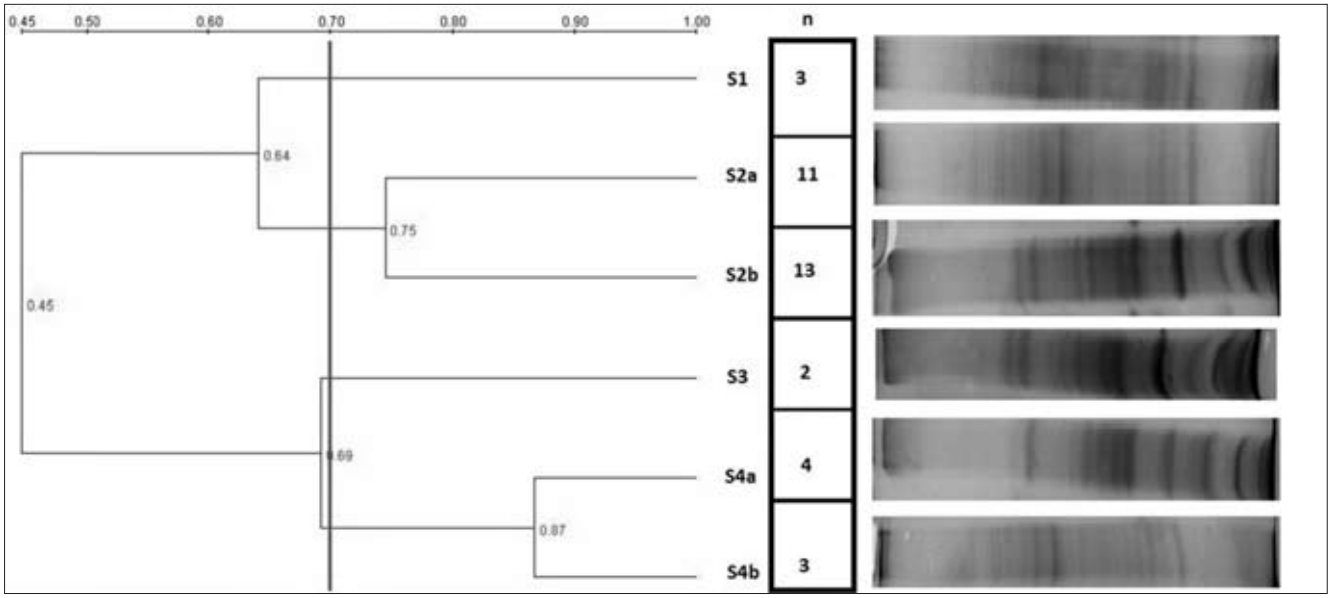

Fig 3. Dendogram based on whole cell protein patterns of *A. sobria* strains
Şekil 3. *A. sobria* suşlarının tüm hücre protein profillerine göre dendogramı

Table 2. *Discriminatory indices (with Confidence intervals) of the typing methods used in this study (70% of cut-off value)*
Tablo 2. *Çalışmada kullanılan tiplendirme metodlarının (%70 eşik değeri) ayrım güçleri (güven aralıkları ile)*

Method	No. of Types	Discrimination Power	Confidence Intervals (%)
Antibiotyping	12	0.83	78-88
SDS-PAGE	4	0.52	38-66
RAPD-PCR	13	0.83	80-87
Antibiotyping+ SDS-PAGE+ RAPD-PCR	22	0.95	93-97

The Typeability, Reproducibility, Discriminatory Power and Confidence Intervals of Typing Methods

All strains (n=36) were typeable and methods used to type *A. sobria* strains phenotypically and genotypically had good reproducibility. Discriminatory indices (D) and confidence interval (CI) of the methods and their combinations were given in *Table 2*.

DISCUSSION

Motile mesophilic *Aeromonas* species including *A. sobria* are Gram negative, ubiquitious bacteria which are frequently isolated from various foods such as fish, shellfish, raw meat and raw milk, vegetables. However, seafood was the most favorable environment for proliferation of these bacteria. FDA considers that motile *Aeromonads* are re-emerging enteric pathogens [25]. These bacteria are also producing warmwater fish diseases [26]. Some factors such as poor water quality, overcrowding and rough handling causing stress are the most common cause the susceptibility of fish to these organisms [27].

Over time, new species has been added to *Aeromonas* genus and however it has been proved that aeromonads were the member of *Aeromonadaceae* family by means of genetic studies. Futhermore, multiple hybridization groups (HGs) were revealed within each of mesophilic *Aeromonas* including *A. sobria* by *DNA* hibridization studies [28]. According to the last edition of Bergey's Manual of Systematic Bacteriology [17], 17 HGs or "genomospecies" and 14 "phenospecies" which refers to a single heterogeneous species (such as *A. sobria*) containing multiple HGs within it are described. Although taxonomic confusions has been tried to solve, it is difficult to see the harmony between phenotypic and genotypic characteristics of *Aeromonas* species. Much of phenotypical methods especially based on biochemical characteristics of *Aeromonas* species are not good enough to characterize and differentiate the species due to their complex taxa. Therefore multiple molecular techniques are required for accurate characterization [3,18]. Some phenotyping methods other than the methods based on biochemical characteristics such as antibiotyping and SDS-PAGE protein analysing have been used to type and/or characterize *Aeromonas* spp [4,8,10]. Das et al.[25] have reported that all *A. sobria* isolates from fresh water fish, frozen fish and fish pickle were 100% resistance to ampicillin. The percentages of resistance against nalidixic acide and neomycin were 12% and 80%, respectively. Awan et al.[29] and Guz and Kozinska [30] have also found that all *A. sobria* strains isolated from food and environmental samples and diseased fish, respectively were resistant against ampicillin. Motyl et al.[31] have also found that all *A. sobria* isolates of human origin were resistant to ampicillin. Guz and Kozinska [30] have also reported that these strains (100%) were sensitive to trimethoprim-sulphamides, oxolinic acid and flumequine. However in this study, 61.1% of *A. sobria* strains were resistant to ampicillin and

the susceptibilies to nalidixic acid and neomycin were also different from their reports. The percentages of resistance against nalixic acid and neomycin were found as 47.2% and 22.2%, respectively. Resistance of our *A. sobria* strains against nalidixic acid were relatively higher in those in other studies [25,32]. Other notable differences in the results between Guz and Kozinka's study [30] and this study were the susceptibilities to flumequine and oxolinic acid. Namely, our *A. sobria* strains were found to be resistant against flumequine (44.4%) and oxolinic acid (97.2%). However while the less percentage (16.7%) of resistance against trimetoprim-sulphamethaxasole was found, resistance against oxytetracycline (69.5%) was higher in this study. Other researchers [29,31] have reported the higher percentages of the susceptibility to tetracycline compared to our results. As for the resistance of the strains against erythromycine, our strains were less resistant (41.7%) than those isolated in other studies [29,30]. The most strains (94.40%) in this study were susceptible to gentamicin as such in other studies [29,31]. Nalidixic acid, flumequine and oxolinic acid which are the first generation quinolons and also oxytetracycline are widely used in farm fish. Resistance against these quinolones especially against oxolinic acid and against oxytetracycline seems to be significant in terms of the difficulties in fish therapy. Similarly resistance against ampicillin (although found to be less than in some other studies) was also noticible. Although antimicrobial therapy is an effective way to control fish diseases, widespread and improper antibiotic use and also other factors such as genetic mutations have all resulted in antibiotic resistance [32-34]. Therefore more effective antimicrobial agents and also vaccines such an alternative way to control disease should be developed.

Whole-cell protein fingerprinting is one of the typing methods used for both taxonomy and differentiation of strains within a species [35]. Several studies have performed to analyse the protein profiles of *Aeromonas* spp and widely varying patterns have been obtained [14,36]. Körkoca and Boynukara [37] have firstly analysed SDS-PAGE protein profiles of *A. hydrophila* and *A. caviae* strains in Turkey. However, studies are seen to be limited and especially no study is available on characterization of protein profiles of *A. sobria* isolates from diseased fish. We analysed the whole cell protein profiles of 36 *A. sobria* strains in order to type and characterize them. Although no very high diversity was found among strains, all of them were typeable by SDS-PAGE analyse and clustered by UPGAMA method. We obtained two clusters (S2 and S4) and 2 unique types and most of the isolates were assigned to S2 cluster. Discriminatory power of SDS-PAGE method determining whole cell profiles was found low (0.52) however, when it was used in combination with another phenotypic method (antibiotyping) and RAPD typing discriminatory power become high (0.95). So using whole cell profiling in combination with other typing techniques may be useful to type and characterize the strains. Maiti et al.[14] have also found that discriminatory power of whole cell profiling was low. Furthermore some researchers [38,39] have reported that although protein fingerprinting has the potential to differentiate *Aeromonas* species, whole cell protein profiles were unsuitable for the characterization of strains within a species because of the low qualitative variation.

Although several phenotypic methods that also mentioned above are effectively used to type different bacterial strains signly or together with another phenotypic and/or genotypic methods, several genotypic techniques such as AP-PCR (arbitrarily primed PCR) or RAPD-PCR, PFGE (Pulsed Field Gel Electrophoresis), DNA sequencing and restriction enzyme digestion with Southern blotting of chromosomal DNA and plasmids are considered to be more powerful and more commonly used to characterize the strains and determine the genetic relatedness between them. However, some criteria such as reproducibility, typeability, discriminatory power, speed, simplicity, ease of interpretation and cost should be considered to optimize these molecular techniques [40-42]. This method has considered to be cheap, simple, speed and to need less equipment. We attempted to determine the genetic relatedness among *A. sobria strains* isolated from diseased fishes by a RAPD-PCR method using ERIC primer. All the strains were able to type and made a cluster analysis. Only one cluster including two subtypes and 12 unique types were found based on 70% similarity indices. Although most of the strains, 33.33% and 22.22% of the strains were assigned to cluster R4 and R1, respectively, the strains showed wide variation among themselves. This may due to their different geophragical origins. Yousr et al.[13] have used RAPD-PCR and ERIC-PCR for molecular typing of *Aeromonas* species and they have found these strains also including *A. sobria* were very diverse. They have grouped *A. sobria* strains into one significant cluster grouped the three strains from the same source and six single isolates at the similarity of 40%. In several studies [12,38], numerous *Aeromonas* isolates from different geographical, environmental and clinical origins have been tried to distinguish and characterize using RAPD-PCR. Although RAPD-PCR has been proved to be useful tool for epidemiological investigation and population genetic analysis of *Aeromonas* spp.[38], RAPD fingerprinting has been reported to allow the identification of strains; but, because of the high variability its potential as an aiding method for species identification was limited [12]. On the other hand being able to detect diversities by this method may allow advantage for vaccine development trials. There is no available study on the phenotypic and genetic diversity of *A.sobria* isolates from diseased fish in Turkey.

In conclusion, we were able to type *A. sobria* strains isolated from diseased fish in different regions of Turkey through both phenotypically based on their antibiotic susceptibilities and whole cell protein profiles and geno-

typically using RAPD-PCR. While high diversitiy among the strains were found by antibiotyping and RAPD-PCR fingerprinting, in SDS-PAGE whole cell protein analysis, less variation were observed in their protein profiles. Compared the discriminatory powers of all three typing methods, RAPD-PCR and antibiotyping was superior to SDS-PAGE. However when used in combination with other techniques SDS-PAGE analysis may contributes the typing goal. Likewise the discriminatory power of triple combination of typing methods we used was higher than when used individulally. The results obtained from anti-biotyping should be guided the antimicrobial therapy efforts in terms of any emergence of antibiotic resistance. The results obtained from this study may give information about variability of the *A. sobria* strains isolated from different regions of Turkey and can be helpful to control disease in fish through guiding the antibiotic therapy and giving information that will be useful to development vaccine.

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Research Article

Japon Bıldırcınlarının Rasyonlarına Çörek Otu *(Nigella sativa* L.) Tohumu veya Çörek Otu Yağı İlavesinin Besi Performansı, Karkas Özellikleri ve Bazı Kan Parametrelerine Etkisi

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Özet

Bu çalışma, bıldırcın rasyonlarına çörek otu tohumu ya da çörek otu yağı ilavesinin besi performansı, karkas özellikleri ve bazı kan parametrelerine etkisini belirlemek amacıyla yapıldı. Araştırmada, 180 adet bir günlük yaşta Japon bıldırcını civcivi kullanıldı. Civcivler 45 hayvandan oluşan 4 ana gruba, her ana grup ise 15 civcivden oluşan 3 alt gruba ayrıldı. Gruplardan birisi başlangıç (1-21. gün) ve büyütme (22-35. gün) dönemlerinde temel yemlerle beslendi (Kontrol). Diğer gruplar aynı yeme %0.1 oksitetrasiklin (Pozitif Kontrol: PK), %1 çörek otu tohumu (ÇOT) veya %0.1 çörek otu yağı (ÇOY) ilave edilerek beslendi. Deneme bitiminde her ana gruptan 15 bıldırcın kesilerek karkas verim özellikleri ve kan serum parametreleri belirlendi. Araştırma sonunda, PK ve ÇOT gruplarının canlı ağırlık ortalaması Kontrol'den önemli derecede yüksek bulundu. Araştırma geneli dikkate alındığında ortalama canlı ağırlık artışı, yem tüketimi ve yemden yararlanma oranı bakımından gruplar arasında farklılık görülmedi. Karkas ağırlığı; Kontrol grubunda diğer tüm gruplardan önemli derecede düşük bulunurken, karkas randımanı, göğüs, but ve kanat oranları bakımından gruplar arasında farklılık oluşmadı. Serum kolesterol konsantrasyonu ÇOY grubunda Kontrol grubundan önemli derecede düşük tespit edilirken, Ca, P, VLDL, HDL, LDL, ALT, ALP, albumin, globulin ve total protein konsantrasyonu bakımından gruplar arasında farklılık görülmedi. Sonuç olarak; bıldırcın rasyonlarına %1 oranında çörek otu tohumu ilavesinin antibiyotik ilaveli grupta olduğu gibi canlı ağırlığı önemli derecede artırdığı, %0.1 oranında çörek otu yağı ilavesinin hipokolesterolemik etki gösterdiği tespit edilmiştir.

Anahtar sözcükler: *Bıldırcın, Çörek otu tohumu, Çörek otu yağı, Besi performansı, Karkas, Kan parametreleri*

Effect of Black Cumin *(Nigella sativa* L.) Seeds or Black Cumin Oil Addition to Japanese Quail Diets on Growth Performance, Carcass Traits and Some Blood Parameters

Abstract

This study was conducted to determine effects of black cumin seeds or black cumin oil addition to quail rations on growth performance, carcass traits and some blood parameters. Totally 180 daily Japanese quail chicks were used. Chicks allocated four main groups each containing 45 chicks, and each main group divided into three subgroups each containing 15 chicks. A group was fed with basal starter diet for 1-21th days and grower diet for 22-35th days (Control). Other groups were fed same diets but their diet added by 0.1% oxytetracycline (Positive control: PC), 1% black cumin seed (BCS) or 0.1% black cumin oil (BCO). At last of study 15 chicks from each main group were slaughtered for determination of carcass traits and blood serum parameters. Final average live weight of quails in PC and BCS groups were significantly higher than C group. There were no differences on live weight gain, feed consumption and feed conversion ratio among the groups on basis of whole study period. Carcass weight was lower in C group than the other groups, while there were no differences on carcass yield, leg, breast and wing ratio among the groups. Serum cholesterol concentration in BCO group was significantly lower than C group but there were no differences on Ca, P, VLDL, HDL, LDL, ALT, ALP, albumin, globulin, total protein concentrations among the groups. In conclusion, it was established that, as being in antibiotic added groups, addition of 1% black cumin seed to quail diet enhanced live weight, and 0.1% black cumin oil had showed hypocholesterolemic affect.

