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Investigating various performance traits of Karakul sheep

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Abstract: Karakul sheep in Turkey is an endangered breed according to the total number of ewes. Two herds of Karakul sheep in Tokat Province, reared similar environmental conditions, kept *in situ in vivo* conservation as a gene resource. This study aimed to determine various performance traits of Karakul sheep reared in breeder conditions as a gene resource. Least squares means for lambing rate (LR), litter size (LS) and lamb production (LP) were found 95.8±0.60%, 1.04±0.01 and 100.0±0.80%, respectively. It was determined that farm and age had significant effect on LR and LP, while farm and year had significant impact on LS. Survival rate in lambs were found 95.2 and 94.1%, respectively on 90th and 180th days. Means of least squares for live weights of lambs at birth and on 90th and 180th days were identified as 3.35±0.02, 21.52±0.27 and 30.34±0.32 kg, respectively. Live weight after shearing and greasy fleece weight values were 40.73±0.12 and 2.04±0.01 kg for females and 62.65±0.53 and 3.48±0.06 kg for males. Lactation milk yield (LMY) was 104.85±3.73 kg in ewes and lactation duration was 159.01±1.70 days. LMY was affected by the lactation number and farm while lactation duration was affected by lactation number. Results revealed that various performance traits of Karakul sheep conserved in breeder conditions were similar to or better than those previously reported for this breed. Also, the breed is similar to medium size native sheep breeds in terms of growth and mature live weight.
Keywords: Genetic resource, Karakul sheep, production traits

Karagül koyunlarında çeşitli verim özelliklerinin araştırılması

Özet: Türkiye'de Karagül koyunu, yok olma tehlikesi altında bulunan bir ırktır. Tokat ilinde birbirine yakın ve benzer çevresel koşullarda yetiştirilen iki Karagül koyun sürüsü, yetiştirici koşullarında gen kaynağı olarak korunmaktadır. Bu araştırma, yetiştirici koşullarında gen kaynağı olarak korunan Karagül koyunlarında çeşitli verim özelliklerinin incelenmesi amacıyla yapılmıştır. Koyunlarda doğum oranı, bir doğuma kuzu sayısı ve kuzu verimi için en küçük kareler ortalamaları % 95,8±0,60; 1,04±0,01 ve % 100,0±0,80 olmuştur. İşletme ve yaşın doğum oranına ve kuzu verimine; işletme ve yılın ise bir doğuma kuzu sayısına etkileri önemli bulunmuştur. Kuzularda 90. ve 180. günde yaşama gücü sırasıyla % 95,2 ve 94,1 olarak tespit edilmiştir. Kuzularda doğum, 90. ve 180. gün canlı ağırlıkları sırasıyla 3,35±0,02; 21,52±0,27 ve 30,34±0,32 kg belirlenmiştir. Kırkım sonrası canlı ağırlık ve yapağı verimi dişilerde 40,73±0,12 ve 2,04±0,01 kg, erkeklerde 62,65±0,53 ve 3,48±0,06 kg tespit edilmiştir. Koyunlarda laktasyon süt verimi 104,85±3,73 kg ve laktasyon süresi 159,01±1,70 gün olmuştur. Laktasyon süt verimine laktasyon sırası ve işletme, laktasyon süresine ise laktasyon sırasının etkisi önemli olmuştur. Sonuç olarak yetiştirici koşullarında korunan Karagül koyunlarında genel olarak çeşitli verim özelliklerinin ırk için bildirilen değerlere benzer veya daha iyi olduğu belirlenmiştir. Ayrıca Karagül ırkı, canlı ağırlık ve büyüme bakımından orta yapılı yerli koyun ırklarına benzerlik göstermektedir.
Anahtar sözcükler: Gen kaynağı, Karagül koyunu, verim özellikleri

Introduction

Karakul is a fat-tailed sheep breed with coarse fleece (1). This breed is called after Karakul town in Turkmenistan (1) or Karagöl located in the city of Bukhara in Uzbekistan (2). The most significant characteristic of this breed is the Astrakhan fur obtained from the newborn lambs. It has been reported that Karakul breed was first brought to Tokat and Antalya in Turkey by the families who migrated from Caucasia at the end of 19th century (22). Later, rams and ewes were brought from Turkistan in 1929 to start breeding (2, 11). Breeding was undertaken in many state institutions led by Çifteler (Eskişehir) and Kazova (Tokat); however, subsequently breeding in these state farms was terminated. Today, Karakul sheep are bred only in Tokat vicinity at a small scale. Because total number of Karakul ewes has decreased considerably, Karakul breed in Turkey was accepted as an endangered breed (8). Therefore, two herds (a total of 320-head) were

conserved in the framework of the project to conserve the genetic resources of domesticated animals. Previous studies, carried out in the 1960s, investigating Karakul sheep in Turkey were mostly related to curl forms and skin structure (6, 7, 11). Some studies performed in 2000s were published on milk (16), fleece (17), fertility (23), and some production traits (13). A number of production traits of ewes and lambs in the Karakul herds during the period of 2005-2008, in which the current research was conducted, were reported (13). The current study includes fertility, live weight after shearing, greasy fleece yield, milk yield and some udder measurements along with lamb survival rates and growth characteristics of the herds from 2011 to 2015.

Karakul sheep breed has been reared for a long time in Anatolia and it is one of components of biodiversity of Turkey. This breed faces a challenge of the need to increase production traits to provide sustainable production. Astrakhan fur, the most important yield of the breed, is not generally utilized in Turkey, and so the existence of the breed depends on the use of other yield characteristics. For this reason, it is important to know the current information about production traits of the breed.

The study aimed to investigate fertility, survival ability, growth, production of milk and wool of Karakul sheep under *in situ in vivo* conservation.

Material and Methods

The study was conducted on two Karakul sheep herds reared in Gülpınar and Ulaş villages of Tokat province (Gülpınar and Ulaş villages are situated between 40° 18' 03"- 40° 18' 48" east longitude and 36° 26' 11"- 36° 23' 07" north latitude and elevation from sea level is 630 m). Data on the number of ewes, the performance traits of which were under investigation are presented in the form of tables. Husbandry and feeding conditions were generally similar in the farms where the project was carried out. The distance between the two farms is 7 km. Ewes in both herds were fed on pasture during the first 3/5 period of gestation, and were offered with 400 g of concentrate feed (14.0% crude protein and 2400 kcal ME/kg) and roughage (400 g sugar beet pulp and 500 g vetch plus barley straw) per ewe daily during the last 2/5 period of gestation. The ewes were kept indoors during the first 6 weeks of lactation period and fed similar to the last period of gestation. After indoor keeping, the ewes were fed on pasture during the rest of lactation period.

Ewes mating was conducted as random mating. Lambing rate (LR) and lamb production (LP) were calculated according to the number of ewes exposed to rams and litter size (LS) was calculated based on the number of ewes lambing. Survival rate (SR) of lambs was based on lambs born alive. The date of birth, gender and age of dam were recorded at birth along with their birth weight no later than 24 hours after the birth. The lambs were received alfalfa hay and lamb grower feed. The growth of lambs was recorded and live weight on the 90th and 180th days was calculated by using interpolation method. Ewes were shorn in June every year. Greasy fleece weight and live weight after shearing were identified with the help of electronic bascules sensitive to 50 g.

Milk yield controls were conducted in 2013 on a total of 30 ewes per herd randomly selected from both herds with single births at the second week of February and were in the 1st, 2nd and 3rd lactation. The first milk control was conducted about on the 45th day after the birth and controls were continued about 90 and 135 days after parturition. The data for the lactation milk yield (LMY) were calculated by interpolation and extrapolation methods. The lactation duration (LD) was calculated as the period between the date of birth and the end of lactation. The end of lactation was determined by extrapolation method based on the last milk control day.

The lambs were separated from their mother one day before the milk control day at 17.^{00} and the ewes were milked by hand on the milk control day around at 08.^{00} and 17.^{00}. Lambs in both herds were not weaned until the last control milking and went to the pasture with the ewes. LMY was calculated by using Fleischmann's method (TrapezII). Udder measurements were collected right before the 1st and the 3rd milk control days of lactation with the help of measuring tape and digital calipers (18).

Statistical analysis: Fertility, greasy fleece weight, lactation milk yield, lactation duration and udder measurements in ewes and growth characteristics in lambs were examined with the Least Squares Method. Duncan's Multiple Comparison Test was used to compare more than two groups with significant differences. Survival rate in lambs was analyzed using Chi-Square Method (9).