Keywords: *Quail, Black cumin seed, Black cumin oil, Growth performance, Carcass, Blood parameters*

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GİRİŞ

Başta broyler yetiştiriciliği olmak üzere çeşitli çiftlik hayvanlarının yetiştirilmesinde 1940'lı yıllardan itibaren büyümeyi uyarıcı yem katkı maddesi olarak çeşitli antibiyotikler kullanılmıştır. Ancak, yem katkı maddesi olarak antibiyotik kullanımının ülkelerin kamuoyunda oluşturduğu rahatsızlıklar, antibiyotiklerin kullanıldığı hayvan dokularında rezidü oluşturma riski, mikroorganizmaların antibiyotiklere karşı direnç geliştirmesi gibi hususlardan dolayı 2006 yılından itibaren yem katkı maddesi olarak kullanılması Avrupa Birliği Ülkelerinde [1] ve Türkiye'de [2] yasaklanmıştır. Bu yasağın ardından antibiyotiklere alternatif olarak kullanılabilecek büyümeyi uyarıcı çeşitli organik maddeler üzerinde yapılan çalışmalar daha da hız kazanmıştır. Bu bağlamda üzerinde durulan maddelerden birisi de aromatik bir bitki olan çörek otu tohumu [3-8] ve bundan elde edilen yağlardır [9-12].

Çörek otu *(Nigella sativa* L.) *Ranunculaceae* (Düğün çiçeğigiller) familyasına ait yıllık bir bitki olup Ortadoğu, Akdeniz ülkeleri, Kuzey Afrika ve Asya kıtasının güneyi başta olmak üzere dünyanın çeşitli ülkelerinde yetiştirilmekte, çörek otundan elde edilen çörek otu tohumu yemeklerde baharat, sağlığın korunması ve şifa amacıyla kullanılmaktadır [4,5,13,14].

Çörek otu tohumunun yapısında temel besin maddeleri olarak %91.50-94.48 kuru madde, %34.49-41.60 yağ, %16.00-26.70 protein, %24.90 azotsuz öz madde, %23.50-33.20 toplam karbonhidrat, %7.94-8.40 selüloz, %3.77-4.86 kül, amino asitler (glutamik asit, arjinin, aspartik asit), mineraller (Ca, P, Na, K, Fe, Cu, Zn, Se, Mg, Mn) ve vitaminler (A, C, tiamin, niasin, pridoksin) bulunmaktadır [13-18]. Çörek otunda farmakolojik anlamda etken maddeler olarak %0.5-1.6 arasında uçucu yağlar (thymoquinon, dithymoquinon, thymohydroquinon, nigellon, thymol, carvacrol, α ve β pinen, d-limenonene, p-cymen), alkoloidler, steroller (betasosterol, sykloeikolenol, sykloartenol, sterol esterler, sterol glukosidler), saponinler ve quinonlar bulunmaktadır [16,18]. Yapılan çalışmalar çörek otu tohumunun antibakteriyel [19-22], antioksidan [23,24] immun sistemi destekleyici [5,25], helmintleri öldürücü [26], antidiabetik [27] ve antitümoral [28] aktivite gösterdiğini ortaya koymuştur.

Broyler rasyonlarına %0.25, 0.50 ve 0.75 oranında çörek otu tohumu ilave edilerek yapılan bir çalışmada [29], çörek otu ilavesinin canlı ağırlık, canlı ağırlık artışı, yem tüketimi ve yemden yararlanma oranını etkilemediği belirlenmiştir. Aynı çalışmada sadece rasyona %0.50 oranında çörek otu tohumu ilavesinin diğer gruplara göre kalçalı but ağırlığını önemli derecede artırdığı, göğüs ve baget but ağırlığı bakımından tüm gruplar arasında farklılık görülmediği tespit edilmiştir.

Broyler rasyonlarına %2 ve 10 oranında çörek otu tohumu ilave edilerek yapılan bir başka çalışmada [3], çörek otu ilave edilen gruplardaki hayvanların canlı ağırlık artışı kontrol grubundan önemli derecede yüksek bulunmuştur. Aynı çalışmada, çörek otu verilen grupların yem tüketimi ve yemden yararlanma oranı kontrol grubundan rakamsal olarak daha yüksek bulunmuştur. Kan serumu AST, ALT, total protein, albumin, globülin, kolesterol ve Ca miktarları bakımından Kontrol grubuna benzer sonuçlar alınmıştır.

Broyler rasyonlarına 10 mg/kg avilamisin, %0.5, 1, 2 ve 3 oranlarında çörek otu tohumu ilavesinin yem tüketimini etkilemediği, ancak canlı ağırlık artışı, yemden yaralanma oranı ve karkas randımanının antibiyotik ve % 1 çörek otu ilave edilen grupta diğer gruplardan önemli derecede yüksek olduğu tespit edilmiştir [4]. Bu araştırma sonunda; kanatlı yemlerinde antibiyotiklere alternatif doğal bir büyüme uyarıcı madde olarak %1 oranında çörek otu tohumu kullanılabileceği bildirilmiştir.

Bıldırcın rasyonlarına antibiyotiklere (10 ppm flavomycin) alternatif olarak 60 ppm çörek otu esansiyel yağı ilavesinin besi performansı ve karkas özellikleri üzerine etkisinin araştırıldığı bir çalışmada [11], besi performansı, karkas ağırlığı ve karkas randımanı bakımından rasyona çörek otu esansiyel yağı ilavesinin kontrole benzer sonuçlar verdiği belirlenmiştir.

Bu çalışma; bıldırcın rasyonlarına antibiyotiklere alternatif olarak, çörek otu tohumu veya çörek otu yağı ilavesinin besi performansı, karkas özellikleri ve bazı kan parametreleri üzerine etkilerini belirlemek amacıyla yapılmıştır.

MATERYAL ve METOT

Bu araştırma, Dollvet A.Ş. Hayvan Deneyleri Etik Kurulu'nun 2014 tarih ve 17 sayılı onayına istinaden yürütüldü. Araştırma, Kafkas Üniversitesi, Hayvancılık Araştırma ve Uygulama Merkezinde bulunan kanatlı ünitesinde yapıldı. Araştırmada hayvan materyali olarak bir günlük yaşta karışık cinsiyette 180 adet Japon bıldırcını *(Coturnix coturnix japonica)* civcivi kullanıldı. Bıldırcın civcivleri her birinde 45 civciv bulunan 4 ana gruba, her ana grup kendi içinde her birinde 15 civciv bulunan 3 alt gruba rastgele ayrıldı. Bıldırcınlar başlangıç (0-3. hafta) ve bitirme (4-5. hafta) döneminde NRC'nin [30] bıldırcınlar için önerdiği normlara göre hazırlanan, bileşimi ve besin madde içeriği *Tablo 1*'de verilen karma yemlerle beslendi. Gruplardan biri söz konusu karma yemle beslenirken (Kontrol) diğer gruplar aynı yemlere araştırma süresince %0.1 oksitetrasiklin (Pozitif Kontrol: PK), %1 çörek otu tohumu (ÇOT) ve %0.1 çörek otu yağı (ÇOY) ilave edilerek beslendi. Deneme süresince gerekli ısıtma ve aydınlatma yapılıp, yem ve su *ad libitum* olarak sağlandı. Araştırma kafes ortamında yürütüldü.

Bıldırcınların canlı ağırlığı, yumurtadan çıkımda ve araştırma süresince haftalık olarak tartılarak belirlendi. İki tartım arasındaki canlı ağırlık değişiminin 7'ye bölünmesiyle günlük canlı ağırlık artışı hesaplandı. Haftalık olarak verilen ve artan yemden hareketle net yem tüketimi belirlendi. Yem tüketiminin hayvan sayısına bölünmesiyle günlük

Tablo 1. *Araştırmada kullanılan karma yemlerin bileşimi ve besin madde içerikleri, %*
Table 1. *Composition and nutrient content of the diets used in the study, %*

Yem Maddeleri	Başlangıç Yemi	Büyütme Yemi
Mısır	34.00	38.00
Buğday	15.70	14.70
Bitkisel yağ	3.53	3.65
Soya fasulyesi küspesi (% 44 HP)	25.00	12.00
Pamuk tohumu küspesi (% 34 HP)	18.51	15.51
Ayçiçeği küspesi (% 36 HP)	-	13.00
DCP	0.94	0.94
Kireç taşı	1.10	1.10
Vit. Min. karması*	0.35	0.35
Tuz	0.30	0.30
DL-Metionin	0.20	0.08
L-Lizin	0.09	0.09
L-Triptofan	0.08	0.08
Sodyum bikarbonat	0.20	0.20
Ayçiçeği küspesi (% 36 HP)	-	13.00
Besin madde içeriği, KM bazında		
Kuru madde	91.70	92.50
Metabolik enerji, kcal/kg**	2962	2896
Ham protein, %	22.10	20.02
Ham yağ, %	6.57	5.12
Ham selüloz, %	5.28	7.52
Ham kül, %	4.28	6.60

** Her kg yemde; vit A, 8.400 IU; vit D_3, 44.800 IU; vit E, 56 mg; vit K_3, 2.24 mg; vit B_1, 1.68 mg; vit B_2, 4.48 mg; niasin, 33.6 mg; cal.D-pantotenat, 10 mg; vit B_6, 2.8 mg; vit B_{12}, 9 µg; D-biotin, 0.112 mg; folik asit, 1.12 mg; vit C, 56 mg; manganez, 59 mg; demir, 47 mg; çinko, 47 mg; bakır, 47 mg; kobalt, 0.112 mg; iyot, 0.56 mg; selenyum, 0.100 mg; molibden, 0.582 mg bulunmaktadır; ** NRC'nin [30] tablo değerlerinden hareketle hesap yoluyla bulunmuştur*

yem tüketimi hesaplandı. Günlük yem tüketiminin, günlük canlı ağırlık artışına bölünmesiyle de yemden yararlanma oranı belirlendi.

Araştırma bitimi olan 35. günde her alt gruptan grup ortalamasına en yakın 5, her ana gruptan 15 adet olmak üzere, toplam 60 bıldırcın kesilerek karkas verim özellikleri belirlendi. Aynı hayvanlardan kesim esnasında kan örnekleri alınarak serumları çıkarıldı. Serum kalsiyum (Ca), fosfor (P), total kolesterol, yüksek dansiteli lipoprotein (HDL), alanin aminotransferaz (ALT), alkalin fosfataz (ALP), albumin, globulin ve total protein miktarları oto analizör (Beckman Coulter AU5800) vasıtasıyla ticari kitler kullanılarak belirlendi. Serum düşük dansiteli lipoprotein (LDL) ile çok düşük dansiteli lipoprotein (VLDL) konsantrasyonları Edwards ve ark.[31] göre hesaplandı.

Araştırmada kullanılan yemlerin besin madde içeriği A.O.A.C.'de [32] bildirilen yöntemlere göre belirlendi.

Araştırmada elde edilen veriler arasında istatistiksel farklılığın olup olmadığı tek yönlü varyans analizi ile belirlendi. Farklılık olan değerler arasındaki farkın önem kontrolü için Duncan Multiple Range testi yapıldı (SPPS 16.0).

BULGULAR

Araştırmada kullanılan çörek otu tohumunun kuru maddesi %89.40, ham proteini %24.13, ham külü %10.27, ham yağı %30.80 ve ham selülozu %9.56 olarak belirlenmiştir.

Gruplardan elde edilen besi performansı parametreleri *Tablo 2*'de verilmiştir. Canlı ağırlık bakımından 1-4. haftalar arasında gruplar arasında istatistiksel bir fark oluşmamıştır. Araştırma sonu olan 5. hafta sonu itibarıyla PK ve ÇOT grubunun canlı ağırlık ortalaması Kontrol grubundan önemli derecede yüksek tespit edilmiştir ($P<0.05$). Deneme geneli dikkate alındığında ortalama canlı ağırlık artışı, yem tüketimi ve yemden yararlanma oranı bakımından gruplar arasında istatistiksel bir farklılık görülmemiştir.

Karkas verim özelliklerinin verildiği *Tablo 3*'te de görüldüğü üzere, kesim canlı ağırlığı ve soğuk karkas ağırlığı PK, ÇOT ve ÇOY gruplarında Kontrol grubundan önemli derecede yüksek tespit edilmiştir. Karkas randımanı ile göğüs, but, kanat ve sırt + boyun oranları bakımından gruplar arasında istatistiksel bir farklılık gözlenmemiştir.

Araştırma gruplarından elde edilen kan serum parametrelerinin verildiği *Tablo 4*'te de görüldüğü üzere ÇOY grubunun serum kolesterol konsantrasyonu Kontrol ve PK gruplarından önemli derecede düşük bulunmuştur. Serum Ca, P, VLDL, HDL, LDL, ALT, ALP, albumin, globulin ve total protein konsantrasyonları bakımından gruplar arasında istatistiksel farklılık görülmemiştir.

TARTIŞMA ve SONUÇ

Araştırma sonu itibariyle antibiyotik ve çörek otu tohumu ilave edilen gruplardaki bıldırcınların canlı ağırlığı kontrol grubundan önemli derecede yüksek bulunmuştur *(Tablo 2)*. Çörek otu yağı ilave edilen gruptaki bıldırcınların canlı ağırlığı ise Kontrol grubuna göre rakamsal olarak daha yüksek bulunmuştur. Rasyona antibiyotik, çörek otu tohumu ya da çörek otu yağı ilavesi araştırma sonu canlı ağırlığında kontrol grubuna göre sırasıyla %5.4, 4.8 ve 4.0 oranında bir artışa sebep olmuştur. Söz konusu bu artışlar ekonomik açıdan olumlu bir durum olarak düşünülebilir. Çörek otu tohumu ilave edilen grubun canlı ağırlığının Kontrol grubundan önemli derecede yüksek oluşu, çörek otu tohumunun protein, yağ bakımından zengin ve yağının ise yağ asitlerinden oleik ve linoleik asitlerce [15,16] zengin olmasıyla, içermiş olduğu farmakolojik bakımdan etkin bileşiklerden dolayı barsaklarda antibakteriyel etki göstermesi, antioksidan özellik taşıması ve sindirim üzerinde olumlu etkiler oluşturması gibi etkenlerin tümünün bir

Tablo 2. *Araştırma süresince gruplardan elde edilen ölçütler*
Table 2. *Traits obtained from groups during the study*

Haftalar	Kontrol	PK	ÇOT	ÇOY	Önem
Canlı ağırlık ortalamaları, g					
Başlangıç	10.32±0.31	10.24±0.29	10.30±0.27	10.24±0.26	-
1	31.32±1.00	33.45±1.07	31.17±0.94	32.68±0.80	-
2	66.39±1.67	67.51±1.90	65.72±1.52	67.88±1.32	-
3	108.97±2.03	110.74±2.21	108.87±1.77	109.61±1.72	-
4	149.63±2.20	152.49±2.68	148.01±2.43	152.38±2.04	-
5	173.98±.2.22b	183.34±2.98a	182.41±2.78a	181.00±2.24ab	*
Canlı ağırlık artışı ortalamaları, g/gün					
1-5	4.68±0.62	4.95±0.53	4.81±0.49	4.88±0.55	-
Yem tüketimi, g/gün					
1-5	14.88±3.87	15.11±3.88	14.93±4.00	15.32±3.83	-
Yemden yararlanma oranı, g/g					
1-5	3.18±1.11	3.05±0.80	3.10±0.76	3.14±0.82	-

*- : Önemsiz; * Aynı satırda farklı harf taşıyan ortalamalar arasında önemli farklılık vardır (P<0.05)*

Tablo 3. *Gruplardan elde edilen karkas verim ölçütleri*
Table 3. *Carcass yield traits obtained from the groups*

Parametre	Kontrol	PK	ÇOT	ÇOY	Önem
Kesim ağırlığı, g	161.80±1.9b	172.25±1.5a	175.20±1.9a	171.15±1.9a	***
Soğuk karkas ağırlığı, g	113.0±1.9b	119.9±1.9a	119.4±1.8a	119.6±1.3a	*
Karkas randımanı, %	70.2±0.6	69.2±1.1	67.7±0.9	69.5±0.8	-
Karkas parçaları ağırlıklarının soğuk karkas ağırlığına oranı, %					
Göğüs	37.7±0.4	38.3±0.4	37.2±0.8	38.0±0.4	-
But	24.3±0.2	24.8±0.3	23.9±0.3	24.7±0.2	-
Kanat	9.6±0.2	9.3±0.2	9.4±0.2	9.2±0.1	-
Sırt + Boyun	28.5±0.6	27.5±0.5	29.2±0.5	28.0±0.5	-

*- : Önemsiz; * Aynı satırda farklı harf taşıyan ortalamalar arasında önemli farklılık vardır (P<0.05); *** Aynı satırda farklı harf taşıyan ortalamalar arasında önemli farklılık vardır (P<0.001)*

Tablo 4. *Deneme gruplarındaki kan serum parametreleri*
Table 4. *Blood serum parameters in the experimental groups*

Parametreler	Kontrol	PK	ÇOT	ÇOY	Önem
Ca, mg/dL	9.5±0.2	9.0±0.4	10.1±0.2	10.3±0.7	-
P, mg/dL	7.0±0.4	6.4±0.5	6.2±0.2	7.6±0.4	-
Total kolesterol, mg/dL	212±7.9a	205±14.1a	201±7.5ab	177±6.2b	*
VLDL, mg/dL	22.1±2.6	21.1±1.4	24.1±2.2	20.3±1.3	-
HDL, mg/dL	113.0±3.8	108.1±14.1	108.5±7.3	100.7±5.5	-
LDL, mg/dL	77.2±3.5	69.0±5.1	68.1±2.8	60.9±4.2	-
ALT, IU/L	3.8±0.3	3.3±0.3	4.7±0.6	3.5±0.4	-
ALP, IU/L	975±55	1007±72	1082±59	879±50	-
Albumin, g/dL	0.9±0.0	0.9±0.0	0.9±0.0	1.0±0.0	-
Globulin, g/dL	1.4±0.1	1.6±0.1	1.5±0.0	1.6±0.0	-
Total protein, g/dL	2.4±0.1	2.5±0.1	2.5±0.1	2.5±0.1	-

*- : Önemsiz; * Aynı satırda farklı harf taşıyan ortalamalar arasında önemli farklılık vardır (P<0.05)*

arada olumlu etki oluşturmasıyla ilişkili olabilir. Kontrol grubuna göre ÇOY grubunda canlı ağırlığın rakamsal olarak yüksek oluşu da çörek otu tohumu için bahsedilen durumlarla ilişkili olabilir. Bu görüşümüzü destekler vaziyette birçok araştırmacı çörek otu tohumunda bulunan farmakolojik olarak aktif maddelerin farklı bakteri türlerine karşı antibakteriyel etki gösterdiğini bildirmektedirler [12,19,33,34]. Bilindiği üzere barsak florasının kontrol altında tutulması hayvanlarda performansı artırıcı bir etki oluşturmaktadır. Diğer taraftan çörek otu yağı ve bu yağda bulunan farmakolojik olarak aktif maddeler (thymoquinon, carvone, anethole, carvacrol, 4-terpineol) antioksidan aktiviteye sahip bileşiklerdir [35,36]. Ayrıca, çörek otu yağı [37] ve çörek otunda bulunan thymoquinone [38] karaciğeri korucu etkiye sahip maddelerdir. Bunlara ilaveten broyler rasyonlarına esansiyel yağ asidi ilavesinin protein, selüloz ve yağ sindirimini artırdığı bildirilmektedir [9]. Bu araştırmaya benzer şekilde rasyona %1 [4,7,39], %1.5 [7], %2, 3 ve 4 [5] ya da %2 ve 10 [3] oranında çörek otu tohumu ilavesinin canlı ağırlık artışı üzerinde olumlu etki yaptığı belirlenmiştir. Erener ve ark.[39] da broyler rasyonlarına %0.1 oranında çörek otu yağı ilavesinin canlı ağırlık artışını önemli derecede artırdığını tespit etmişlerdir. Yine, Abdel-Hady ve ark.[40], tarafından soya fasulyesi küspesi yerine rasyona %4, 8, 16 ve 32 oranında çörek otu tohumu küspesi ilave edilerek bıldırcınlarda yapılan bir çalışmada %4 ve 8 oranındaki ilavelerin canlı ağırlığı ve canlı ağırlık artışını önemli ölçüde artırdığı belirlenmiştir. Bu sonuçlardan farklı olarak, bıldırcın rasyonlarına 60 ppm çörek otu esansiyel yağı [11] ya da broyler rasyonlarına %0.25, 0.50, 0.75 [28] veya %0.5, 2 ve 3 [4] oranında çörek otu tohumu ilavesinin canlı ağırlığı ve canlı ağırlık artışını etkilemediği şeklinde sonuçlarda bulunmaktadır. Talha ve Mohamed [8] ise broyler rasyonlarına %1 ve 2 oranında çörek otu tohumu ilavesinin canlı ağırlık artışını olumsuz yönde etkilediğini bildirmişlerdir.

Rasyona çörek otu tohumu ya da yağının ilave edilmesi yem tüketimi ve yemden yararlanma oranı üzerinde kontrole benzer sonuçlar vermiştir *(Tablo 2)*. Bu durum bıldırcın rasyonlarına %1 çörek otu tohumu ya da %0.1 çörek otu yağı ilavesinin olumsuz bir etki oluşturmadığını göstermektedir. Bu çalışmaya benzer şekilde, broyler rasyonlarına %0.25, 0.50 ve 0.75 [29], %1 ve 1.5 [7] veya %2 ve 10 [3] oranında çörek otu tohumu ya da bıldırcın rasyonlarına 60 ppm çörek otu yağı [11] ilavesinin yem tüketimi ve yemden yararlanma oranını etkilemediği belirlenmiştir. Abdel-Hady ve ark.[40] ise bıldırcın rasyonlarına soya fasulyesi küspesi yerine %4 ve 8 oranında çörek otu küspesi kullanılmasının yem tüketimini artırdığını, ancak yemden yararlanma oranını etkilemediğini tespit etmişlerdir. Bu sonuçlarla birlikte, broyler rasyonlarına %1 [4,7,39] ve %1.5 [7] oranında çörek otu tohumu ya da yağı [39] ilavesinin yemden yararlanma oranını önemli derecede iyileştirdiği şeklinde sonuçlar da bulunmaktadır. Talha ve Mohamed [8] ise broyler rasyonlarına %1 ve 2 oranında çörek otu tohumu ilavesinin yem tüketimini Kontrol grubuna göre önemli derecede azalttığını, fakat yemden yararlanma oranı bakımından gruplar arasında önemli bir farklılığın görülmediğini bildirmişlerdir.

Bu çalışmada kesim canlı ağırlığı ve soğuk karkas ağırlığı Kontrol grubunda diğer tüm gruplardan önemli derecede düşük bulunmuş, karkas randımanı bakımından gruplar arasında önemli bir farklılık görülmemiştir *(Tablo 3)*. Antibiyotik, çörek otu tohumu ve yağı ilave edilen gruplarda kesim canlı ağırlığı ve karkas ağırlığının yüksek oluşu bu gruplardaki hayvanların deneme sonu canlı ağırlıklarının kontrol grubundan yüksek oluşundan kaynaklanabilir. Bu durum ekonomik anlamda olumlu bir husus olarak düşünülebilir. Bu çalışmaya benzer şekilde Erener ve ark.[39] broyler rasyonlarına %1 oranında çörek otu tohumu ilavesi durumunda karkas ağırlığının önemli derecede arttığını belirlemişlerdir. Bıldırcınlarda yapılan bir başka çalışmada ise rasyona %4 çörek otu küspesi ilavesinin kesim canlı ağırlığını ve karkas ağırlığını değiştirmediği belirlenmiştir [40]. Bu çalışmada rasyona çörek otu tohumu ilavesine bağlı olarak karkas randımanının değişmediği şeklindeki tespitimiz diğer bazı araştırma sonuçlarıyla [4,11,39] uyum halindedir. Karkas parametrelerinden göğüs, but, kanat ve sırt+boyun ağırlığının karkas ağırlığına oranı bakımından araştırma grupları arasında önemli bir farklılık görülmemiştir. Güler ve ark.'nın [4] broyler rasyonlarına %0.5, 2 ve 3 oranında çörek otu tohumu ilavesinin göğüs, but, kanat ve boyun ağırlığını, Lymia ve ark.'nın [29] broyler rasyonlarına %0.25 ve 0.75 oranında çörek otu ilavesinin göğüs ve but oranını, Talha ve Mohamed'in [8] broyler rasyonlarına %2 oranında çörek otu tohumu ilavesinin karkas randımanını değiştirmediği şeklindeki sonuçlar bu araştırma sonuçlarıyla uyum halindedir. Ancak broyler rasyonlarına % 0.5 oranında çörek otu tohumu ilavesinin but oranını [29], %1 oranında çörek otu tohumu ilavesinin but, göğüs, kanat ve boyun oranını [4], %2, 3 ve 4 oranında çörek otu tohumu ilavesinin karkas randımanını, but ve göğüs oranını önemli derecede artırdığı [5] şeklinde araştırma sonuçları da bulunmaktadır. Talha ve Mohamed [8] ise broyler rasyonlarına %1 oranında çörek otu ilavesinin karkas randımanını olumsuz yönde etkilediğini tespit etmişlerdir.

Bıldırcın rasyonlarına çörek otu yağı ilavesi serum total kolesterol düzeyinde Kontrol ve antibiyotik ilave edilen gruplara göre önemli azalmaya sebep olurken, çörek otu tohumu ilavesinde ise rakamsal bir azalma gerçekleşmiştir *(Tablo 4)*. Serum total kolesterol düzeyindeki söz konusu azalmalar ve bu gruplarda serum LDL konsantrasyonundaki rakamsal azalmalar çörek otu ve yağında bulunan linoleik asitten kaynaklanabilir. Zira linoleik asit perifer kan dolaşımında bulunan kolesterol fraksiyonlarını karaciğere taşıyarak metabolize edilmesini sağlamakta, böylece de kolesterol konsantrasyonunda azalmaya neden olmaktadır [41]. Bıldırcın rasyonlarına %4, 8, 16 ve 32 oranında çörek otu küspesi [40] ve broyler rasyonlarına %2, 4, 6, 8 ve 10 oranında çörek otu tohumu ilave edilerek yapılan çalışmalarda da kontrol grubuna göre diğer tüm gruplarda serum total kolesterol düzeyinde

önemli bir azalma tespit edilmiştir [42]. Yumurtacı tavuklarda rasyona 1.5, 2.5 ve 3.5 ml/kg miktarında çörek otu yağı ilave edilerek yapılan bir çalışmada sadece 3.5 ml/kg yağ ilave edilen grupta Kontrole göre serum kolesterol düzeyinde önemli bir azalma tespit edilmiştir [10]. Benzer şekilde yumurta tavuklarında rasyona %0.5, 1.0 ve 1.5 oranında çörek otu tohumu ilave edilerek yapılan bir çalışmada sadece %1.5 oranında çörek otu tohumu ilave edilen grubun serum kolesterol düzeyinde önemli bir azalma tespit edilmiştir [43]. Rat rasyonlarına 800 mg/kg çörek otu yağı ilave edilerek yapılan bir çalışmada [44] serum total kolesterol, LDL ve trigliserid konsantrasyonunda önemli düşmeler tespit edilmiştir. Al-Homidan ve ark.[3] tarafından broylerlerde yapılan bir çalışmada ise çörek otu ilavesinin serum kolesterol düzeyini etkilemediği tespit edilmiştir. Bu çalışmada rasyona çörek otu tohumu veya yağı ilavesi serum LDL konsantrasyonunda da dikkati çekici bir azalmaya sebep olmuş, ancak azalış istatistiksel olarak önemli bulunmamıştır (P>0.068). Üzerinde durulan diğer kan parametreleri (Ca, P, VLDL, HDL, ALT, ALP, Albumin, Globulin, Total protein) açısından gruplar arasında istatistiksek bir farklılık görülmemiştir. Bu çalışmaya benzer şekilde Al-Homidan ve ark.[3] tarafından yapılan çalışmada da rasyona çörek otu ilavesinin serum AST, ALT, total protein, albumin, globulin ve Ca konsantrasyonunu değiştirmediği belirlenmiştir. Bıldırcınlarda yapılan bir başka çalışmada, rasyona %4 oranında çörek otu küspesi ilavesinin serum total protein ve albumin miktarını artırdığı, ancak %8, 16 ve 32 oranındaki ilavelerin ise söz konusu parametreleri değiştirmediği bildirilmiştir [40]. Shewita ve Taha [42] ise broyler rasyonlarına %2, 4, 6, 8 ve 10 oranında çörek otu tohumu ilavesinin serum trigliserid oranını önemli derecede azalttığını, %2 ve 4 oranında ilavesinin total protein oranını azalttığını, %10 ilavesinde artırdığını tespit etmişlerdir. Aynı araştırmacılar rasyona %8 ve 10 oranında çörek otu tohumu ilavesinde serum albumin düzeyinde kontrole göre önemli bir artış belirlemişlerdir.

Sonuç olarak; bıldırcın rasyonlarına %1 oranında çörek otu tohumu ilavesinin antibiyotik ilave edilen grupta olduğu gibi canlı ağırlığı kontrole göre önemli derecede artırdığı, çörek otu tohumunun ya da yağının canlı ağırlık artışı, yem tüketimi ve yemden yararlanma oranını etkilemediği, karkas randımanını değiştirmediği, çörek otu yağı ilavesinin ise hipokolesterolemik etki gösterdiği tespit edilmiştir.

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Research Article

Classification of Holstein Dairy Cattles in Terms of Parameters Some Milk Component Belongs by Using The Fuzzy Cluster Analysis

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Abstract

This study was carried out on classification of Holstein Friesian breed dairy cattles in terms of some milk component parameters and on investigating the relevant parameters in the resulting cluster structures. Within the scope of this study, thirteen different criteria were used including somatic cell count (SCC), milk fat (%), milk protein (%), milk lactose (%), casein (%), urea (%), dry matter (%), non-fat dry matter (%), density (g/cm^3), acidity (°SH), free fatty acids (mmol/10L), citric acid (%) and freezing point (°C). As a result of the analysis using Fanny algorithm based on the principle of fuzzy equality, the fuzziness level was found to be minimum when a total of 282 cattles were divided into 2 clusters with the accuracy rate of 97.5%. Accordingly, the cattles were classified in terms of the investigated characteristics in 2 different clusters in which 25 cattles were in Cluster 1 and the rest of the cattles were in Cluster 2. When the resulting cluster structures were studied, it was found that Cluster 2 has a more stable clustering than Cluster 1. When evaluating the change in milk components according to the clusters, it was concluded that somatic cell count, dry matter (%), milk fat (%) and density (g/cm^3) have significant differences between clusters (P<0.05), while the other parameters were found statistically non-significant (P>0.05).