Results

Table 1 presents the ewes fertility characteristics of which were assessed and Table 2 presents the least squares means for fertility characteristics. Mean least squares for LR, LS and LP in ewes were 95.8±0.60%, 1.04±0.01 and 100.0±0.80%, respectively. It was found that farm and age ($P < 0.05$) affected LR, farm ($P < 0.001$) and year ($P < 0.01$) affected LS and farm ($P < 0.001$) and age ($P < 0.05$) affected LP (Table 2).

Survival rates of lambs on the 90th and 180th days were found 95.2 and 94.1% (Table 3) and the impact of the examined factors were generally insignificant. Table 4 presents the means of the live weights of the lambs at different stages. Live weights at birth and the 90th and 180th days of lambs were found 3.35±0.02, 21.52±0.27 and 30.34±0.32 kg, respectively. The effects of the examined factors on the live weight of lambs at different periods were found significant at different levels ($P < 0.05$; $P < 0.01$; $P < 0.001$).

Table 1. Number of ewes investigated for the fertility traits by the farm and year subclasses

Farm	Age	2011-2012	2012-2013	2013-2014	2014-2015	General
Gülpınar	2	38	26	16	35	115
	3	46	34	27	16	123
	4	24	47	33	27	131
	5	22	21	43	32	118
	6≥	20	29	38	67	154
	Total	150	157	157	177	641
Ulaş	2	35	18	27	25	105
	3	35	36	18	27	116
	4	24	31	35	18	108
	5	22	23	30	35	110
	6≥	24	32	39	53	148
	Total	140	140	149	158	587
General	2	73	44	43	60	220
	3	81	70	45	43	239
	4	48	78	68	45	239
	5	44	44	73	67	228
	6≥	44	61	77	120	302
	Total	290	297	306	335	1228

Table 2. Numerical values and the least squares means (±SEM) for fertility traits

Items	NME	NLE	NSLE	NMLE	NLBA	LSM±SE		
						LR (%)	LP (%)	LS (x100)
Farm						*	***	***
Gülpınar	641	606	592	14	620	94.4±0.80	96.7±1.10	102.4±0.80
Ulaş	587	571	535	36	607	97.1±0.80	103.4±1.20	106.5±0.80
Age						*	*	-
2	220	202	197	5	207	91.9±1.30^{a}	94.4±1.90^{a}	102.6±1.40
3	239	232	224	8	240	97.3±1.30^{b}	100.3±1.80^{b}	103.1±1.30
4	239	228	215	13	241	95.5±1.30^{b}	100.9±1.80^{b}	105.6±1.30
5	228	221	206	15	236	96.8±1.30^{b}	103.9±1.90^{b}	107.3±1.30
6≥	302	294	285	9	303	97.2±1.20^{b}	100.6±1.70^{b}	103.6±1.20
Year						-	-	**
2011-2012	290	275	264	11	286	95.1±1.20	99.2±1.70	104.3±1.20^{ab}
2012-2013	297	283	260	23	306	95.2±1.20	103.1±1.70	108.3±1.20^{b}
2013-2014	306	296	288	8	304	96.5±1.10	98.9±1.60	102.4±1.20^{a}
2014-2015	335	323	315	8	331	96.3±1.10	98.9±1.60	102.6±1.10^{a}
Interactions								
FxA						-	-	-
FxY						-	-	-
AxY						-	-	-
General	1228	1177	1127	50	1227	95.8±0.60	100.0±0.80	104.4±0.60

F: Farm, A: Age, Y: Year, - P>0.05, * P<0.05, ** P<0.01, *** P<0.001

a, b Differences between the means with unlike letters in the same column are significant at P <0.05.

LSM: Least squares means, SE: Standard error, NME: Number of mating ewes, NLE: Number of lambing ewes, NSLE: Number of single lambing ewes, NMLE: Number of multiple lambing ewes, NLBA: Number of lambs born alive, LR: Lambing rate, LP: Lamb production, LS: Litter size

Table 3. Number of lambs born alive and survival rates at different periods

Items	**Number of lambs (n)**			**Survival rate (%)**	
	Live birth	**90th day**	**180th day**	**90th day**	**180th day**
Farm				*	*
Gülpınar	620	602	596	96.9	96.0
Ulaş	607	567	560	93.4	92.3
Dam Age				-	-
2	207	194	192	93.7	92.8
3	240	230	227	95.8	94.2
4	241	233	232	96.7	96.3
5	236	228	226	96.2	95.8
6≥	303	284	279	93.7	92.1
Birth Year				-	-
2012	286	271	268	94.8	93.4
2013	306	288	283	94.1	92.5
2014	304	294	293	96.4	96.4
2015	331	316	312	95.5	94.3
Gender				-	-
Female	603	572	565	94.7	93.7
Male	624	597	591	95.7	94.6
Birth Type				-	-
Single	1127	1077	1066	95.5	94.5
Twin	100	92	90	92.0	90.0
General	1227	1169	1156	95.2	94.1

- P>0.05, * P<0.05

Table 4. The least squares means (±SEM) for live weight at different ages (kg)

Items	n	Birth	n	90th day	n	180th day
Farm		**		*		**
Gülpınar	620	3.28±0.04	602	22.01±0.41	596	29.55±0.49
Ulaş	607	3.41±0.03	567	21.03±0.30	560	31.13±0.35
Dam Age		-		-		-
2	207	3.22±0.07	194	21.55±0.71	192	29.67±0.84
3	240	3.34±0.05	230	21.90±0.48	227	30.88±0.57
4	241	3.42±0.04	233	22.15±0.44	232	30.92±0.53
5	236	3.34±0.04	228	21.58±0.44	226	30.92±0.54
6≥	303	3.41±0.04	284	20.42±0.46	279	29.32±0.57
Birth Year		***		***		***
2012	286	3.18±0.04[a]	271	19.14±0.47[a]	268	28.08±0.57[a]
2013	306	3.41±0.03[b]	288	22.41±0.35[b]	283	30.00±0.43[b]
2014	304	3.48±0.05[b]	294	22.78±0.53[b]	293	30.88±0.63[b]
2015	331	3.32±0.05[c]	316	21.75±0.54[c]	312	32.40±0.63[c]
Gender		***		*		***
Female	603	3.23±0.03	572	21.16±0.33	565	29.53±0.39
Male	624	3.47±0.03	597	21.88±0.35	591	31.15±0.42
Birth Type		***		*		***
Single	1127	3.76±0.01	1077	22.10±0.11	1066	31.72±0.13
Twin	100	2.93±0.05	92	20.94±0.53	90	28.96±0.63
Interactions						
FxDA		***		-		-
FxBY		***		***		***
FxG		-		*		***
FxBT		-		-		-
DAxBY		**		-		-
DAxG		-		-		-
DAxBT		-		-		-
BYxG		**		-		-
BYxBT		***		-		-
GxBT		-		-		-
Regression				1.603±0.291***		1.564±0.356***
General	1227	3.35±0.02		21.52±0.27		30.34±0.32

F: Farm, DA: Dam Age, BY: Birth Year, G: Gender, BT: Birth Type, - P>0.05, * P<0.05, *** P<0.001

[a, b, c] Differences between the means with unlike letters in the same column are significant at P<0.05.

Regression: Partial regression of live weight on birth weight.

Live weight after shearing was 40.73±0.12 and 62.65±0.53 and greasy fleece weight were 2.04±0.01 and 3.48±0.06 kg in ewes and rams, respectively. The impact of the examined factors were found significant in general (P <0.05; P <0.01; P <0.001), (Table 5).

Table 6 presents the least squares means for LMY and LD, and Table 7 shows the least squares means for udder measurements. LMY and LD were determined to be 104.85±3.73 kg and 159.01±1.70 days, respectively. Udder circumference on the 45th day of lactation was found 44.20±0.37 cm and teat-floor distance 26.48±0.15 cm. It was identified that lactation number and farm affected LMY (P <0.01) and lactation number affected the LD (P <0.05). The udder measurement values except teat-floor distance was found to decrease when lactation progressed (Table 7).

Table 5. The least squares means (±SEM) for live weight after shearing and greasy fleece weight (kg)

Items	Live weight after shearing				Greasy fleece weight			
	n	Ewes	n	Rams	n	Ewes	n	Rams
Farm		***		***		***		*
Gülpınar	692	40.23±0.13	49	60.78±0.73	685	1.88±0.02	49	3.43±0.09
Ulaş	628	41.23±0.14	60	64.52±0.79	628	2.20±0.02	60	3.54±0.08
Age		***		***		***		***
1.5	164	35.54±0.28^{a}	48	42.59±0.95^{a}	164	1.91±0.03^{a}	48	2.86±0.10^{a}
2.5	206	40.50±0.23^{b}	26	63.29±0.99^{b}	206	2.03±0.03^{b}	26	3.46±0.11^{b}
3.5	229	42.60±0.22^{c}	18	69.72±1.15^{c}	228	2.12±0.03^{bc}	18	3.97±0.12^{c}
4.5	233	42.43±0.22^{c}	17	74.99±1.23^{d}	230	2.14±0.03^{c}	17	3.65±0.13^{b}
5.5	248	42.01±0.22^{c}			248	2.03±0.02^{b}		
6≥	240	41.29±0.24^{bc}			237	2.00±0.03^{b}		
Year		***		***		***		***
2012	329	39.59±0.19^{a}	18	57.96±1.15^{a}	329	1.99±0.02^{a}	18	3.23±0.12^{a}
2013	328	40.82±0.18^{b}	29	62.31±1.09^{b}	327	2.05±0.02^{ab}	29	3.24±0.12^{a}
2014	331	40.44±0.20^{b}	31	63.52±1.12^{b}	327	1.97±0.02^{a}	31	3.70±0.12^{b}
2015	332	42.07±0.20^{c}	31	66.81±0.92^{c}	330	2.15±0.03^{b}	31	3.76±0.10^{b}
Interactions								
FxA		-		***		***		***
FxY		***		***		***		-
AxY		**		-		***		-
General	1320	40.73±0.12	109	62.65±0.53	1313	2.04±0.01	109	3.48±0.06

F: Farm, A: Age, Y: Year, - P>0.05, * P<0.05, ** P<0.01, *** P<0.001
[a, b, c, d] Differences between the means with unlike letters in the same column are significant at P<0.05.

Table 6. Some descriptive values and the least squares means (±SEM) for lactation milk yield and lactation duration

Items	n	LMY (kg)	Min	Max	LD (day)	Min	Max
LN		**			*		
1	20	92.12±4.56^{a}	44	128	153.41±2.91^{a}	130	169
2	19	112.09±4.68^{b}	74	174	158.24±2.99^{ab}	136	187
3	20	110.33±4.56^{b}	85	141	165.38±2.91^{b}	143	202
Farm		**			-		
Gülpınar	29	97.50±3.79	44	128	156.21±2.42	130	202
Ulaş	39	112.19±3.73	74	174	161.81±2.38	136	187
Interaction							
LNxF		-			-		
General	59	104.85±3.73	44	174	159.01±1.70	130	202

LMY: Lactation milk yield, LD: Lactation duration, LN: Lactation number, F: Farm, Min: Minimum, Max: Maximum, - P>0.05, * P<0.05, ** P<0.01
[a, b] Differences between the means with unlike letters in the same column are significant at P<0.05.

Table 7. The least squares means (±SEM) for some udder measurements (cm)

Items	n	Days					
		45th day	135th day	45th day	135th day	45th day	135th day
		Udder width		**Udder depth**		**Udder circumference**	
LN		*	**	-	-	-	-
2	22	13.17±0.21^{a}	9.48±0.28^{a}	14.70±0.18	11.64±0.56	42.98±0.63	28.73±0.88
3	20	13.95±0.22^{b}	10.09±0.30^{ab}	15.25±0.18	11.35±0.60	44.78±0.65	30.34±0.95
4	22	13.92±0.21^{b}	10.74±0.27^{b}	14.94±0.18	12.37±0.56	44.84±0.62	31.14±0.88
Farm		**	-	-	-	***	-
Gülpınar	31	13.31±0.18	9.99±0.24	14.86±0.15	11.16±048	45.74±0.53	30.50±0.75
Ulaş	33	14.05±0.17	10.22±0.22	15.06±0.14	12.41±0.45	42.66±0.51	29.64±0.72
Interaction							
LNxF		**	-	-	-	*	-
General	64	13.68±0.12	10.10±0.16	14.96±0.10	11.79±0.33	44.20±0.37	30.07±0.52
		Length of right teat		**Length of left teat**		**Diameter of right teat**	
LN		**	*	*	-	*	*
2		2.93±0.12^{a}	2.25±0.07^{a}	3.00±0.13^{a}	2.34±0.07	1.64±0.05^{a}	1.31±0.03^{a}
3		3.41±0.13^{b}	2.46±0.07^{ab}	3.38±0.13^{ab}	2.48±0.07	1.87±0.05^{b}	1.43±0.04^{b}
4		3.53±0.12^{b}	2.54±0.07^{b}	3.47±0.12^{b}	2.50±0.06	1.80±0.05^{b}	1.42±0.03^{b}
Farm		-	-	-	-	***	-
Gülpınar		3.27±0.10	2.36±0.06	3.24±0.11	2.41±0.06	1.88±0.04	1.40±0.03
Ulaş		3.31±0.10	2.47±0.06	3.32±0.10	2.47±0.05	1.63±0.04	1.38±0.03
Interaction							
LNxF		-	*	-	-	-	**
General		3.29±0.07	2.42±0.04	3.28±0.073	2.44±0.04	1.76±0.03	1.39±0.02
		Diameter of left teats		**Distance between teats**		**Teat-floor distance**	
LN		-	**	-	-	-	-
2		1.61±0.05	1.27±0.03^{a}	16.83±0.38	13.22±0.23	26.64±0.26	29.94±0.46
3		1.71±0.05	1.41±0.03^{b}	17.71±0.40	13.29±0.25	26.45±0.27	29.66±0.49
4		1.72±0.05	1.42±0.03^{b}	17.75±0.38	13.76±0.23	26.34±0.26	28.77±0.45
Farm		***	-	-	-	-	*
Gülpınar		1.80±0.04	1.40±0.03	17.29±0.32	13.27±0.20	26.53±0.22	30.04±0.39
Ulaş		1.56±0.04	1.34±0.02	17.56±0.31	13.58±0.19	26.43±0.21	28.87±0.37
Interaction							
LNxF		-	*	-	-	-	-
General		1.68±0.03	1.37±0.02	17.43±0.22	13.42±0.14	26.48±0.15	29.46±0.27

LN: Lactation number, F: Farm, - P>0.05, * P<0.05, ** P<0.01, *** P<0.001

$^{a, b}$ Differences between the means with unlike letters in the same column are significant at P<0.05.

Discussion and Conclusion

Lambing rate (LR) (95.8%) obtained in this study is a rather high value for native sheep breeds. As a matter of fact, LR values were found higher than the values reported for the same breed (85.0 - 91.4%) (13, 15, 23) and some of the other fat tailed breeds (Akkaraman, Morkaraman) (67.6 - 90.5%) (3, 12). The high lambing rate in both farms indicates that environmental conditions were taken into consideration during the mating period. Fertility characteristics were considered to be the lowest in two years old ewes. Litter size (LS) obtained from Karakul ewes (1.04) was identified similar to the values reported for the same breed (1.00 - 1.18) (11, 13, 15, 23). The Karakul breed has of low value in terms of LS and is similar to the Dağlıç (1.05) (10) and Karayaka (1.03 - 1.08) (5, 20) in this respect. The low LS in Karakul herds is due to the low rate of twin birth; this shows that although the breed has a high LR, it is not a prolific breed.

There is only one study in the literature on the survival rate (SR) of Karakul lambs in Turkey (13). In the present study, SR values at 90th and 180th days (95.2 and 94.1%) were found similar to those identified for the same herds from 2006 to 2008, reported as 96.0 and 90.0%, in general (13). SR values obtained from lambs on the 90th and 180th days can be regarded as optimal and this is important for the sustainability of the herds.

The findings of the birth weight for female lambs (3.23 kg) and male lambs (3.47 kg) and the average live weight on the 180th days (30.34 kg) in this study were similar (3.24 and 3.47 kg) (11) or higher (3.03 and 3.23 kg) (13) than the those of values for birth weight of female and male lambs and live weight on the 180th day (24.62 kg) (13) of the same breed. When the live weights at birth, 90th and 180th days of the breed are evaluated together, it could be said that Karakul breed is similar to medium size breeds (Bafra, Dağlıç, Karayaka), but lower than the large size breeds (Akkaraman, Chios) in terms of the growth (1, 4, 5, 12, 19, 21).

The means obtained for the ewes live weight after shearing (40.73 kg) and greasy fleece weight (2.04 kg) were consistent with the those of results reported for the same breed (36.81 - 42.95 kg and 1.84 - 2.84 kg) (11, 13). In addition, live weight after shearing value was similar to the lower limit of range (42.70 - 62.60 kg) reported for some native breeds (5, 19).