Keywords: *Fuzzy clustering, Milk composition, Fanny algorithm, Holstein*

Bulanık Kümeleme Analizi İle Siyah Alaca Süt Sığırlarının Bazı Süt Bileşenlerine Ait Parametreler Bakımından Sınıflandırılması

Özet

Bu çalışma, Siyah Alaca ırkı süt sığırlarının bazı süt bileşenleri bakımından bulanık kümeleme analizi ile sınıflandırılması ve oluşan küme yapılarında ilgili parametrelerin incelenmesi üzerine yürütülmüştür. Araştırma kapsamında somatik hücre sayısı (SHS), süt yağı (%), süt proteini (%), süt laktoz (%), kazein (%), üre (%), kuru madde (%), yağsız kuru madde (%), yoğunluk (g/cm^3), asitlik (°SH), serbest yağ asidi (mmol/10L), sitrik asidi (%) ve donma noktası (°C) olmak üzere on üç farklı ölçüt kullanılmıştır. Bulanık kümeleme analizinde bulanık eşitlik ilkesine dayalı Fanny algoritması kullanılarak yapılan analiz sonucunda ise toplam 282 adet inek %97.5 doğru sınıflandırma oranı ile 2 ayrı kümeye ayrıldığında bulanıklık düzeyinin minumum olduğu görülmüştür. Buna göre inekler incelenen özellikler bakımından 25 tanesi küme 1'de, 257 tanesi de küme 2'de yer alacak şekilde 2 farklı kümede sınıflandırılmıştır. Oluşan küme yapıları incelendiğinde ise küme 2'nin küme 1'e göre daha kararlı bir küme oluşturduğu tespit edilmiştir. Kümelere göre süt bileşenlerinin değişimi değerlendirildiğinde ise SHS, süt yağı, kuru madde (%), süt yağı (%) ve süt yoğunluğunun (g/cm^3) kümeler arası önemli bir (P<0.05) farklılık gösterdiği, diğer parametrelerin ise istatistiksel açıdan önemli bir farklılık göstermediği (P>0.05) sonucuna varıldı.

Anahtar sözcükler: *Bulanık kümeleme, Süt kompozisyonu, Fanny algoritması, Siyah Alaca*

INTRODUCTION

In animal breeding, knowing the differences or similarities of the individuals in a population based on the investigated characteristics is important for both for breeding studies and for revealing the genetic profiles of the individuals in the herd [1]. One of the common methods used in animal breeding for this purpose is the clustering analysis. This method has been applied successfully in many subjects, such as distinguishing breeds or populations, determining the genotype similarities of the individuals and the classification of morphological characteristics [1-5].

Cluster analysis is a method which is used to classify according to similarities or dissimilarities of the ungrouped and scattered data or independent variables [6-9]. The purpose of this method, which is based on unsupervised learning, is to provide a grouping of the units showing similar characteristics in a way that homogeneous within

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theirselves by bringing them together [10-12]. In this way, both the process time is shortened and the scattered data is provided in a more general form (group), and also useful and summary information can be presented to the researchers [9]. In the cluster analysis, each unit is assigned to a cluster with a final decision, and according to the classic cluster concept, a unit is either a member of the relevant cluster (Membership 1) or remain outside the cluster without being a member (Membership 0) [13]. Therefore, researchers may encounter some unstable situations.

As reported in the literature by several researchers, different clustering methods, depending on the distance criteria used may give different results. In addition, it can be seen that some units which are taking place in different clusters in clustering algorithms in which approximately the same results are obtained, may be in the condition of being instabile (uncertain) in their cluster membership. Similarly, not knowing how to group the data before the analysis in the cluster analysis can reveal the uncertainty in making a final decision while classifying according to the similarity of some units. So, in one way, the identification of the outliers which are difficult to assign to a cluster will help making more reliable interpretations [9,14-16]. Fuzzy clustering analysis is recommended as a more suitable method in detection of cases involving such uncertainties. In this method, there is a situation that each unit in the cluster belongs to a cluster with the membership degrees ranging between [0,1]. Thus, a unit can belong to multiple clusters with different membership degrees. In this context, it can also said that the fuzzy clustering analysis, unlike the classical cluster analysis, provide flexibility to cluster limits and contains more detailed information [13,17-19]. Therefore, in the study, fuzzy clustering analysis was used to classify the dairy cattles in terms of analyzed characteristics, and it was aimed to study how these characteristics changed in the resulting cluster structures.

There are limited number of studies in the literature related to the fuzzy clustering analysis which is only recently used in animal breeding. For example, some of these studies in which the fuzzy clustering was used are; in determining phylogenetic relationships in sheep by Geng et al.[20] in the classification of body measurements of sheep by Kılıç and Özbeyaz [15] and Karakaya and Bafra; the examination of animal behavior by Cohen et al.[21], Görgülü [22] in the classification of cattle in terms of some milk yield characteristics.

The purpose of this study was to classify the dairy cattles according to thirteen different milk component [somatic cell count (SCC), milk fat (%), protein (%), lactose (%), casein (%), urea (%), dry matter (%), non-fat dry matter (%), density (g/cm^3), acidity (ºSH), free fatty acid (mmol/10L), citric acid (%) and the freezing point (ºC)] traits and to determine the criteria effective in clustering by analyzing the change in the resulting cluster structures.

MATERIAL and METHODS

Material

The study was conducted in experimental farm of East Mediterranean Agricultural Research Institute in Adana, TURKEY. The animals included in the study consisted of 282 Holstein cows. The Holstein cows were 5 to 6 years of age and weighed between 500 and 550 kg. The feed ration contained silage, wheat straw, alfaalfa dry hay, with an addition of feed concentrate. The experiment was continued for 4 months.

The SCC in milk samples obtained from cows in the morning was analyzed by DCC which is a De Laval brand measuring instrument. Firstly, the SCC was determined separately for each breast lobe. After necessary cleaning, the udder to be measured the first milk was discharged by milking a few times, then milk sample was taken to plastic sample tubes. This taken sample was taken to the measuring tapes, after performing its homogenization by turning upside-down a few times by closing the mouth of the tube.

The SCC was evaluated in the barn in a very short time (about 45-60 seconds) by taking De Laval measuring tape from the plastic cups by which the samples do to not interfuse in the mammary lobes of milk samples. Determining the number of cells was done by measuring according to the principle of counting somatic cells stained with a DNA-specific fluorescent probe propidium iodide, approximately 60 µl milk samples were taped, installed tape is placed in the measuring window of the De Laval cell counter, and the SCC was determined by evaluating 1 µl [23].

The milk analyzes were performed with the FOSS MilkoScan™ 120 instrument, and the values of the milk fat was determined by the Röse Gottlieb; the milk protein by the Kjeldahl method; the non-fat dry matter by the heating oven method; the lactose by the Boehringer Mannheim Enzymatic kit; the density by the Anton Paardan DMA 38 density measuring instrument; the acidity by titration with 0.25 M NaOH; free fatty acid, fat by titration using a pH electrode and the citric acid was determined by the Boehringer Mannheim Enzymatic Kit [24].

Methods

Fuzzy clustering analysis is emerging as a suitable method when the clusters does not separated from each other clearly or the some units are creating unstable conditions for cluster membership [25-27]. There are two main methods of fuzzy clustering analysis. The first one is Fuzzy c-means (FCM) which is based on c-partition and the second one is the hierarchical clustering method based on the fuzzy equality principle [28-30]. In this study, the Fanny algorithm which is based on the fuzzy equality principle was used. The fuzzy clustering technique used

in this algorithm aims to minimize the objective function as shown in *Equation 3* below, and has some limitations. These were given in *Equation 1* and *2*, respectively. The limitations are,

1. $U_{iv} \geq$ i=1,2,…,n and v= 1,2,…, k (1)

$$2.\ \sum_{v=1}^{k} u_{iv} = \%100 \quad \text{i=1,2,…,n} \tag{2}$$

The objective function is expressed as follows:

$$C = \sum_{v=1}^{k} \frac{\sum_{i,j=1}^{n} u_{iv}^2 u_{jv}^2 d_{(ij)}}{2\sum_{j=1}^{n} u_{jv}^2} \tag{3}$$

Where in, $d_{(i,j)}$: the distance between the i^{th} and j^{th} units (similarity), u_{iv}: the unknown membership of the i^{th} unit to the v^{th} cluster, u_{jv}: unit the unknown membership of the j^{th} unit to the v^{th} cluster, k: number of clusters, and n: the total number of units [31,32].

In the study, the degree of fuzziness in the cluster was determined by using Dunn's partition coefficient (F_k). Dunn's partition coefficient (F_k) also known as a coefficient that is used to understand how far is the fuzzy clustering away from the exact clustering [13]. The general mathematical expression of the Dunn partititon coefficient is indicated as in *Equation 4*. The value of this coefficient can be 1/k at minimum and can be 1 at maximum. Accordingly, the value range of the Dunn partititon coefficient is defined as [1/k, 1] [33].

$$F_k = \sum_{i=1}^{n}\sum_{v=1}^{k} \frac{u_{iv}^2}{n} \tag{4}$$

The Normalized Dunn Coefficient $F'_k(u)$ is obtained when this coefficient is normalized regardless of the number of clusters, and is calculated by the equation given in *Equation 5*. This coefficient, also known as t he non-fuzziness index, is in the range of [0,1] [14].

$$F_k^{'}(u) = \frac{F_k(u) - \left(\frac{1}{k}\right)}{1 - \left(\frac{1}{k}\right)} = \frac{kF_k(u) - 1}{k - 1} \tag{5}$$

In determining the number of clusters in the study, Kaufman partition coefficient D(U) and the Normalized Kaufman coefficient $D_c(U)$ was used as well as the Normalized Dunn Coefficient $F'_k(u)$. These coefficients are calculated by using the equations given in Equation 6 and Equation 7, respectively [34]. In determining the appropriate number of clusters, the Normalized Dunn Coefficient $F'_k(u)$ was considered to be high and the value of Normalized Kaufman coefficient was considered to be low as the critera in the study [34].

$$D(U) = \frac{1}{n}\sum_{v=1}^{k}\sum_{i=1}^{n}(h_{iv} - u_{iv})^2 \tag{6}$$

$$D_c(U) = \frac{D(U)}{1 - \left(\frac{1}{k}\right)} \tag{7}$$

The other coefficient which was considered in the study in order to determine the number of clusters was the Silhoutte Coefficient (SC_i), and this coefficient is also a widely used index to determine the stability of the cluster structures [7]. It was determined according to the Mean Silhouette index *(SC)* obtained by calculating the mean of these values that how well all of the units clustered such as in k number of cluster. It is considered to be appropriate clustering when this value is above 0.50, and the number of clusters corresponding to the Maximum *(SC)* value is taken as the optimal number of clusters [16]. All analyzes in this study was performed using the NCSS 2001 software package [35].

RESULTS

In the research, the numbers of clusters between k=2 and 10 were increased one by one in order to define appropriate cluster number in fuzzy clustering analysis. For this purpose, Mean Silhouette coefficient values ($\overline{SC}$) for each cluster number were obtained as in *Table 1* containing Silouette coefficient values ($\overline{SC_i}$) and average of all. When the average Silhouette coefficient values ($\overline{SC}$) at *Table 1* were analyzed, it can be said that if the number of cluster were k=2, cluster 2 (SC_i=0.8773) had more stable structure than cluster 1 (SC_i=0.2033).

The maximum ($\overline{SC}$) coefficient value was obtained with the two fuzzy clusters. Accordingly, it can be said that the appropriate number of clusters according to the ($\overline{SC}$) coefficient is k=2. In addition, the ($\overline{SC}$) coefficient value was generally found higher than 0.5 for the number of other clusters, and this also showed that the appropriate number of clusters for clustering structure has been reached.

When the Dunn partititon coefficient (F_k) and Normalized Dunn Coefficient $F'_k(U)$ values in *Table 2* were analyzed in order to determine the degree of the fuzziness in the cluster, it was found that when the cluster number is k=2, F_k value was found as 0.94; and the $F'_k(u)$ value was found as 0.89. According to these values, it can be said that the cluster is closer to fuzzy clustering when the cluster number is k=2. To determine the number of clusters, when Normalized Kaufman coefficient $D_c(U)$ and Normalized Dunn Coefficient $F'_k(u)$ coefficient values were analyzed; the $F'_k(u)$ value was at its highest and the

Table 1. *The ($\overline{SC_i}$) and ($\overline{SC}$) values according to the number of clusters*
Tablo 1. *Küme sayılarına göre ($\overline{SC_i}$) ve ($\overline{SC}$) değerleri*

No of Clusters	Average Silhouette Cofficients SC_i										
	1	2	3	4	5	6	7	8	9	10	($\overline{SC}$)
2	0.2033	0.8773									0.82
3	0.3463	0.3398	0.7066								0.60
4	0.4123	0.5920	0.5610	0.2047							0.50
5	0.8522	0.3265	0.6104	0.6645	0.5546						0.55
6	0.5360	0.8522	0.5455	0.5540	0.5831	0.5782					0.57
7	0.8522	0.5742	0.4621	0.5451	0.5110	0.5360	0.6201				0.54
8	0.8457	0.5123	0.4728	0.6317	0.4604	0.4460	0.5652	0.5878			0.53
9	0.7978	0.6177	0.8352	0.5054	0.4604	0.6431	0.6599	0.2709	0.5878		0.56
10	0.7978	0.5294	0.6599	0.6227	0.6070	0.3653	0.8352	0.6177	0.2709	0.5550	0.54

Table 2. *The partition coefficients and the accurate classification rates according to the number of clusters*
Tablo 2. *Küme sayılarına göre ayrıştırma katsayıları ile doğru sınıflandırma oranları*

Number of Clusters (k)	F_k	$F'_k(u)$	D(U)	$D_c(U)$	ACR(%)
2	0.94	0.89	0.01	0.03	97.5
3	0.85	0.79	0.04	0.07	95.7
4	0.80	0.74	0.06	0.08	95.8
5	0.82	0.78	0.04	0.06	92.7
6	0.81	0.78	0.06	0.08	95.8
7	0.80	0.76	0.06	0.08	95.7
8	0.79	0.76	0.06	0.08	94.9
9	0.80	0.78	0.06	0.07	95.7
10	0.79	0.77	0.06	0.07	95.7

F_k: *Dunn Coefficient,* ***$F'_k(u)$:*** *Normalized Dunn Coefficient,* ***D(U):*** *Kaufman Coefficient,* ***$D_c(U)$:*** *Normalized Kaufman Coefficient,* ***ACR (%):*** *Accuracy classification rate*

$D_c(U)$ was at its lowest for k=2. It can be said that the appropriate number of cluster is 2. In addition, as seen in *Table 2*, the accurate classification rate was determined as 97.5% as a result of discriminant analysis conducted by using cluster membership values for the number of clusters k=2. The accurate classification rate being high also showed that the number of clusters is 2 can be also considered as an indication for this number to be appropriate.

The optimal cluster number was determined to be k=2 according to the results of the evaluations. As a result of the fuzzy clustering analysis, a total of 282 cattles were divided into two clusters, 25 cattles were in Cluster 1 a nd 257 cattles were in Cluster 2. The mean values of milk component parameters which are analyzed within the study for both clusters were given in *Table 3*.

When the cluster structures in *Table 3* were examined, the values of investigated parameters for Cluster 1 and Cluster 2 were found, SCC: 420.32 -77.71, Dry matter (%): 12.86-12.36; Non-fat dry matter (%): 8.60-8.69; Fat (%): 4.34-3.71; Protein (%): 3.23-3.24, Lactose (%): 4.53-4.62; Casein (%): 2.55-2.58; Urea (%): 0.02-0.03; Density (g/cm^3): 1.02-1.03; Free

Table 3. *The mean and standard deviation values of milk components in the cluster parameters*
Tablo 3. *Kümelerdeki süt bileşen parametrelerinin ortalama ve standart sapma değerleri*

Criteria	Cluster 1 (n_1 = 25)	Cluster 2 (n_2 = 257)
The somatic cell count (SCC)	420.32±207.92	77.71±36.37**
Dry matter, %	12.86±1.26	12.36±1.22*
Non-fat dry matter, %	8.60±0.24	8.69±0.45^{ns}
Fat, %	4.34±1.18	3.71±1.11**
Protein, %	3.23±0.25	3.24±0.37^{ns}
Laktose, %	4.53±0.15	4.62±0.23^{ns}
Casein, %	2.55±0.18	2.58±0.29^{ns}
Urea, %	0.02±0.004	0.03±0.005^{ns}
Density, g/cm^3	1.02±0.001	1.03±0.001*
Free fatty acid, mmol/10L	3.62±1.33	3.05±1.52^{ns}
Citric acid, %	0.13±0.03	0.13±0.03^{ns}
Freezing point, °C	0.53±0.02	0.53±0.03^{ns}

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

fatty acid (mmol/10L): 3.62-3.05; citric acid (%): 0.13-0.13 and the freezing point (ºC): 0.53-0.53, respectively.

The differences between clusters for each of these parameters were determined by independent samples t-test, and the homogeneity of variance was determined by Levene's test.

As a result of this study, the differences of SCC, dry matter (%), density (g/cm^3) and milk fat (%) which are one of the investigated the parameters between clusters were found statistically significant (P<0.05) and the differences of the clusters between the other parameters were found non-significant (P>0.05).

DISCUSSION

In this study which was carried out on Holstein dairy cattle breeds, cattles are classified in terms of some milk component parameters by using fuzzy clustering analysis. As a result of fuzzy clustering analysis, cattles were divided into 2 cluster with 97.5% correct classification rate to be minimum fuzzy level. Accordingly, cattles were grouped as 25 of them were in cluster 1, 257 of them were in cluster 2. When the changes in the cluster structure of the milk parameters studied in this research were examined, it is determined that somatic cell count (SCC) showed a significant (P<0.01) difference. When the cluster structures obtained by fuzzy clustering analysis, the Somatic cell count in Cluster 2 was found as SCC <100.000 cell/ml, and was found SCC <500.000 cell/ml in Cluster 1 as seen in *Table 3*.

Somatic cell count (SCC) is one of the important criteria that can be used in determining milk quality and in revealing whether the cattles in the herd have the mastitis case [36-39]. The SCC value in a regular milk is generally required to be SCC <200.000 cell/ml, and it is it has been considered to be abnormal when it is above this value [38,40]. Within the framework of this information, as a result of fuzzy clustering analysis, that the SCC value was found above 200.000 cell/ml in Cluster 1.

The milk fat rate was determined as 4.34% in Cluster 1, and as 3.71% in Cluster 2, and a significant difference (P<0.01) was determined between clusters *(Table 3)*. The study results for the milk fat which has importance in terms of the pricing of the milk [41] was found to be higher than the value of 3.5% which is declared in Turkish Food Codex [42]. Milk dry matter was another milk component which showed a significant (P<0.05) decrease between the clusters in the study.

The value of milk dry matter in Cluster 1 was determined as 12.86% and as 12.36% in Cluster 2, and was found lower in Cluster 2 than Cluster 1 (P<0.05). The values within both clusters of milk dry matter which is important for the nutritional value of milk [43] were found lower than the value of 13.62% reported by Sahin and Kasıkcı [44], and found in compliance with the value of 12% which is declared in Turkish Food Codex [42]. The milk density which gives information whether any cheating was done on milk [37] was found 1.02 g/cm^3 in Cluster 1, and 1.03 g/cm^3 in Cluster 2, and a statistically significant (P<0.05) difference was found between clusters *(Table 3)*. When the milk density values for both clusters were analyzed, it was found in compliance (TS1018) with the values of 1.028 - 1.039 g/cm^3 reported for raw milk standard [45]. Differences between clusters in terms of dry matter, milk fat and density may affected by SCC. There are many research results on this manner [37,46,47]. Although no statistically significant difference was found between clusters, the milk protein value (%) was found higher than the values reported by many researchers on this subject as being 3.23%-3.24%, respectivly [37,48].

The milk casein was found as 2.55%-2.58% in the clusters, and was observed in the amount of less casein in Cluster 1. The value of the non-fat dry milk matter was found as 8.60 - 8.69% in the clusters and was found compatible with the rate of 8.5% declared in Turkish Food Codex [42]. It was found that the freezing point and the citric acid (%) values had the same value in both clusters, and were determined as 0.53ºC and 0.13%, respectively *(Table 3)*. The amount of milk urea (%) in the custers was found between the values of 0.03%-0.02%. The free fatty acid was found as 3.62 mmol/10L%-3.05 mmol/10L% in the clusters, the amount of milk lactose was determined as 4.53%-4.62%.

Consequently, as a result of classifying the dairy cattles by fuzzy clustering analysis according to some milk components, it has been understood that the main diversity criteria for the clusters was the SCC. The other characteristics [dry matter (%), density (g/cm^3) and milk fat (%)] were determined to have minor effects on the clustering. In this respect, in the classification studies conducted in the field of animal breeding, it has been thought that using the fuzzy clustering analysis would enable the researchers to make a more realistic classification, especially in situations involving uncertainty.

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Research Article

The Effects of Different Zinc Sources and Microbial Phytase Supplementation on the Tibial Bone Properties, Strength and Zn Mineralization Broilers Fed with Diet Low Phosphorus [1]

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Abstract

This experiment was conducted to evaluate the effects of different Zn sources and microbial phytase supplemented to low available P (aP) corn-soy diets on morphometric indices of tibiotarsi, bone strength and Zn accumulation. The experimental period lasted 42 days. A total of 875 one-day-old Ross 308 male broiler chicks were randomly assigned to seven treatment groups (positive, negative and five experimental), each with five identical subgroups of 25 birds. The positive control (PC) group was fed a diet containing adequate concentration (0.45%) of available phosphorus (aP) due to mineral premix (except zinc) and feeds; the negative control (NC) group was fed a basal diet including low concentration (0.30%) of available phosphorus (aP) due to mineral premix (except zinc) and feeds; 0.30% aP and 500 FTU phytase kg-1; 0.30% aP and 75 mg Zn-proteinate kg-1; 0.30% aP and 75 mg ZnSO4 kg-1; 75 mg Zn-proteinate and 500 FTU phytase kg-1; 75 mg ZnSO4 and 500 FTU phytase kg-1 were added to the experimental groups of phytase (PH), organic zinc (OZ), inorganic zinc (IZ), organic zinc + phytase (OZ+PH) and inorganic zinc + phytase (IZ+PH) respectively. There were no significant differences among the groups in mean tibiotarsal diaphysis diameter, thickness of the medial wall, tibiotarsal index, medullary canal diameter, modulus of elasticity or breaking stress. However, diet with OZ + PH and IZ + PH supplementation had a greater influence on tibiotarsal weight and tibiotarsal weight/length index when compared to those measurements in broilers in both control groups ($P<0.001$). In addition, feed additives were seen to have a significant effect on tibiotarsal length ($P<0.01$), robusticity index ($P<0.001$), thickness of the lateral wall ($P<0.05$) and Zn content ($P<0.05$). In conclusion, the study indicated that the use of organic and inorganic Zn alone or in combination with microbial phytase improved tibial bone traits and Zn content.

Keywords: *Phytase, Zinc,Tibia, Morphometric indices, Bone strength, Broiler*

Değişik Çinko Kaynakları ve Mikrobial Fitaz Katkısının Düşük Düzeyde Fosfor İçeren Etlik Piliçlerin Tibia Kemik Özellikleri, Dayanıklılığı ve Çinko Mineralizasyonu Üzerine Etkileri

Özet

Bu araştırma, düşük yararlanılabilir fosfor (Py) içeren mısır-soya temeline dayanan diyetlere farklı çinko kaynakları ile mikrobiyal fitaz ilavesinin broylerlerde tibia morfometrik indeksler, kemik direnci ve çinko birikimi üzerine etkisini değerlendirmek amacıyla yapılmıştır. Araştırma kırk iki gün sürmüştür. Toplam 875 adet günlük Ross 308 civciv, yedi farklı deneme grupları ve her grup 25 adet erkek hayvan içeren beş alt gruptan oluşturulmuştur. Pozitif kontrol grubu, çinko içermeyen mineral ön karması ve yeterli miktarda yararlanılabilir fosfor (%0.45) içeren rasyonla beslenmiştir. Negatif kontrol grubu ise, çinko içermeyen mineral ön karması ve düşük fosfor (%0.30) içeren temel bir rasyonla beslenmiştir. Deneme grupları; Fitaz, organik çinko, inorganik çinko, organik çinko + fitaz ve inorganik çinko + fitaz rasyonlarına sırasıyla %0.30 Py ve 500 FTU fitaz/kg; %0.30 Py ve 75 mg/kg Zn-proteinat; %0.30 aP ve 75 mg/kg ZnSO4; 75 mg/kg Zn-proteinat ve 500 FTU fitaz/kg; 75 mg/kg ZnSO4 ve 500 FTU fitaz/kg katılmıştır. Tibiotarsal diafiz çapı, medial duvar kalınlığı, tibiotarsal indeksi, medullar kanal çapı, esnekliği ve kırılma direnci bakımından gruplar arasında bir farklılık bulunmamıştır. Bununla beraber, OZ + PH and IZ + PH rasyon grubuna ait tibiotarsal ağırlık, tibiotarsal ağırlık/uzunluk indeksi değerleri her iki kontrol grubundan önemli düzeyde farklılık ($P<0.001$) bulunmuştur. Ayrıca, yem ilavelerinin tibiatarsi uzunluğu ($P<0.01$), sağlamlık indeksi ($P<0.001$), lateral duvar kalınlığı ($P<0.05$) ve çinko içeriği ($P<0.05$) üzerine önemli etkisi olduğunu göstermiştir. Sonuç olarak, bu çalışma, organik ve inorganik çinkonun tek başına ya da mikrobiyal fitaz ile kombinasyon halinde kullanılması, tibia kemik özellikleri ve çinko içeriğini geliştirmede etkili olduğunu göstermiştir.

Anahtar sözcükler: *Fitaz, Çinko, Tibia, Morfometrik indeksler, Kemik direnci, Broyler*

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INTRODUCTION

Zn is an essential trace mineral that is required for growth, bone development, feathering, enzyme systems, reproduction, maintaining correct insulin levels, thyroid function and the immune system, appetite, DNA, RNA and protein production in all avian species. Zn must be added to most poultry diets to meet requirements because of the poor availability of Zn in plant feed ingredients caused by the binding of Zn by phytate [1]. The National Research Council (NRC) [2] has estimated the Zn requirement for broiler chickens to be 40 mg kg^{-1} in the diet. Burrell *et al*.[3] reported improved performance when broilers consumed diets formulated to contain 110 mg Zn kg^{-1}. Furthermore, it is common practice in the U.S. broiler industry to formulate diets that contain 100-120 mg supplemental Zn kg^{-1} [3]. Zinc is usually added to broiler diets as inorganic feed grade zinc sulfate, zinc chloride, zinc oxide, or one of the organic forms complexed to amino acids, proteins, or carbohydrates. In recent years, organic zinc sources have been increasingly used, due to their potentially higher zinc bioavailability [4] and lower manure loading [5]. However, some studies have indicated little or no difference in the bioavailability of Zn between organic and inorganic sources [6,7].

The major portion of phosphorus in plant-derived ingredients is primarily present in the form of phytate, which is a mixed salt of phytic acid and which is unavailable to poultry because they have a limited capability to utilize phytate phosphorus. In addition, phytate is capable of binding di- and trivalent cations such as Ca, Zn, Cu and Mn in very stable complexes [8], thus reducing the availability of these minerals to the animal [9]. Supplemental microbial phytase has been shown to be a very effective and practical method for improving dietary phytate P bioavailability [10]. Studies have shown that the inclusion of phytase in chicken diets improved the bioavailability of dietary Zn [11,12].

Normal bone development in birds is also influenced by nutritional factors, genetics, gender, aging and the absolute growth rate, but the most relevant factor for poultry bone strength is nutrition. Calcium and phosphorus are the primary inorganic nutrients in the bone that may be important for bone health and strength [13]. Reichmann and Connor [14] showed that the extent of bone mineralization affects bone strength, and poor mineralization has been associated with increased risk of fractures. Weak bones result in breaking during processing and lower meat grade. Also, weak legs often result in reduced feed intake, thus affecting weight gain and feed conversion ratio [15]. Thus, bone problems in poultry can have an economic cost of several hundred million dollars a year.

Bone condition is commonly used as an indicator of mineral adequacy in poultry diet [13]. A number of invasive (bone ash, breaking strength, weight, and bone volume), and noninvasive methods (ultrasound) exist to determine bone mineralization in poultry [16]. The cortical index (tibiotarsal index) was first proposed by Barnet and Nordin [17] to indicate bone mineralization by morphometric measurements. Virtama and Telkka [18] showed a significant positive correlation between this method and bone mineral content in bone mineralization. Reisenfeld [19] and Seedor *et al*.[20] similarly used the robusticity index and the bone weight/bone length index to describe bone mineralization. Tests of bone strength in the literature [21] typically report the kilograms of force required to break various bones. However, Patterson *et al*.[22] reported that stress and modulus of elasticity are better terms to use than force in making bone strength comparisons between groups of birds that may differ in body size and bone dimension.

As far as we know, there is very little information available concerning the effects of the addition of dietary organic or inorganic zinc and microbial phytase on the bone robusticity index, bone weight/bone length index and tibiotarsal index, or the comparison of these results with the mechanical properties of tibiotarsi in broilers. Therefore, the aim of this study was to determine the effects of the interaction between Zn sources and microbial phytase on the morphometric and mechanical properties and Zn content of the tibia of broiler chickens fed low-P diets.

MATERIAL and METHODS

The study was approved by the Local Ethics Committee on Animal Experiments of Abant Izzet Baysal University (AİBÜ, 01.07.2009, HADYEK/23).

Housing, Birds and Diets

This study was conducted in the poultry research farm of the Mudurnu Sureyya Astarci Vocational School of Higher Education of Abant Izzet Baysal University in Bolu, Turkey. The poultry were housed in an environmentally controlled room with 35 floor pens of 2 m × 2 m for 42 days. The temperature of the animal facility was maintained at 31-33°C on the first day of life and lowered by 2-3°C every week to 22-23°C in the final week. The lighting program was 23L: 1D during the entire trial, and the duration of the experiment was 42 days.