LMY (104.85 kg) obtained in the current study was found higher than the means reported for the same breed (61.5 and 60.0 kg) (16, 22). The herd in Ulaş village is regularly milked each year; however, the herd in Gülpınar village was milked for the first time in the framework of the study. The fact that Ulaş herd gave 14.69 kg more milk than Gülpınar herd might be due to accustomed to hand milking. LMY and LD differed from the lactation number groups, while the first lactation ewes had numerically the lowest values than those of the other groups. This is in the line with the general understanding that milk production of ewes generally increases by lactation numbers. In this research, high lactation milk yield of the ewes shows that breeders can utility from Karakul breed in terms of milk yield. LD determined in this study (159.01 days) was found in the range of values reported for the same breed (137.9 - 168.0 days) (2, 16, 22). On the other hand, LD was similar in both herds. There was a wide variation in LMY and LD in both herds. This shows that it is possible to make improvement in the direction of milk yield in Karakul breed.

The longer the lactation duration was in Karakul sheep, the more decreasing was observed in udder measurement values other than the teat-floor distance. This is related to decreased milk yield due to progression of lactation. As a matter of fact, similar situation has been reported for various native breeds (3, 18). In general, udder measurement values obtained for Karakul ewes in this study were higher than those found for Tushin and Morkaraman ewes (14). Udder measurement values obtained for Bafra sheep (18) on the 42nd day of lactation were similar to or higher than the values found in the current study other than the values for right and left teat length and the distance between teats.

In conclusion, the performance traits of Karakul sheep were similar to or better than the those of results reported before for the same breed, and the breed was alike to medium size native breeds in terms of growth and mature live weight.

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Conflict of Interest

The authors declared that there is no conflict of interest.

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Determination of 8-hydroxy-2'deoxyguanosine, malondialdehyde levels and antioxidant enzyme activities in Kangal dogs with venereal tumour

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Abstract: Canine transmissible venereal tumour (TVT) is a contagious cancer and sexually transmitted one dog to another by allogenic transfer of living cancer cells. The aim of this study is to examine the relationship between antioxidative metabolism and venereal tumour at Kangal Dogs and to show the size of oxidative damage it causes through 8-hydroxy-2'deoxyguanosine (8-OHdG). The materials of this study included the blood sera of 15 Kangal Dogs with TVT, and as a control group of 15 healthy Kangal Dogs. The sera levels of 8-OHdG were determined by competitive enzyme linked immunosorbent assay (ELISA) method. The level of malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) which are antioxidative enzymes were investigated by spectrophotometric methods as described. Serum 8-OHdG and MDA levels of dogs with TVT were found to be significantly increased compared to the control group (P<0.001). The levels of GPx were statistically different (P<0.05). In the light of the findings, which are obtained in this study, the transmissible venereal tumour, which is an infectious and treatable type of cancer, can lead to new studies that may be caused by oxidant-antioxidant metabolism.
Keywords: Antioxidant enzyme, Kangal dog, transmissible venereal tumour, 8-OHdG.

Venereal tümörlü Kangal köpeklerinde 8-hidroksi-2'deoksiguanozin, malondialdehit düzeyleri ile antioksidan enzim aktivitelerinin belirlenmesi

Özet: Transmissible (bulaşıcı) venereal tümör (TVT), canlı kanser hücrelerinin allojenik transferiyle bir köpekten diğer bir köpeğe çiftleşme ile bulaşan bir kanser türüdür. Bu çalışmada, Kangal köpeklerinde önemli bir bulaşıcı kanser türü olan venereal tümör ile antioksidatif metabolizmanın arasındaki ilişkinin incelenmesi ve oksidatif hasarın boyutunun 8-hidroksi-2'deoksiguanozin (8-OHdG) düzeyleri üzerinden gösterilmesi amaçlandı. Bu amaçla, 15 adet TVT'li, kontrol grubu olarak 15 adet sağlıklı Kangal köpeklerinden alınan toplam 30 adet kan serumu çalışmanın materyalini oluşturdu. Bu hayvanlara ait kan serumlarında 8-OHdG kompetitif enzim bağımlı immunosorbent analiz (ELISA) yöntemi ile belirlendi. Malondialdehit (MDA), antioksidan enzimler olan süperoksit dismutaz (SOD), glutatyon peroksidaz (GPx) ve katalaz (CAT) seviyeleri ise spektrofotometrik yöntemlerle analiz edildi. TVT teşhisli köpeklerin serum 8-OHdG ve MDA düzeyleri kontrol grubundaki köpeklere göre istatiksel olarak anlamlı derecede artış gösterdiği tespit edildi (P<0,001). Antioksidan enzim olan GPx düzeylerinde istatiksel farklılık tespit edilmiştir (P<0,05). Bu çalışmada elde edilen sonuçlar neticesinde bulaşıcı ve tedavi edilebilir bir kanser türü olma niteliğindeki köpek bulaşıcı venereal tümörün oksidan antioksidan metabolizma kaynaklı yapılacak yeni çalışmalara yön gösterebilecek niteliktedir.
Anahtar sözcükler: Antioksidan enzim, bulaşıcı venereal tümör, Kangal köpeği, 8-OHdG.

Introduction

Transmissible venereal tumour (TVT) is a contagious tumour that affects female and male dogs by mating. The tumour is transmitted effectively by contact of mucosa. The surface membranes enable to the transmission because of disruption of mucosal integrity, abrasions or ruptures. In addition to genital contact, extragenital TVT cases can also be seen such as skin, face, nasal cavity, oral cavity, rectum and perineum are transmitted by social behavior (eg, sniffing and licking). The metastasis rate is 5% and usually occurs in regional lymph nodes (3, 18, 27).

The fact that contagious venereal tumour is a cancer type which can be transmitted easily from one dog to

another by the allogenic transfer of live cancer cells that increase the importance of this disease (22). While deaths can usually be seen in cancer patients, it should not be ignored that in many of the TVT cases can gain of life-time immunity and this may contribute to cancer studies by revealing the mechanism (29).

The increase in the cancer incidence can be due to the increased DNA damage or reduced repair mechanism. The oxidative damage occurs because of altered DNA's. The DNA damage products are resulting in endogenously formed by oxygen radicals. These products are known to cause degenerative diseases such as aging, cancer and heart disease (7). Many studies that aim to research the markers for early diagnosis of a disease with poor prognosis such as cancer have focused on free radicals and oxidative stress especially in recent years (8). Endogenic formation of reactive oxygen species (ROS) leads to oxidation of DNA. Beside the factors that cause cancer, the cancer formation mechanism includes the DNA damage formed by ROS (17). 8-hydroxy-2'-deoxyguanosine (8-OHdG) which is one of the DNA damage products of reactive oxygen species, is a commonly used marker in determining the extent of the DNA damage (31). Although there are over more than 20 oxidative damage products, due to its potential for susceptibility and mutagenicity are focused on most and have been the subject of many studies that 8-hydroxyguanine or its deoxynucleotide 8-OHdG (6, 14, 16, 20, 28, 29).

Oxygen radicals, usually products of metabolism *in vivo* are also formed by phagocyte cells and lipid peroxidation (7). The measurement markers of lipid peroxidation are pentane, malondialdehyde (MDA), lipid hydroperoxides and isoprost (26). MDA which is a lipid peroxidation product is known as an important and most commonly used marker in evaluating oxidative stress (25). The free radicals that form the oxidative damage are defused by catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and nonenzymatic antioxidants which are enzymes of defense system (26).

TVT is an important cancer type of cancer among cancer types because of a kind of cancer that is transmitted by mating of dogs from one to another by means of allogenic transfer of living cancer cells and also it shows fast endemic spread and resulting in restraints dogs for breeding. At the same time, it can that by enlightening the mechanism of a cancer with this etiology can contribute to studies conveyed on other types of cancer. Therefore, this study aims to examine the relationship between venereal tumour cases that are clinically significant and known as contagious cancer and antioxidative metabolism on Kangal Dogs peculiar to Sivas province which are grown in public farms and raised by people and to show the extent of oxidative damage via 8-OHdG.

Material and Methods

This study was approved by the Sivas Cumhuriyet University Animal Research Ethics Committee dated 23.02.2016 and numbered 23. The materials of this study included the blood sera of 15 dogs with TVT which are cytological (Figure 1) and clinically examined, and of 15 healthy dogs, all in Kangal breeding farm and people in Sivas.

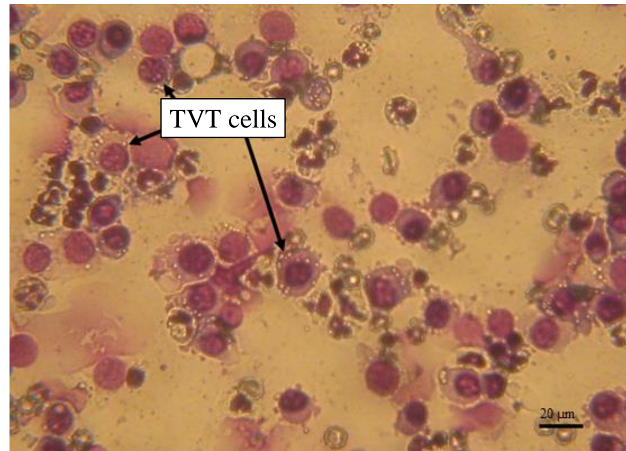

Figure 1. TVT cell via cytological.
Giemsa staining technique microscopy magnification 400x, scale bar: 20µm.

Blood for biochemical analysis was taken from each dog by *vena cephalica antebrachii* into biochemistry tubes, brought to the Sivas Cumhuriyet University Faculty of Veterinary Medicine Department of Biochemistry lab and centrifuged at 4,000 rpm for 10 min. Sera were stored at -20 °C until the day of analysis.

The serum levels of 8-OHdG were determined by competitive ELISA (enzyme linked immunosorbent assay) method. Analyzes were performed with the ELISA device (Thermo Scientific, Multiskan Go, USA), according to the kit procedures using SUNRED Canine 8-OHdG (Cat. No. DZE201151217) assay.

According to test procedure, added to microplate wells from 50 µl each standard (4, 8, 16, 32, 64 ng/ml) and 50 µl streptavidin-HRP included in the kit. And 40 µl of each sample was transferred to micro wells and 10 µl of 8-OHdG antibody and 50 µl of streptavidin-HRP were added. The microplate was covered and incubated for 1 hour at 37°C in a dark environment. At the end of the incubation, the wells were washed 3 times with wash buffer. After washing, were added 50 µl of chromogen solution A and 50 µl of chromogen solution B to each well and incubated for 10 min. At the end of the incubation, 50 µl of stop solution was added to each well to change the blue color to yellow. Finally, the absorbance of the standard and samples was measured on an ELISA device at a wavelength of 450 nm. Calibration curve was calculated for the absorbance values obtained and the 8OHdG levels were calculated as ng/ml against the absorbance of the samples.

MDA measurement was performed according to the Janero (11) method. The principle of this method is based on measuring the absorbance of the color produced by MDA with thiobarbituric acid (TBA) in acidic medium. For this purpose, 50 µl of sample was taken, 250 µl of TCA and 100 µl of TBA solutions were added and incubated at 95°C for 30 minutes. After cooling the samples, the absorbance at 535 nm was measured spectrophotometrically in a microtiter plate reader (Thermo Scientific, Multiskan Go, USA). Standards were prepared using 1,1,3,3, tetramethoxypropane, formed by hydrolysis of this compound. The standard graph was drawn using these results and MDA results were calculated with this graph.

Determination of SOD activity according to Sun et al. (23) and Durak et al. (5) which the superoxide produced by the xanthine / xanthine oxidase system is based on reduction of nitro blue tetrazolium (NBT). The resulting superoxide radicals (O_2) reduce NBT and form a colored formazan. This complex gives maximum absorbance at 560 nm. When SOD is present in the medium, there is no NBT reduction and does not turn blue-violet and a light color is formed depending on the amount and activity of the enzyme. Determined in a microtiter plate reader (Thermo Scientific, Multiskan Go, USA).

Determination of GPx activity according to Paglia and Valentine (19) was determined by kinetic, spectrophotometric analysis in which the incubation mixture containing phosphate buffer solution (PBS), nicotinamide adenine dinucleotide phosphate (NADPH), glutathione disulfide (GSSG), reduced glutathione (GSH), Na-azide and ethylenediaminetetraacetic acid (EDTA) was pre-incubated for 10 minutes at 37°C. The addition of hydrogen peroxidase and the decrease in absorbance at 340 nm were determined kinetically in a microtiter plate reader (Thermo Scientific, Multiskan Go, USA).

Catalase activity was calculated using the method of Aebi (1). 50 mM phosphate buffer was prepared with pH 7.0; further, 30 mM hydrogen peroxide solution was prepared. 1 mL of H_2O_2 was added to a 2 mL sample, 1 mL of phosphate buffer was added to the resulting solution and measurements were made at 230 nm in a microtiter plate reader (Thermo Scientific, Multiskan Go, USA).

The data were compared in SPSS 22.00 statistical analysis program (21). The normality of data distributions was investigated by Kolmogorov Smirnov test. Student *t*-test was used for parametric variables for the comparison of the studied parameters whether there is a statistical difference or not. Differences were considered with significant at P <0.05.

Results

The serum levels of 8-OHdG, MDA, SOD, GPx and CAT the dogs with TVT and control group shown in Table 1. According to Table 1, it was found that serum levels of 8-OHdG dogs (55.46±5.02 ng/ml) with TVT diagnosis were significantly increased compared to the control group (8.78±0.70 ng/ml) dogs (P <0.001).

The levels of MDA were found to be statistically significant increase in dogs (3.32±0.29 nmol/ml) with TVT diagnosis compared to control dogs (1.12±0.14 nmol/ml) as shown in Table 1 (P <0.001).

The levels of GPx were statistically significant between TVT (0.565±0.122 U/ml) and control groups (1.512±0.362 U/ml) (P <0.05). The levels SOD did not show any statistical difference between TVT (66.2±2.31 U/L) and control groups (71.09±1.0 U/L) (P >0.05). The levels CAT did not show any statistical difference between TVT (43.43±4.13U/ml) and control groups (48.93±8.79U/ml) (P >0.05).

Discussion and Conclusion

Antioxidant defense system is in a condition of equilibrium with free radicals formed in various ways in the organism. However, in cases where free radicals cannot be cleaned adequately from the organism it damages to the DNA and damage products are formed. These damage products can lead to various metabolic diseases, such as aging, cancer and even modifications in DNA structure. The most well-known damage product is 8-OHdG which is mutagenic (9).

Table 1. Serum 8-hydroxy-2'-deoxyguanosine (8-OHdG), malondialdeyde (MDA), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) levels of transmissible venereal tumour (TVT) and control groups.

Parameters	**TVT group** (Mean±SE)	**Control group** (Mean±SE)	**P**
8-OHdG (ng/ml)	55.46±5.02	8.78±0.70	0.001
MDA (nmol/ml)	3.32±0.29	1.12±0.14	0.001
SOD (U/L)	66.2±2.31	71.09±1.0	0.065
CAT (U/ml)	43.43±4.13	48.93±8.79	0.576
GPx (U/ml)	0.565±0.122	1.512±0.362	0.020

Data are presented as (Mean±SE). SE: Standard error. Statistical comparisons were performed using Student *t*-test. Differences were considered statistically significant at P < 0.05.

The increase of reactive species can raise the development of malignancy and can be responsible for the rise in growth of cancer risk. The ineffectiveness of various antioxidant defense system enzymes can further increase the levels of oxidative damage and increase the cancer development in animals. Particular attention should be paid on DNA oxidative damage formed by a reactive kind such as hydroxyl radical (9). In many studies in the field of humanities and veterinary medicine increased oxidative damage in cancer patients has been associated with increased cancer risk (6, 10, 14, 16, 20, 28, 29, 30).

In a study which determines the malondialdehyde, antioxidant activity and adenosine levels in serum of dogs with TVT, serum MDA levels were found significantly higher in dogs with TVT than in healthy dogs but antioxidant activities were lower. MDA levels were found as 2.347nmol/mL in dogs with TVT and 1.512nmol/mL in healthy dogs (2). In this study, MDA levels at TVT diagnosed dogs were found to be 3.32nmol/mL and 1.12nmol/mL at healthy dogs. The results of Aydın et al. (2) are similar to the results of this study with increased MDA levels in TVT patients.

It is claimed that oxidant-antioxidant balance is an important factor in cancer formation and progression. An 8-OHdG level that is product of DNA oxidation and SOD, GPx and glutathione S transferase (GST) activities were measured as antioxidant activity in patients with bladder cancer. By means of serum 8-OHdG level and GPx activities, there were not any significant differences between the patient and control group. However, SOD activity were significantly lower and GST activity was significantly higher in the patient group (4).

In a study conducted by Macotpet et al. (15), malondialdehyde levels were evaluated in blood samples from dogs having cancer (n=80) and clinically healthy dogs (n=101). Serum MDA was significantly higher in dogs with cancer than in clinically healthy dogs.