A total of 875 one-day old male broiler chickens (ROSS 308) were purchased from a local commercial hatchery, Organic Zn (Bioplex, Alltech, Inc., Nicholasville, KY), inorganic Zn (zinc sulphate-$ZnS0_4.7H0_2$) and microbial phytase (Karyzyme® P 500, Kartal Kimya AS, Istanbul, Turkey) were obtained from commercial suppliers. The chicks were randomly assigned to seven groups of 125 birds each, each group being further separated into five replicates of 25 birds each. The feeding program consisted of a starter diet until 21 days of age and a finisher diet until 42 days of age. Chicks were given *ad libitum* access

to feed and tap water containing no detectable Zn. Diets were formulated to meet or exceed the requirements of the NRC [2] for broilers of this age. The PC group was fed a diet containing an adequate concentration (0.45%) of aP in a mineral premix without zinc. The NC group was fed a basal diet including a low concentration (0.30%) of aP in a mineral premix without zinc. This level of aP was selected to maintain the dietary available P of current NRC [2] recommendations and to ensure responses with phytase addition. For treatment groups PH, OZ, IZ, OZ+PH and IZ+PH, the basal diet was supplemented respectively with 0.30% aP and 500 FTU phytase, 0.30% aP and organic zinc (75 mg/kg of Zn from Zn-proteinate), 0.30% aP and inorganic zinc (75 mg/kg of Zn from ZnSO4), 0.30% aP, organic zinc and 500 FTU phytase, and 0.30% aP, inorganic zinc and 500 FTU phytase. Dietary treatments included the basic diet or basic diet supplemented with 75 mg/kg of Zn as feed-grade Zn sulfate from conventional inorganic sources or Zn-methionine inorganic Zn compounds.

Performance, some blood parameters, nutrient digestibility for broilers, and experimental treatments were described previously by Midilli *et al.*[23]. Organic and inorganic zn and phytase levels of diets used in the experiment are presented in *Table 1*. Chemical composition and Ingredients of the diets are shown in *Table 2*.

Sample Collection and Analysis

In this trial, 70 right tibiotarsi of Ross 308 male broilers were used to investigate the effects of Organic or inorganic Zn and microbial phytase supplement in corn-soybean meal based diets on bone characteristics. At 42 days, 10 broilers from each group (two chicks from each of the five replicates) were randomly selected and slaughtered by cut-ting the carotid arteries with subsequent exsanguination.

The right tibia of each bird was removed as a drumstick with the flesh intact. The drumsticks were labeled and immersed in boiling water (100°C) for 10 min. After cooling to room temperature, the drumsticks were defleshed by hand and the patella was removed. They were then air-dried for 24 h at room temperature.

The tibiotarsal length was measured with a dial caliper and the tibia bones were weighed on a precision balance. The thickness of the medial and lateral walls was measured as close as possible to the midpoint using a dial caliper. The diameter of the medullary canal was computed from the difference between internal and external diameter of the diaphysis. The bone weight/length index was obtained by dividing the tibia weight by its length [20]. The tibiotarsal and the robusticity indexes were determined using the following formulae:

Tibiotarsal index = diaphysis diameter - medullary canal diameter/diaphysis diameter x 100 [17] robusticity index = bone length/cube root of bone weight [19].

Following morphometric measurements, the modulus of elasticity and breaking stress and were determined by the method described by Timoshenko and Goodier [25]. The three-point bending test was applied to determine the elasticity moduli of the tibia bones. Prior to testing, the outer diameter and length of each bone were measured. During the test, the maximum load carrying capacities of the bonds were determined by applying an increasing load in conditions of 3-point bending on a universal testing machine operating with a load cell of 10 kN and a platter speed of 2.0 mm/min. Breaking stress was determined automatically by the device and the deflections of the bones were also measured. The elasticity modulus of each bone was then calculated by using the following

$$E = \frac{\left(\frac{P}{y}\right)L^3}{48I}\left(1 + 2.85\left(\frac{h}{L}\right)^2 - 0.84\left(\frac{h}{L}\right)^3\right)$$

force-deformation relationship [25]:

Where E = modulus of elasticity; P/y = initial slope of load-displacement curve; L = length; h = height of specimen; I = area moment of inertia. The inner diameters of the bones were also measured from the cross-sections of the fractured bones in addition to the outer diameters to determine their area moment of inertia (I).

The broken tibias were later used for other measurements. They underwent a 48-h defatting process under the action of finally evaporating hexane. The bones were dried at 105°C for 24 h, placed in a desiccator, and weighed to determine their fat-free dry weight. The bones were then placed in a muffle furnace at 600°C for 24 h and cooled in a desiccator, and the ash weight was recorded. The resultant

Table 1. *Organic and inorganic Zn and phytase levels of diets used in the experiment, mg/kg*
Tablo 1. *Denemede kullanılan yemlerin organik ve inorganik çinko ve fitaz düzeyleri, mg/kg*

Supplemental	Positive Control (0.45% aP)	Negative Control (0.30% aP)	Phytase (0.30% aP)	Organic Zn (0.30% aP)	Inorganic Zn (0.30% aP)	Organic Zn + Phytase (0.30% aP)	Inorganic Zn + Phytase (0.30% aP)
Phytase, FTU/kg	-	-	500 FTU	-	-	-	-
Zn-proteinate	-	-	-	75	-	75 + 500 FTU	
$ZnSO_4$	-	-	-	-	75		75 + 500 FTU

FTU = phytase unit, Available Phosphorus = aP

Table 2. *Chemical composition and ingredients of the diets (%)*
Tablo 2. *Yemlerin bileşimi ve kimyasal kompozisyonu (%)*

Ingredients	Starter (1-21 d)		Grower (22-42 d)	
	PC	NC (Low P)	PC	NC (Low P)
Maize	48.70	48.50	53.00	54.00
Wheat	1.20	2.10	2.00	2.00
Soybean meal (46.50% CP)	41.20	41.00	34.40	34.00
Soybean oil	5.30	5.10	7.00	6.70
Limestone	1.00	1.55	1.00	1.55
Dicalcium phosphate[1]	1.85	1.00	1.85	1.00
Vitamin premix[2]	0.10	0.10	0.10	0.10
Zn-free mineral premix[3]	0.25	0.25	0.25	0.25
Salt	0.25	0.25	0.25	0.25
DL-Methionine	0.15	0.15	0.15	0.15
Total	100.00	100.00	100.00	100.00
Chemical composition (Analysed)				
Dry matter	90.37	90.42	90.65	90.28
Metabolizable energy[4], kcal/kg	3035	3063	3179	3185
Crude protein, %	22.80	23.20	19.86	20.15
Ether extract, %	7.80	7.50	9.82	9.35
Starch, %	29.00	30.05	31.00	32.00
Sugar, %	5.93	5.80	5.90	5.75
Crude fiber, %	3.79	3.84	3.48	3.25
Ash, %	5.94	5.58	5.61	5.34
Ca, %	0.87	0.89	0.92	0.85
$P_{Available}$, %	0.43	0.31	0.47	0.28

[1] Contains 23% Ca and 18.10% available P; [2] Supplied per kilogram of diet: Vitamin A, 15.000 IU; cholecalciferol, 1500 ICU; vitamin E, 30.0 IU (dl-α-tocopheryl acetate); menadione, 5.0 mg; thiamine, 3.0 mg; riboflavin, 6.0 mg; niacin, 20.0 mg; panthotenic acid, 8.0 mg; pyridoxine, 5.0 mg; folic acid, 1.0 mg; vitamin B_{12}, 15 mcg; [3] Supplied per kilogram of diet : 80 mg of iron as $FeSO_47H_2O$, 6 mg of copper as $CuSO_45H_2O$, 60 mg of manganese as $MnSO_4H_2O$, 0.35 mg of iodine as KIO_3, and 0.15 mg of selenium as sodium selenite; [4] Metabolizable energy was calculated using the equation of Carpenter and Clegg [24]

ash was dissolved on a sand heater (300VC 15 min) in 10 ml 6 N HCl and 30 ml demineralized water. The solution was transferred after filtration (ashless filters) into a 100 ml volumetric flask. The tibia Zn concentrations were measured by Perkin Elmer AAnalyst 100 Atomic Absorption spectrophotometry (Perkin Elmer Inc., Waltham, MA, USA). The components of the samples were analyzed according to the standard procedures of AOAC [26].

Statistical Analysis

All statistical analyses were performed using SPSS® (Version 14.0 for Windows, SPSS Inc., Chicago, IL, USA). Data are given as means ± standard error (SE). The normality and homogeneity of variances were checked for all variables tested by means of a Shapiro-Wilks test and Bartlett-Box test. For normally-distributed data, differences between groups were compared using one-way ANOVA [27] and means were separated using a Duncan's post hoc test [28]. All statements of significance are based on $P<0.05$.

RESULTS

Morphometric Parameters of Tibia Bone

The effects of feed additives on the morphometric parameters of tibia bone at 6 weeks of age are shown in *Table 3*. The use of organic and inorganic Zn alone or in combination with microbial phytase significantly increased ($P<0.001$) tibiotarsal weight and length ($P<0.01$) in comparison to the control groups. The tibiotarsal weight/length index was found to be significantly higher ($P<0.001$) in the five experimental groups than in the control groups, with the highest readings coming from the organic zinc and microbial phytase diet group. The robusticity index

was lowest in the organic zinc and microbial phytase diet group (P<0.001). The diaphysis diameter data revealed no significant difference between the groups. There were no differences between the groups as to the thickness of the tibia medial wall. In contrast, the greatest thickness of the lateral wall was found in the inorganic zinc and microbial phytase diet-supplemented group (P<0.05). No significant differences between experimental treatments were observed in the medullary canal diameter or in the tibiotarsal index.

Mechanical Measurements and Zn Content of Tibia Bone

The effects of organic or inorganic Zinc and phytase, alone or in combination on the mechanical measurements and tibia Zn content of broilers fed diets low in available phosphorus are presented in *Table 4*. The tibia bone moduli of elasticity and breaking stress were not affected by dietary treatments. Dietary treatments significantly increased the percentage of Zn in tibias in comparison with the control groups (P<0.05).

DISCUSSION

The present study demonstrated that morphometric properties and Zn content of the tibia of broilers are affected when they are fed organic or inorganic Zn with or without microbial phytase supplementation.

Tibia Bone Morphometric Measures

Dietary PH, OZ, IZ, OZ +PH and IZ +PH supplementation significantly increased tibiotarsal weight (P<0.001) and length (P<0.01) when compared with the control groups in the current study *(Table 3)*. In agreement with this, Qian *et al*.[29] reported that the tibias of broilers fed with supplemental phytase (400, 600 and 800 U of phytase/kg) and inorganic P were longer and wider than those of broilers fed P-deficient diets. However, Sahraei *et al*.[30] reported that tibiotarsal weight and length were not influenced by adding 100, 150 or 200 mg/kg of either zinc oxide (72% Zn) or Bioplex Zn (15% Zn). The index of weight to length of bone was first introduced by Seedor *et al*.[20] to show significant variations in bone mineralization. In fact, this index indicates bone density, so that the higher the index the denser the bone [31]. The tibiotarsal weight/length index was found to be significantly higher (P<0.001) in the five experimental groups than in the control groups. The reason for the increased index with the supplemented diet compared to the control diet may be attributed to a significant increase in tibia weight. Also, Kocabagli [32] demonstrated that tibiotarsal weight/length index was increased when microbial phytase was supplemented at levels of 300, 500 and 700 U of phytase/kg in the diet. The result of this study contrasts with the results of Sahraei *et al*.[30], who found that a diet supplemented with 100, 150 or 200 mg/kg of either zinc oxide (72%Zn) or Bioplex Zn (15% Zn) had no significant effect on the tibiotarsal weight/length index.

There was no difference between the groups with regard to the thickness of the medial wall of the tibia. In contrast, the thickness of the lateral wall was different and the highest values for that were found in the IZ + PH and OZ + PH supplemented groups (P<0.05) *(Table 3)*. The lowest robusticity indices were found in the OZ + PH and IZ + PH diet groups (P<0.001). A low robusticity index indicates a strong bone structure [19]. A high value of the tibiotarsal index shows a high mineralization level of the bone [29]. The present study indicated that there were no significant differences (P>0.05) between the control and treatment groups with regard to mean tibiotarsal index *(Table 3)*. In contrast to these findings, Sahraei *et al*.[30] and Kocabagli [32] reported that phytase or zinc supplementation to diet significantly increased tibiotarsal index (P<0.05). The results of the present study indicated that diaphysis diameter and medullary canal diameter were not significantly different (P>0.05) between the control and treatment groups *(Table 3)*.

Mechanical Measurements and Zn Content of Tibia Bone

The skeletal integrity in poultry is affected by numerous factors, including nutritional regime, genetic factors, sex, age, management conditions and production system [30]. The stress at yield reflects the rigidity of bones as a whole, whereas the slope of the linear region of the stress versus strain curve (Young's modulus or elastic modulus) reflects the intrinsic stiffness or rigidity and material properties of bone. A high modulus values indicate the bone to be more rigid, whereas a low modulus could mean the bone is more ductile [15].

Although some small improvement in tibia breaking stress and the modulus of elasticity were observed due to supplemental organic and inorganic Zn alone or in combination with microbial phytase, these differences were not significantly different *(Table 4)*. These results are in agreement with the findings of Kocabagli [32], Perney *et al*.[33] and Sohail *et al*.[34] who reported that the inclusion of phytase in the diet improves bone strength. On the other hand, Patterson *et al*.[22] showed that the bone strength and modulus of elasticity were reduced in the tibiotarsi of broilers when they were fed a low Ca and low P diet. Further, our results agree with those of Shelton and Southern [35] and Scrimgeour *et al*.[36] who reported an influence of Zn on the mechanical properties of bones. These authors described that lower zinc content in animals' feed caused a reduction of bone integrity, bone density, and bone length, deterioration of compact bone formation, changes in the biomechanical competency of bone tissue and a decrease in the density of bones. These observations were supported by tibia Zn content data.