In another study, two control and patient groups were formed according to healthy female dogs (n=13) and female dogs (n=16) diagnosed with pyoderma and healthy male dogs (n=14) and male dogs (n=12) diagnosed with. MDA were measured in plasma and 8-hydroxy-2’deoxyguanosine levels were measured in urine. MDA and 8-OHdG levels were found to be statistically significant in male and female dogs diagnosed with pyoderma (6).

The results of high MDA and 8-OHdG levels found in TVT diagnosed dogs obtained from this study are similar to the results of Macotpet et al. (15) and Ercan and Fidancı (6). It leads to the conclusion that oxidative stress can be associated with cancer types in dogs.

Antioxidant levels were researched at multicentric lymphoma, oral fibrosarcoma, mast cell tumours, malignant melanoma, appendicular ostosarcoma, nasal tumours and peripheral ameloblastoma at dogs with cancer. Total antioxidant capacity (TAC), antioxidant enzymes which are GPx, CAT and SOD levels were measured. As a result, except for patients with nasal tumour, increased levels of SOD in patients with malignant melanoma, mast cell tumour, multicentric lymphoma and oral fibrosarcoma, increased levels of GPx and total antioxidant capacity in osteosarcoma and multicentric lymphoma patients, increased in levels of CAT and SOD at 42 dogs with cancer (24). In this study, it was observed that there was no increase at the levels of SOD, CAT and GPx in dogs with TVT but the levels were lower compared to the control group. The decrease, which is not reflected to the statistical difference, composes the result of increasing oxidative damage.

In a study in which lipid peroxidation and antioxidant enzyme levels were compared between dogs diagnosed with breast tumour and healthy dogs. The levels of lipid peroxidation have shown a significant increase in dogs diagnosed with breast tumour. It was determined that glutathione reductase levels showed significant increase in breast tumour patients when the antioxidant levels are evaluated (12).

Kumaraguruparan et al., (13) have observed in their study about breast tumour diagnosed dogs that increased antioxidant levels have an effect of reducing the peroxidation levels. SOD, CAT and GPx levels in dogs with tumours were found to be doubled compared to the normal tissues.

This study, in means of antioxidant enzyme levels, does not resemble to the results of studies of Jayasri et al., (12), Kumaraguruparan et al., (13). This study indicates that increased oxidative damage in TVT patients leads to a decrease in antioxidant enzyme.

In conclusion, the levels of 8-OHdG and MDA were found high in dogs with transmissible venereal tumours. Although a statistical difference was detected only at GPx levels, the significant decrease in antioxidant enzymes of SOD, GPx and CAT levels in dogs with TVT was remarkable. Increased oxidative damage and lipid peroxidation levels affect the changes in formation of antioxidant enzyme levels. In the light of the findings, which are obtained in this study, the transmissible venereal tumour, which is a contagious and treatable type of cancer, can lead to new studies that may be caused by oxidant-antioxidant metabolism. It can be concluded that the use of additional oxidative stress markers and antioxidant parameters may be useful in order to reveal the unknown activation mechanisms with more patients and more studies although activation of oxidant-antioxidant defense is observed in dogs with TVT.

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Conflict of Interest

The authors declared that there is no conflict of interest.

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Determination of serum amyloid A, haptoglobin and hepcidin levels in calves with endemic viral pneumonia

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Abstract: The aim of this study was to investigate serum amyloid A, haptoglobin and hepcidin levels in respiratory infections caused by various viral agents in calves. Pneumonia is an infectious or non-infectious disease affecting the lungs of all animals, and during the inflammation, blood levels of acute phase proteins increase rapidly. Hepcidin is a hormone that has many functions and is known as type II acute phase reactant. The blood samples were collected from a total of 20 calves to form the study group. The control group consisted of 10 healthy animals. Sera were analyzed with ELISA for antigen detection. According to the results, Bovine herpesvirus type-1 (BHV-1) was positive in 3 calves, Bovine viral diarrhea virus (BVDV) was positive in 3 calves, Bovine respiratory syncytial virus (BRSV) was positive in 1 calf, and Bovine parainfluenza virus type 3 (BPI3) was positive in 1 calf. Adenovirus infection was detected in 5 animals together with other viruses. Mixed infections were observed in 12 calves. Haptoglobin, serum amyloid A and hepcidin values were measured in sera. According to the results, statistically significant differences were observed between the two groups for the haptoglobin ($P<0.01$), serum amyloid A ($P<0.05$) and hepcidin ($P<0.05$). It was concluded that these parameters can be used to support the diagnosis of respiratory infections of the calves.
Keywords: Calf, haptoglobin, hepcidin, serum amyloid A, viral pneumonia.

Yaygın viral pnömonili buzağılarda serum amiloid A, haptoglobin ve hepsidin düzeylerinin belirlenmesi

Özet: Bu çalışmanın amacı, buzağılarda çeşitli viral etkenlerinin neden olduğu solunum yolu enfeksiyonlarında serum amiloid A, haptoglobin ve hepcidin düzeylerini araştırmaktır. Pnömoni, tüm hayvanların solunum sistemlerini etkileyen, enfektif veya non-enfektif sebeplere bağlı olarak gelişen bir hastalık olup, yangı sırasında akut faz proteinlerinin kandaki değerleri hızla artmaktadır. Hepsidin; birçok fonksiyona sahip olan bir hormon olup aynı zamanda tip II akut faz reaktant olduğu bilinmektedir. Araştırmanın çalışma grubunu oluşturmak için, toplam 20 buzağıdan kan örnekleri alındı. Kontrol grubu ise, sağlıklı 10 hayvandan oluşturuldu. Kan serumları, antijen tespiti için ELISA testi ile tarandı. Elde edilen sonuçlara göre 3 hastada Bovine herpes virüs tip 1 (BHV-1), 3 hastada Bovine viral diarrhea virus (BVDV), 1 hastada Bovine respiratory syncytial virüs (BRSV) ve 1 hastada Bovine parainfluenza virüs tip 3 (BPI3) pozitifti. Adenovirüs enfeksiyonu 5 hayvanda diğer virüslerle birlikte tespit edildi. Oniki hastada miks enfeksiyon saptandı. Haptoglobin, serum amiolid A ve hepsidin değerleri toplanan serum örneklerinde ölçüldü. Sonuç olarak; iki grup arasında, haptoglobin ($P<0,01$), serum amiloid A ($P<0,05$) ve hepsidin ($P<0,05$) farkları istatistiksel açıdan önemli bulunarak, buzağıların solunum sistemi enfeksiyonlarının teşhisini desteklemede, belirtilen parametrelerin kullanabileceği saptandı.
Anahtar sözcükler: Buzağı, haptoglobin, hepsidin, serum amiloid A, viral pnömoni.

Introduction

Pneumonia is an infectious and non-infectious originated disease affecting lungs of all animals. There are many factors that lead the disease. It is a disease which causes serious financial losses especially in cattle farms. Pneumonia is the inflammation of the lung parenchyma. It is usually associated with inflammation of bronchioles and pleuritis. Clinically; it is characterized by fever, nasal discharge, increased respiratory rate, changes in depth and characteristics of breaths, cough, abnormal breathing sounds in auscultation, lacrimation, depression and partial anorexia (2, 3). In dairy calf pneumonia; in acute outbreaks, many animals have been reported to be depressed and have a high fever. In some calves; there are visible signs in the respiratory system (22).

In the acute phase of the inflammation, proteins that show significant changes in blood levels are called acute phase protein (APP). Acute phase proteins are used to

assess the response of the body's immune system to inflammation or trauma (15, 19). Their secretion is regulated by the proinflamatory cytokines, in particular interleukin 6 (IL-6) (15). In addition to diarrhea, respiratory diseases are one of the major health problems affecting young dairy calves. In order to prevent outbreaks of disease, early detection, isolation and treatment of sick animals are important. The results obtained in several different studies have shown that APPs are useful for the detection and monitoring of respiratory diseases. In calves with respiratory tract diseases, the following APPs were determined: haptoglobib (Hp), fibrinogen (Fb), serum amyloid A (SAA), transferrin (Tf), lipopolysaccharide binding protein (LBP), α1-acid glycoprotein (AGP), α1-antitrypsin (α1-AT), seromucoid (Sm), ceruloplasmin (Cp), albumin and α2-macroglobulin (21).

Hepcidin is a hormone that has multiple functions and a peptide structure (14). Hepcidin has been studied in human and many animal species (mice, rats, pigs, fish, dogs) to date. In a study on healthy canine tissues, hepcidin was found to be secreted mostly in the liver and less in the lungs and kidneys but not in other tissues (9). Hepatic hepcidin production is under the influence of many stimulants such as low levels of iron and erythropoietic activation. Also some cytokines, especially IL-6, increase the level of hepcidin (13). Hepcidin also assists host defense due to its direct antimicrobial properties (8, 23). Inflammation states stimulate hepcidin production and increase its release, leading to a decrease in iron secretion from macrophages and a decrease in plasma iron levels (5, 16).

The aim of this study was to investigate serum amyloid A, haptoglobin and hepcidin levels in respiratory infections caused by various viral agents in calves.

Material and Methods

This research was carried out on the basis of the permission of Mehmet Akif Ersoy University Local Animal Ethics Committee dated 05.10.2016 and numbered 214. The research material consisted of Holstein breed calves from the farms in Burdur Province that were brought to Veterinary Hospital of Mehmet Akif Ersoy University. The owners of the animals in the study were informed about the applications. The blood samples were collected from the animals of the study group with high respiratory rate, wheezy respiratory, cough and seroprulant nasal discharge.

Blood samples were collected from a total of 20 calves according to the criteria mentioned above. These samples were gathered out of Holstein calves with 8 females and 12 males (aged 2 - 6 months). The control group consisted of 10 healthy calves (aged 2-6 months) from another farm. Complete blood counts of the calves were performed in both groups (Diatron Abacus Junior Vet Hematology Analyzer, S / N 130702 model).

The blood samples were coagulated, and their sera were separated in the cooled centrifuge at 4000 rpm for 5 min. The resulting serum samples were transferred to Eppendorf tubes (1.5 ml) evenly using micropipette. The tubes were recorded with their sample numbers, and stored at -20°C until used.

Twenty blood serum collected from suspected animals with clinical signs of pneumonia were analyzed with ELISA test for the detection of causative agents antigens (Bio-X Diagnostics S.A ELISA kit for serodiagnosis of Bovine herpesvirus type-1 (BHV-1)), Bovine viral diarrhea virus (BVDV), Bovine respiratory syncytial virus (BRSV), Bovine parainfluenza virus type 3 (BPI3) and Adenovirus 3. Sera were diluted 1/100 before inoculation. The samples were analyzed with ELISA according to the manufacture’s recommendations. In the ELISA reader, the percentages of the agents in the blood were determined numerically. The results were evaluated according to the evaluation of the agent positivity given in the kit procedure. Bovine specific haptoglobin, serum amyloid A and hepcidin ELISA (Enzyme-Linked Immunosorbent Assay) kits (MyBioSource, San Diego (USA)) were used for the measurement of their levels in sera of study and control group animals. In order to increase the reliability of the study, serum samples were run in duplicate.

Statistical analysis: The obtained data were evaluated by the 2-Sample t test method between the study and control groups. Data were given as mean and standard error. P value of <0.05 was considered significant.

Results

Agent positivity evaluation intervals are shown in Table 1. According to the results, BHV-1 was positive in 3 calves, BVDV was positive in 3 calves, BRSV was positive in 1, and BPI3 was positive in 1 calf. Mixed infections were determined in 12 calves (Table 2).

The common symptoms of calves were cough, wheezing, seroprulent respiratory nasal discharge and depression. The clinical findings of calves in study and control groups are given in Table 3. While there was no difference in the body temperature between the two groups, the number of respiratory (P<0.01) and pulse rate (P<0.05) of the calves in study group were found to be higher than in those in control group. When the hematological parameters of the control and study groups were evaluated, the difference between the values of leukocyte (P<0.01), monocyte (P<0.01), neutrophils (P<0.01), and hematocrit (P<0.05) were found to be significant. No significant difference was found between lymphocyte, erythrocyte, hemoglobin and platelets values (P>0.05) (Table 4).

Table 1. Agent positivity evaluation intervals.

	0	+	++	+++	++++	+++++
BHV-1	V≤30	<V≤67	<V≤104	<V≤141	<V≤178	178<
BVDV	V≤20	<V≤40	<V≤60	<V≤80	<V≤100	100<
BRSV	V≤20	<V≤40	<V≤60	<V≤80	<V≤100	100<
BPI3	V≤20	<V≤40	<V≤60	<V≤80	<V≤100	100<
Adeno 3	V≤20	<V≤33	<V≤56	<V≤79	<V≤102	102<

V: Value, +: Strength of positivity.

BHV-1: Bovine herpesvirus type 1; BVDV: Bovine viral diarrhea virus; BRSV: Bovine respiratory syncytial virus; BPI3: Bovine parainfluenza virus type 3; Adeno 3: Adebovirus type-3.

Table 2. Percentages of pneumonia agents in calves of study group.

Calves (No)	**BHV-1 (% agent)**	**BVDV (% agent)**	**BRSV (% agent)**	**BPI3 (% agent)**	**Adeno3 (% agent)**
1	17.2	22.9	36.77^{+}	65.62^{+++}	15.34
2	30.5	36.3	59.16^{++}	10.61	15.6
3	119.0	170.2^{+++++}	101.1^{+++++}	108.7^{+++++}	81.6^{++++}
4	20.7	16.4	58.38++	55.3++	52.1++
5	96.7^{++}	9.09	16.44	15.3	10.5
6	22.9	161.3^{+++++}	70.94^{+++}	69.1^{+++}	16.3
7	15.6	11.7	9.5	31.97^{+}	13.9
8	17.6	32.88^{+}	23.05^{+}	19.07	15.9
9	100.7^{++}	13.54	15.11	10.6	138.3^{+++++}
10	31.0	84.319^{++++}	85.72^{++++}	103.3^{+++++}	56.7^{++}
11	32.0	156.25^{+++++}	98.61^{++++}	110.6^{+++++}	108^{+++++}
12	26.3	86.45^{++++}	21.22	14.5	10.9
13	18.8	167.6^{+++++}	67.27^{+++}	14.5	18.9
14	36.9^{+}	7.74	18.8	20.1	7.4
15	82.1^{++}	18.8	17.27	17.3	11.9
16	129.6^{++++}	168.3^{+++++}	68.61^{+++}	80.7^{+++}	15.8
17	20.6	45.6^{++}	17.2	18.87	12.9
18	11.1	58.08^{++}	13.38	20.16	9.22
19	15.5	30.75^{++}	25.33^{+}	33.8++	19.5
20	19.7	85.11^{++++}	54.83^{++}	77.7^{+++}	13.7

+ : Strength of positivity.

Table 3. Comparison of clinical findings of calves in control and study groups.

Parameters	**Study group (n= 20) Mean±SD**	**Control group (n= 10) Mean±SD**	**P**
Respiratory rate/ min	72.8±4.6	34.8±1.6	<0.001
Pulse rate/ min	101.9±4.4	84.1±3.3	0.005
Body temperature/ °C	38.49±0.21	38.64±0.10	0.504

Table 4. Mean values of some hematological parameters of calves in control and study groups.

Parameters	**Study group (n=20)**	**Control group (n=10)**	**P**
WBC (10^9/l)	18.42±2.07	9.185±0.691	<0.001
LYM (10^9/l)	6.22±0.70	6.60±0.56	0.674
MON (10^9/l)	0.963±0.2	0.092±0.011	<0.001
NEU(10^9/L)	11.24±1.6	2.73±0.39	<0.001
RBC (10^12/l)	8.38±0.5	9.17±0.32	0.192
HGB(g/dl)	8.35±0.43	8.77±0.39	0.480
HCT (%)	26.17±1.7	30.0±1.2	0.036
MCV(fl)	31.75±1.2	33.60±1.0	0.250
PLT (10^9/l)	923±393	707±51	0.593

WBC: Leukocyte, LYM: Lymphocyte, MON: Monocytes, NEU: Neutrophil, RBC: Erythrocyte, HGB: Hemoglobin, HCT: Hematocrit, MCV: Mean cell volume, PLT: Platelets.

Table 5. Statistical evaluation of haptoglobin, hepcidin and serum amyloid A values in both groups.

Parameters	Study group (n=20)	Control group (n=10)	P
Haptoglobin (ng/ml)	197±20	1.00±1.6	<0.001
Serum amyloid A (ng/ml)	4.89±1.2	0.66±0.73	0.005
Hepcidin (ng/ml)	0.833 ±0.055	0.073±0.800	0.005

A moderate difference was found between hepcidin and serum amyloid A values of the control and study groups (P<0.05). The most significant difference was observed in the haptoglobin (P<0.01). The values of haptoglobin, hepcidin and serum amyloid A were showed in Table 5.

Discussion and Conclusion

In this study, while there was no difference in body temperature between the two groups, the number of breath and pulse rates of the calves in study group per minute was found to be higher than in those in control group (P<0.01, P<0.05, respectively). Our results were similar to the results of Yılmaz and Gökçe (24). The lack of fever in the animals in the study group is thought to be due to the passing of the viremia period.