Table 3. *The effect of feed additives on morphometric parameters of tibia bone at 6 wk of age (mean ± SE)*
Tablo 3. *Altı haftalık yaşta tibia kemiklerinin morfometrik parametreler üzerine yem ilavelerinin etkisi (ortalama ± SE)*

Parameters	PC	NC	PH	OZ	IZ	OZ + PH	IZ + PH	P
Tibia weight mg	6754.10±355.18^{a}	7546.30±245.76^{a}	7811.20±201.81^{a}	7733.00±2.13^{a}	7681.50±287.98^{a}	10786.20±333.81^{b}	10717.40±532.88^{b}	***
Tibia length, mm	96.56±2.13^{a}	101.01±1.13^{ab}	102.00±0.57^{b}	103.06±2.93^{b}	102.13±1.03^{b}	102.99±0.92^{b}	103.08±0.89^{b}	**
Tibiotarsi Weight / Lenght ındex mg / mm	69.75±2.93^{a}	74.54±1.80^{a}	76.49±1.73^{a}	74.58±0.08^{a}	75.82±2.29^{a}	104.72±3.24^{b}	103.91±5.12^{b}	***
Robusticity İndex	5.10±0.08^{a}	5.14±0.04^{a}	5.14±0.04^{a}	5.22±0.31^{a}	5.20±0.04^{a}	4.65±0.06^{b}	4.70±0.08^{b}	***
Diaphysis diameter, mm	10.21±0.31	10.51±0.22	10.65±0.17	10.11±0.10	10.06±0.21	9.98±0.24	10.12±0.12	NS
Thickness of the medial wall, mm	1.60±0.10	1.84±0.09	1.74±0.14	1.88±0.08	1.77±0.16	1.68±0.13	1.56±0.11	NS
Thickness of the lateral wall, mm	2.23±0.08^{ab}	1.92± 0,14^{ab}	1.89±0.18^{b}	1.81± 0.37^{b}	1.79±0.19^{b}	2.24±0.12^{ab}	2.37±0.13^{a}	*
Medullary canal diameter mm	6.34±0.37	6.68±0.29	6.73±0.19	6.32±2.44	6.77±0.33	6.03±0.26	6.35±0.20	NS
Tibiotarsal index	38.20±2.44	36.42±2.58	36.70±1,85	38.43±1.56	32.00±2.49	39.66±1.84	37.25±1.67	NS

NS: *Non significant; * P<0.05;* ***ab:*** *The mean values within the same row with different superscript differ significantly *(P<0.05); **(P<0.01); ***(P<0.001)* ***PC:*** *Positive control,* ***NC:*** *Negative control,* ***PH:*** *Phytase,* ***OZ:*** *Organic zinc,* ***IZ:*** *Inorganic zinc,* ***OZ+PH:*** *Organic zinc + phytase,* ***IZ+PH:*** *Inorganic zinc + phytase*

Table 4. *The effects of organic or inorganic Zinc and phytase, alone or in combination on the mechanical measurements and tibia Zn content of broilers fed diets low in available phosphorus (mean ± SE)*
Tablo 4. *Düşük fosforlu diyetlere organik ve inorganik çinko ile fitaz ilavesinin broylerlerde tibia kemiği mekanik özellikleri ve çinko içeriği üzerine etkileri (ortalama ± SE)*

Parameters	PC	NC	PH	OZ	IZ	OZ + PH	IZ + PH	P
Modulus of elasticity, kg /cm^{2}	3420±368	2454±225	2639±253	2894±276	2817±308	3095±160	2799±171	NS
Breaking stress, kg / cm^{2}	170±16	139±8	168±12	156±12	150±9	174±10	164±9	NS
Zn, mg/kg	279.20±9.13^{a}	277.20±18.91^{a}	329.90±14.28^{b}	291.10±10,83^{ab}	325.20±5.99^{b}	317.90±1.70^{b}	300.50±16.40^{ab}	*

NS: *Non significant; * P<0.05;* ***a,b:*** *The mean values within the same row with different superscript differ significantly (P<0.05);* ***PC:*** *Positive control,* ***NC:*** *Negative control,* ***PH:*** *Phytase,* ***OZ:*** *Organic zinc,* ***IZ:*** *Inorganic zinc,* ***OZ+PH:*** *Organic zinc + phytase,* ***IZ+PH:*** *Inorganic zinc + phytase*

According to Jongbloed *et al*.[37], bone and pancreatic zinc are the best response criteria to assess the biological value of zinc sources in monogastrics. Underwood and Suttle [38] found a gradual decrease in Zn concentration in wool, feathers and bones caused by lower zinc content in the feed. Scrimgeour *et al*.[36] found a positive correlation between dietary and bone zinc level.

The tibia Zn content was found to be significantly higher ($P<0.05$) in three of the experimental groups than in the control groups. The highest values for tibia Zn content were found in the PH (329.90), IZ (325.20), OZ + PH (317.90), and IZ + PH (300.50) groups. These results are consistent with the data published by Roberson and Edwards [39], Yi *et al*.[11], Mohanna and Nys [40], Ao *et al*.[41], Aksu *et al*.[42], Shelton and Southern [35]. Roberson and Edwards [39] showed that adding 600 U of phytase/kg of diet to the basal diet significantly increased bone Zn concentration (21 to 23 vs 23 to 25 mg/g), and Yi *et al*.[11] reported that the concentration and amount of Zn in tibias were linearly increased by the dietary addition of phytase and Zn. Results reported by Mohanna and Nys [40] indicated that tibia and plasma Zn concentrations increased linearly with increasing levels of dietary Zn up to 75 mg/kg. Ao *et al*.[41] demonstrated that the total tibia Zn content was linearly increased by dietary supplementing from both inorganic (ZnSO4.7H2O,) and organic Zn (a chelated zinc proteinate) sources. Aksu *et al*.[42], who reported that tibia Zn concentrations decreased with decreasing levels of dietary Zn.

Moreover, data from Shelton and Southern [35] determined that dietary inclusion of microbial phytase increased the availability of Zn and tibia Zn concentrations in broiler chicks. These beneficial effects of phytase could possibly be due to the release of minerals such as Zn from phytate complexes. As a conclusion, the results of this study indicate that microbial phytase (500 FTU/kg^{-1}) is effective in improving bone morphometric traits and Zn content in broilers fed with diets based on corn-soybean meal and containing 75 mg/kg from organic or inorganic (not deficiency) sources and low available phosphorus.

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Short Communication

Comparison of Rectal, Eye and Ear Temperatures in Kangal Breed Dogs [1]

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Abstract

The aim of this research was to compare rectal, eye and ear temperature of 30 healthy *Kangal* dogs. Rectal temperature was measured by use of standard rectal digital thermometer. Right and left eye temperatures were detected by infrared thermal camera from the *carancula lacrimalis* region. Tympanic thermometer was used to determine right and left ear temperatures. Data were analyzed by Pearson correlation test. The comparative analysis showed no significant differences between the measurement methods. Measuring of ear and eye temperature could be an alternative method to obtain body temperature in *Kangal* dogs.

Keywords: *Body temperature, Rectal temperature, Eye, Ear, Kangal dog*

Kangal Irkı Köpeklerde Kulak, Göz ve Rektal Isıların Karşılaştırılması

Özet

Bu çalışmanın amacı 30 sağlıklı kangal köpeğinin kulak, göz ve rektal ısılarını karşılaştırmaktı. Rektal ısı standart digital rektal termometre kullanılarak ölçüldü. Sağ ve sol göz ısısı *carancula lacrimalis* bölgesinden kızılötesi termal kamera ile belirlendi. Timpanik termometre sağ ve sol kulak ısı değerlerini belirlemede kullanıldı. Veriler Pearson korelasyon testi ile analiz edildi. Karşılaştırmalı analizler tüm metodlar arasında önemli bir farklılık olmadığını gösterdi. Kangal köpeklerinde kulak ve göz ısısının ölçülmesi, vücut ısısının belirlenmesi için alternatif bir yöntem olabilir.

Anahtar sözcükler: *Beden ısısı, Rektal ısı, Göz, Kulak, Kangal köpeği*

INTRODUCTION

Measuring of the body temperature is the crucial part of the canine physical examination. Digital rectal thermometer is the most commonly used tool to detect body temperature in dogs. However, it has some disadvantages such as time consuming, difficult to achieve in aggressive dogs, which could result with rectal injury, and impossibity in cases of atresia ani [1]. Alternative methods to detect body temperature are useful both patients and clinicians. Thermal microchips and infrared thermometers are used to measure body core temperature in dogs. However, the major disadvantage of microchip thermometry is necessity of equipment [2].

Infrared thermography is a noninvasive technique that detects temperature differences in a region. It has been pointed that eye temperature can reflect the core body temperature. Moreover infrared thermography can be used to detect body temperature without touching or disturbing the animal [3].

Measuring of the ear temperature in veterinary medicine is a new, rapid and also economic technique. It has superiority on rectal temperature (RT) in some instances such as fractious dogs or those with recto-anal disease. However, the major limitations include ear diseases or inflammation and some dogs refuse placement of the probe [4].

Even though numerous researches have been conducted on *Kangal* dogs [5,6], to the authors' knowledge, there is no scientific data about measurement results of their eye and ear temperatures. Therefore, the aim of this study was to determine the difference between rectal, eye and ear temperatures in *Kangal* dogs.

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MATERIAL and METHODS

Animals

Thirthy male *Kangal* dogs (6-12 months old, mean body weight 40±9 kg) were enrolled in the study, which were brought to veterinary clinics at Ataturk University for various purposes; including routine physical examination, vaccinations and neutering. Each dog was healthy based on the physical examination. Measurements were collected at time between 9 am and 3 pm.

Measurements

Temperature measurements were obtained in a room, which had 25.3±1.1°C mean temperature, 58.0±8.0% relative air humidity and no direct sunshine. Before measurements, each dog was acclimatized in this room for 20 min.

Three different sources were used to determine dog temperature: rectal, eye and ear. Rectal temperatures were collected with a conventional digital thermometer from the dogs restrained by their owners. Digital rectal thermometer was inserted about 2 cm into the rectum, and it remained until the audible beep was heard.

Eye temperatures were obtained with infrared thermal camera (IR FlexCam S, Infrared Solutions Inc., Plymouth, MN, USA). Measurements were recorded when the infrared thermal camera was placed at a 1 meter distance from each dog. The dog was restrained by owners for 2 seconds to measure the eye temperature, and then eye temperature was obtained focusing on *carancula lacrimalis* region by thermal camera.

Ear temperatures were obtained by infrared tympanic thermometer (Genius 2 Tympanic Thermometer, Covidien Ilc, Mansfield, USA) when the animal was restrained by owner. To measure the tympanic temperature of each ear, tympanic thermometer was inserted in the ear canal descending to the eardrum. After pressing the activation button, auricular temperature was appeared within 2 seconds.

Statistical Analysis

All data were analyzed using the SPSS 19 (IBM Company, Version 19.0, SPSS Inc, USA, 2010) statistical package. Data were presented as the average of the right tympanic temperature (RTT), left tympanic temperature (LTT), right carancula lacrimalis temperature (RCT), left carancula lacrimalis temperature (LCT) and RT. Differences among RTT, LTT, RCT, LCT and RT were analyzed by Pearson correlation test.

RESULTS

The consistency of RTT, LTT, RCT and LCT measurements versus to rectal temperatures are given in *Fig. 1*. The comparative statistical analysis results are shown in *Table 1*, which is indicated not significantly different results of

Fig 1. The consistency of the right-left carancula temperatures (RCT-LCT), right-left tympanic temperatures (RTT-LTT) versus to rectal temperature measurements of dogs

Şekil 1. Köpeklerde rektal ısı ölçümlerinin, sağ-sol timpanik ısı ve sağ-sol carancula ısılarına göre yoğunluğu

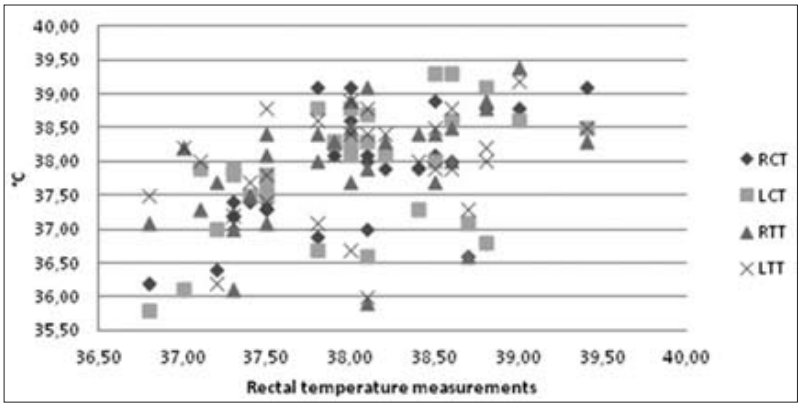

Table 1. *Means, standard errors (SE), correlations and level of significance (P) of the right tympanic temperature (RTT), left tympanic temperature (LTT), right carancula temperature (RC), left carancula temperature (LCT) and rectal temperature (RT) in dogs*

Tablo 1. *Köpeklerde rektal, sol carancula, sağ carancula, sol timpanik ve sağ timpanik ısıların ortalamaları, standart hataları, korelasyonları ve önem seviyesi*

Variable	Mean± SE (°C)	95% CI		RCT	LCT	RTT	LTT	RT
		Lower Bound (°C)	Upper Bound (°C)					
RCT	37.682±0.149	37.354	38.011	-	0.906**	0.485**	0.490**	0.569**
LCT	37.750±0.179	37.357	38.143		-	0.409*	0.459*	0.506**
RTT	37.940±0.168	37.570	38.311			-	0.739**	0.431*
LTT	37.920±0.191	37.499	38.341				-	0.368*
RT	37.997±0.119	37.754	38.239					-

** P<0.001, * P<0.005

RTT, LTT, RCT, LCT and RT. *Fig. 1* and *Table 1* observe high positive correlations between RTT, LTT, RCT, LCT and RT.

DISCUSSION

Measuring of the RT is a conventional method to reveal the body temperature in dogs. It has emphasized that measurement of RT may not be possible in 1.9% dogs due to intolerance [4]. This ratio may be higher if there is a rectal or perianal disease. It was reported that tympanic temperature measurement (TTM) was well tolerated than RT in dogs [2,4]; moreover it has also been reported that several dogs displeased the aural probe more than the rectal probe [7]. In the current study all measurement methods were well tolerated by all dogs. The prior study [8] has revealed that, similiar to our results, TMMs' is easy to collect and rapid compared with RT.

Infrared thermography has been gaining popularity instead of surface temperature, and it does not need to direct contact with the patient [9]. Eye temperature is a more accurate and useful indicator of core body temperature [10]. It has been stated that direct sunlight could result in increased eye temperature [11]. We performed all thermographic measurements in a room without sunlight. As reported previously mean eye temperatures of horses was 32.6°C [11]; however, another study reported this ratio as 38.2°C, which was normal limit for horse' temperature [3]. This diversity is due to measurements of different parts of the eye. Central corneal temperatures of dogs were ranged between 34.4°C and 35.2°C [9]. Moreover it has been pointed out that temperature of nasal limbus tends to be higher than other regions of eye because of increased blood flow [10]. In this study, we also noticed lower central cornea temperature than nasal limbus, but we did not collect the temperature from the central cornea which had lower temperatures than body core temperature [12]. Our temperature results obtained from carancula lacrimalis region correspond with rectal and tympanic temperatures.

In this study right and left tympanic temperature values showed strong similarity with RT. It has been reported that auricular temperature can be used to detect core body temperature in dogs [13]. On the other hand, a study on different breed dogs identified auricular temperatures have lower values than RT [14], which is disagreement with our findings. This discrepancy can propably due to breed differences and/or ear anatomy. Breed differences in body temperature have been reported in dogs [4,14]. It has been stated that sex do not affect the ear temperature [15], the current study was carried on only male dogs to prevent possible effects of temperature alterations.

Although current study did not focus on the selection of an advantegous method for obtaining body temperature, it was obvious that restriction of the dog by an assistant required in all methods. Measuring of eye temperature from the nasal limbus (carancula lacrimalis region) seems to more comfortable and safer than RT and TMM because it can be achived without touching the animal. Moreover, TMM is easier than RT due to rapid detection of body temperature.

Measuring ear and eye temperature could be an alternative method to detect body temperature in *Kangal* dogs. It can be pointed out that further studies about body temperature techniques should be planned on other breeds and species.

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Short Communication

Determination of Aflatoxin Levels in Maize Grain by High Performance Liquid Chromatography Using an Immunoaffinity Column Cleanup [1]

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[1] *The results of this study were presented in part as an abstract at the 3rd National Veterinary Pharmacology and Toxicology Congress from 29th September to 2nd October, 2010 in Kusadasi/Aydin, Turkey*

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Abstract

The aim of this study was to determine aflatoxin contamination levels in maize grain produced in Samsun Province, Turkey. Forty samples of maize grain intended for animal feed in Samsun Province were analyzed. The amounts of the aflatoxins, B_1, B_2, G_1 and G_2, were quantified with a liquid chromatography (HPLC-FLD) and post-column derivatization. Recoveries ranged between 89 and 98%. The detection limits were 0.007 and 0.02 ng/g and the corresponding quantification limits were 0.022 and 0.063 ng/g. As a result, it has been shown that the accurate and sensitive HPLC-FLD method was confirmed as being appropriate for the detection of AFs in maize grain, besides, the incidence of aflatoxins in maize was very low (2.5%).

Keywords: *Aflatoxin, Immunoaffinity column, High performance liquid chromatography, Maize*

Tane Mısır Örneklerinde Aflatoksin Düzeylerinin Yüksek Performanslı Likit Kromatografi-İmmunoafinite Kolon Yöntemi İle Belirlenmesi

Özet

Bu çalışmada Samsun ilinde üretilen tane mısırlarda aflatoksin kontaminasyonunun araştırılması amaçlandı. Bu amaçla, Samsun ilinde hayvan yemi olarak kullanıma sunulan 40 adet tane mısır örneği analiz edildi. Aflatoksin B_1, B_2, G_1 ve G_2 düzeyleri kolon sonrası türevlendirmenin yapıldığı likit kromatografi (HPLC-FLD) sistemi ile ölçüldü. Aflatoksinlerin geri kazanım oranları %89-98, belirleme alt limitleri (LODs) 0.007-0.02 ng/g ve hesaplama alt limitleri (LOQs) 0.022-0.063 ng/g aralığında belirlendi. Sonuç olarak, tane mısırlarda aflatoksinlerin tespitine yönelik olarak uygulanan immunoafinite kolon temizleme ve kolon sonrası brom türevlendirmesini içeren doğruluk ve hassasiyeti yüksek HPLC-FLD metodunun, aflatoksinlerin tespitinde uygun bir yöntem olarak kullanılabileceği, bunun yanında, Samsun ilinden elde edilen tane mısır örneklerinde aflatoksinle kontaminasyon oranının düşük düzeyde (%2.5) olduğu ortaya konulmuştur.

Anahtar sözcükler: *Aflatoksin, İmmunoafinite kolon, Yüksek performanslı likit kromatografi, Mısır*

INTRODUCTION

Aflatoxins (AFs) are toxic metabolites produced by a large number of *Aspergillus* species that include *Aspergillus flavus*, *A. parasiticus*, *A. nomius, A. pseudotamarii, A. parvisclerotigenus, A.bombycis, A. ochraceoroseus and A. rambellii.* Although 18 types of AFs have been identified, the well-known ones are AFB_1, AFB_2, AFG_1 and AFG_2 [1]. *Aspergillus* spp. grow in hot and humid environments, producing AFs during harvesting and storage that affect the quality and safety of food and feedstuffs [2]. The ingestion of AFB_1 and AFB_2-contaminated food or feed by female mammals leads to the excretion in milk of the partially detoxified hydroxylated analogues, aflatoxin M_1 (AFM_1) and aflatoxin M_2 (AFM_2). In dairy cows, these contaminants can occur in commercial milk and be processed in cheese, and in many developing countries, the presence of AFM_1 in human milk can lead to ingestion by infants [3].

Maize is a major cereal crop for both livestock and human nutrition worldwide. In Turkey, maize is produced in large quantities in the Black Sea Region; in 2013, 271.808 tonnes of maize were produced in the Black Sea Region

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and Samsun Province was the top producer with 81.209 tonnes [4].

A reliable risk assessment of mycotoxin contamination for humans and animals relies on their identification and accurate quantification in food and feedstuffs [5]. Aflatoxins are low molecular mass polar compounds which possess UV absorption and fluorescence properties. For this reason, liquid separation techniques have predominated in their analysis, initially thin layer chromatography (TLC), but subsequently LC [3]. However, immunoaffinity column clean-up, a modern sample clean-up technique, has been applied to increase method specificity and sensitivity by the selective enrichment and isolation of the target aflatoxins [5,6].

In the present study, aflatoxin contamination levels in maize grain in Samsun Province, were determined to test whether the method has the appropriate level of sensitivity for the task and to survey the level of contamination of samples from various sources.

MATERIAL and METHODS

Collection of Maize Samples

Maize grain samples were obtained from feed sellers in Bafra, Carsamba, Terme and Samsun districts of Samsun Province. A total of 40 samples, with 10 from each district, were collected.

Extraction of Aflatoxins and Immunoaffinity Column Clean-up

The extraction and clean-up procedures were performed according to the AOAC method [7], with some modifications. Each sample (500 g) was ground to a fine powder with a laboratory mill (IKA®, Staufen, Germany). A 50 g sub-sample was taken and 4 g sodium chloride (Merck, Darmstadt, Germany) and 100 ml of ultrapure water (Millipore Simplicity®, Molsheim, France) were added. They were mixed vigorously for 1 min with a magnetic stirrer (WiseStir®, Daihan Scientific, Seul, South Korea). One hundred and fifty millilitres of methanol (Merck, Darmstadt, Germany) were added and the mixture was stirred vigorously for 2 min. The extract was filtered through a filter paper (Whatman® No.4) and 5 ml of clear filtrate (equivalent to 1 g of product) was transferred to a beaker and 15 ml of phosphate buffered saline (PBS) (Merck, Darmstadt, Germany) solution was added.

The Aflaprep® immunoaffinity column (R-Biopharm, Glasgow, Scotland) were at room temperature prior to conditioning. The column was loaded with 10 ml of PBS which was passed through the column at a speed of 2-3 ml/min under gravity. Twenty millilitres of diluted filtrate was then passed through the column at a flow rate of 3 ml/min under gravity. The column was then washed with two, 10 ml volumes of water at a flow rate of 5 ml/min and dried by applying a light vacuum for 10 s.

One millilitre of methanol was then passed through the column under gravity. Following that, 1 ml of ultrapure water was passed through the column under gravity. The remainder of the elution solvent in the column was collected by forcing pressurised air through the column after most of the eluent had passed through under gravity. The eluent was filtered through a disk filter unit (0.45 µm) prior to HPLC injection.

HPLC-FLD Analysis

The mobile phase consisted of methanol-water (45+55, v/v). One hundred and twenty milligrams of potassium bromide and 350 µl of 4 M nitric acid (Merck, Darmstadt, Germany) were added to each liter of the mobile phase. The flow rate was 1 ml/min isocratically. Post-column derivatization was carried out with bromine in the KobraCell® (R-Biopharm, Glasgow, Scotland) with an electrochemical reaction current of 100 µA. Fluorescence detection (RF-10AXL, Prominence LC 20A, Shimadzu, Kyoto, Japan) was performed at an excitation wavelength of 360 nm and emission wavelength of 430 nm. A reversed-phase C18 column (Inertsil® ODS-3V, 5 µm, 4.6 x 250 mm, GL Science, Tokyo, Japan) was used for separation. The column's oven temperature was set at 40°C and the run time was 30 min per analysis [7].

Calibrant solutions were prepared by diluting the total aflatoxin standard stock solution (Aflastandard®, 1.000 ng/ml, R-Biopharm, Glasgow, Scotland) with the mobile phase at concentrations of 0.0625, 0.125, 0.25, 0.625, 1.25, 1.875 and 2.5 ng/ml. They were injected into the HPLC at a volume of 100 µl. Recovery studies were carried out by spiking uncontaminated samples with three different levels (0.5, 1 and 2 ng/ml) of each aflatoxin.

RESULTS

The values of R^2, LOD, LOQ and RSD% (Relative Standard Deviation) are shown in *Table 1*.

Average recoveries were 89, 89, 98 and 93% and retention times were 11.8, 15.0, 18.3 and 23.6 min for AFG_2, AFG_1, AFB_2 and AFB_1, respectively *(Fig. 1)*.

Table 1. *R^2, LOD, LOQ and RSD% values of aflatoxins*
Tablo 1. *Aflatoksinlerin R^2, belirleme, hesaplama alt limitleri ve %RSD değerleri*

Aflatoxin	R^2	LOD (ng/g)	LOQ (ng/g)	RSD%
B_1	0.999	0.013	0.038	8.0
B_2	0.999	0.007	0.022	4.4
G_1	0.999	0.021	0.063	8.1
G_2	0.999	0.016	0.049	2.8

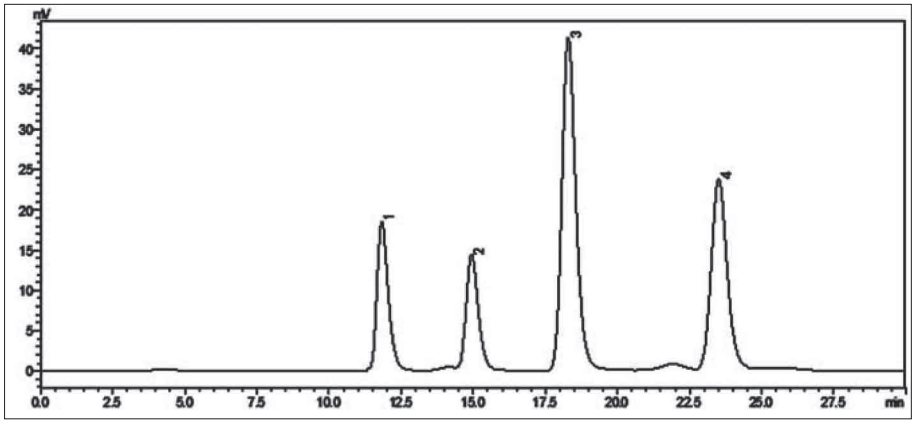
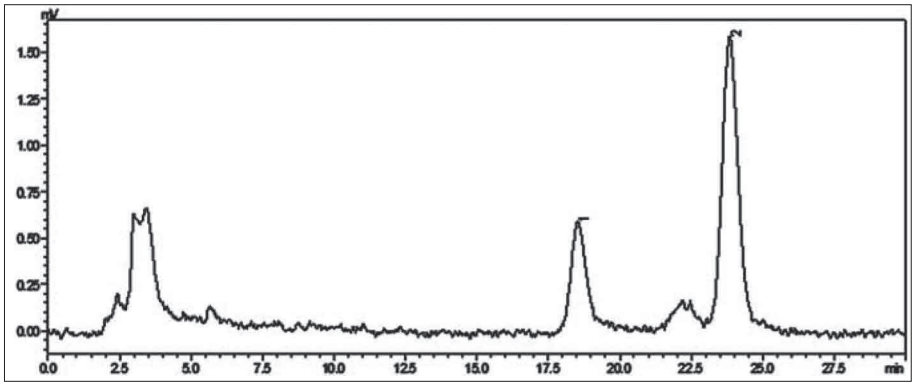


Fig 1. HPLC-FLD chromatogram of total aflatoxin standards at a concentration of 2.5 ng/ml. **1-** AFG_2, **2-** AFG_1, **3-** AFB_2, **4-** AFB_1

Şekil 1. 2.5 ng/ml konsantrasyonunda total aflatoksin standartına ait HPLC-FLD kromatogramı, **1-** AFG_2, **2-** AFG_1, **3-** AFB_2, **4-** AFB_1

Fig 2. HPLC-FLD chromatogram of the aflatoxin positive sample, **1-** AFB_2, **2-** AFB_1

Şekil 2. Aflatoksin pozitif numuneye ait HPLC-FLD kromatogramı, **1-** AFB_2, **2-** AFB_1



Only one maize sample collected from Samsun Province was contaminated and the contaminants were AFB_1 (0.193 ng/g) and AFB_2 (0.055 ng/g) *(Fig. 2)*.

DISCUSSION

In the present study, only one sample of a total of 40 maize grain samples from Samsun Province was contaminated with aflatoxins (AFB_1 and AFB_2); the AFB_1 concentration was lower than the limit set by the EU and Turkey (20 ng/g), but an AFB_2 limit has not been set [8,9]. Various mycotoxin screening studies of food and feedstuffs have been carried out in Turkey but there have been few undertaken with HPLC-FLD. Our results for aflatoxin levels were lower than in several previous studies performed in the same region or in other regions of Turkey. Specifically, Giray et al.[10] used ELISA to determine AFs and ochratoxin A (OTA) levels in 47 corn samples collected from various street bazaars and market outlets in different regions of Turkey. The AFs contamination range was between 0.625 and 8.57 ng/g for the Black Sea region, with 30% of samples contaminated. Oruc et al.[11] analyzed for AFB_1, T-2 toxin, fumonisin, deoxynivalenol and zearalenone with ELISA in various feedstuffs collected from a feed manufacturer that had obtained the samples from different regions of Turkey. They found seven samples of maize grains contaminated with AFB_1 in the range of 1.86 to 58 ng/g.

Previous reports indicate that certain volatile compounds produced by plants inhibit AF formation [12]. Plant lipoxygenase in maize and peanuts and its product, the 13(S)-hydroperoxide derivate, were also shown to interfere with AF formation [13]. Genetic modification of mold susceptible plants also plays a role in food safety. This involves increasing production of compounds such as anti-fungal proteins, hydroxamic acids and phenolics that reduce fungal contamination [14]. In the present study, the low AF contamination levels in maize may be related to genetic modification and/or proper harvesting, handling procedures and storage by agricultural producers.

The monitoring of AFs depends on their detection with precise analytical methods. While common analytical methods employ different detection techniques (ELISA, LC or TLC), all procedures require a suitable sample extraction step [15]. The extracts of most matrices are unsuitable for direct chromatographic analysis due to the large number of co-extracted impurities [5]. An immunoaffinity method is highly advantageous, as it is rapid and inexpensive [16]. In the present study, the KobraCell® was also used. It is an electrochemical cell that generates a reactive form of bromine for derivatization of aflatoxins, resulting in more sensitive detection. We achieved more precision in recovery of LODs and LOQs than several studies that used ultraviolet detection and/or TFA derivatization. Fu et al.[17] used an ultra-high pressure LC method with ultraviolet detection for the determination of AF levels in corn and peanuts but did not include a derivatization process. Their LODs for AFB_1, AFB_2, AFG_1 and AFG_2 were 0.32, 0.19, 0.32 and 0.19 ng/g and quantification limits were 1.07, 0.63,

1.07 and 0.63 ng/g, respectively. We achieved detections at 11 to 26 fold lower levels than their results and also better recoveries. Majeed et al.[18] determined AF and OTA levels in rice, corn and corn products in Pakistan with HPLC-FLD by using an AflaTest® immunoaffinity column and TFA derivatization. Their LODs and LOQs for AFB_1, AFB_2, AFG_1 and AFG_2 were 0.05/0.15, 0.10/0.30, 0.05/0.15 and 0.10/0.30, respectively, which were higher than in the present study.

The HPLC-FLD method with immunoaffiniy column clean-up involving post-column bromination that we applied to maize for detection of AFs is sensitive and accurate. AF contamination levels in the maize sampled in Samsun Province were very low but should be checked regularly because AFs threaten both human and animal health. For the future, the systematic use of new analytical techniques to measure AF levels with greater precision can help ensure that public health standards are improved.

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