When the hematological parameters of the animals in the study and control groups were evaluated, it was determined that leukocytosis was related to acute respiratoric inflammation due to infection and it was found to be similar to the other study findings (24). There was a difference in neutrophil counts (P<0.01) and hematocrit values (P=0.036), but no significant difference was found in other parameters. Since no bacterial agents were investigated in the present study, secondary bacterial infection is highly possible.

Acute phase proteins have been accepted as a valuable indicator in determining the importance of pathological conditions in humans and animals. In cattle, SAA and Hp take place at the forefront of important acute-phase proteins, but their responses differ from individual animals.

In the case of viral and bacterial infections, acute phase response can be differ. For example, acute phase response to bacterial infections is reported to be more severe than viral infections in bovine mastitis (6).

Alsemgeet et al. (1) reported that haptoglobin was not detected in healthy cows and newborn calves. In the same study serum amyloid A level was found to be lower in healthy calves compared to adult cows, whereas in calves with inflammatory diseases; it has been reported to be significantly high. Niine et al. (17) reported that SAA and Hp levels were significantly increased in calves with Giardia and Cryptosporidium infections compared to healthy calves. Kabu et al. (12) reported a statistically significant increase in the concentrations of serum Hp and SAA in water buffaloes with dermatosis compared to the control group. Sahinduran et al. (20) reported that serum Hp and SAA concentrations, in cattle with BVD and BHDV-1 in single and dual infection, have shown statistically significant increase compared to healthy cattle. In the other study, significant increases were observed in serum Hp and SAA concentrations in calves suspected from Bovine respiratory disease (10). Also significant increase in the SAA concentration had been observed in calves with enteritis and pneumonia compared to the control group (11). Coşkun et al. (4) reported that SAA and Hp levels in both of the blood serum and the bronchoalveolar lavage were higher in calves with bronchopneumonia than in healthy calves. In our study, SAA and Hp levels were significantly higher in calves with pneumonia than in healthy calves (P<0.05 and P<0.00, respectively). It was thought that the increase in the values in these parameters was the result of acute phase response. These results are in agreement with above mentioned studies. Especially in cattle, SAA and Hp levels were found to be a suitable marker for site conditions in early detection of the diseases with viral pneumonia. In addition, the levels of these parameters were higher in patients with BVDV and in animals with mixed infections in this study.

Hepcidin plays a fundamental role in the regulation of iron metabolism. Hepcidin is an acute phase protein that is synthesized to restrict the body's iron stores and to prevent iron being requisitioned by invading microorganisms (18) and inflammation states stimulate hepcidin production (5). Hepcidin also helps host defense due to its direct antimicrobial properties (8, 23). Erkilic et al. (7), reported that serum hepcidin levels were very high in calves suspected of septicemia, and these levels decrease drastically after treatment. In our previous study on 56 cattle with single and dual infection (BHV-1, BVDV), hepcidin levels were compared. It was found that there was a significant difference between the serum hepcidin concentrations of cattle with single and dual infection and the control group of cattle (20). The results of this study were similar to the above mentioned study and the difference between serum hepcidin concentration of calves in study group and serum concentrations of calves in control group was significant (P<0.05).

Considering all three parameters (SAA, Hp and hepcidin) in our study, haptoglobin level was higher than

the other two parameters. Many studies have indicated the significance of Hp as a clinically useful parameter for measuring the occurrence and severity of inflammatory responses in cattle with mastitis, pneumonia, enteritis, peritonitis, endocarditis, abscesses, endometritis and other natural or experimental infectious conditions (6). Accordingly, haptoglobin which is important acute phase protein in bovine, can have more importance in respiratory infections in calves.

As a conclusion, hepcidin, serum amyloid A and especially haptoglobin were found to be significantly increased in calves with viral pnemonia.

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Conflict of Interest

The authors declare that there have no conflict of interests.

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Antioxidant activities, total phenolic and flavonoid contents of honey collected from different botanical origins

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Abstract: In this study, it is aimed to determine the presence of antioxidant capacity, total phenolic and flavonoid contents in six different types (multiflora, pine, chestnut, sunflower, acacia, citrus) and eventually 65 samples of honey from different parts of Turkey. Pollen analysis of all honey samples in the laboratory was carried out to determine the purity (> 65-70%) of the plant source. Total phenolic content determined in honey samples was found the highest value in pine honey 166.46 ± 5.80 (mgGAE / 100 g honey) while the lowest value was found in flower honey with (106.04 ± 9.55). The level of flavonoid contents of the groups was lowest on the flower and citrus honey (1.3 ± 0.2 and 1.6 ± 0.1) and the highest value were on chestnut and pine (2.7±0.4 and 2.8 ± 0.2) were detected. Comparing the radical scavenger activity in honey groups, the activity of chestnut honey was the highest (100.54 ± 22.72). The results of this study show that the phytochemical structure and biological activity of honey are completely different from each other depending on the plant source. On the other hand, it is possible to say that the antioxidant, phenolic, and flavonoid values are high, which is a good indicator of the quality and naturalness of honey.
Keywords: Antioxidant, flavonoid, honey, honey bee, phenolic acid.

Farklı botanik kaynaklardan elde edilen balların antioksidan aktiviteleri, toplam fenolikleri ve flavonoid içerikleri

Özet: Bu çalışmada, Türkiye'nin çeşitli bölgelerinde üretilmiş 6 farklı çeşit (yayla, çam, kestane, ayçiçeği, akasya, narenciye) toplam 65 bal numunesinde toplam fenolik asit, flavonoid düzeyi ve total antioksidan aktivitesi incelenmiştir. Laboratuvarda tüm bal numunelerinin polen analizi yapılarak bitki kaynağının saflık derecesi (>%65-70) tespit edilmiştir. Bal örneklerinde belirlenen total fenolik içeriği 166,46 ± 5,80 (mgGAE/100g bal) ile en yüksek değer çam balına ait iken, en az 106,04 ± 9,55 ile çiçek balında bulunmuştur. Gruplar arası flavonoid içeriği en düşük düzey çiçek ve narenciye balında sırasıyla (1,3 ± 0,2 ve 1,6 ± 0,1), en yüksek değer ise kestane ve çam balında (2,7±0,4 ve 2,8 ± 0.2) tespit edilmiştir. Bal gruplarında yapılan radikal süpürücü aktivitesinin karşılaştırılmasında ise kestane balın aktivitesinin en yüksek olduğu (100,54 ± 22,72) ortaya konulmuştur. Çalışmanın sonuçları, balın fitokimyasal yapısı ve biyolojik aktivitesinin bitki kaynağına bağlı olarak birbirinden tamamen farklı olduğunu göstermektedir. Diğer taraftan bu değerlerin yüksek olması balın kalitesinin ve doğallığının iyi bir göstergesi olacağını söylemek mümkündür.
Anahtar sözcükler: Antioksidan, bal, balarısı, fenolik asit, flavonoid.

Introduction

For centuries, bees and bee products have been used for therapeutical purposes. Today, they maintain their actuality in the field of supportive therapy and show a fast development under the name of apitherapy. Honey, which is one of the apitherapy products and one of the oldest nutritional sources of mankind, is a sweet product which bees collect from flower nectars or from secretions of some insects living on plants and store in honeycombs by exposing them to enzymatic change (3, 6, 22). The formation and composition of honey produced naturally differ significantly according to regions and sources of the plant (23). The composition of plant nectar differs according to the geography the plant is grown in, soil fertility, rainfall, light, altitude and a great number of other environmental factors (14, 24). In other words, the quality and biochemical characteristics of honey differ according to the source of nectar (8, 13, 22). Studies conducted show that the antioxidant activity of natural foods is higher than food products with synthetic structure (18). For this reason, daily intake of antioxidant food is important and required for free radicals which form as a metabolism product in the organism. In addition to being a food product and source of energy, honey is also important for human health as a natural antioxidant source due to various phytochemicals it includes (3, 12, 17).

Antioxidant compositions of honey are enzymatic (glucose oxidase, catalase, peroxidase) or non-enzymatic (phenolic acids, flavonoids, ascorbic acid, tocopherol, carotenoids) (18, 19). Phenolic acids contribute significantly to the antioxidant activity of honey (1, 17). Flavonoids have a significant antioxidant and anti-inflammatory function by providing free-radical scavenging, inhibiting cyclooxygenase and lipooxygenase enzymes, chelating transition metals such as iron and copper, protecting α-tocopherol at LDL and providing oxidizable ascorbic acid regeneration (16). The rates of these compositions in honey differ significantly depending on the source flora honey is collected from and the structure of the geographical areas (8, 12, 23). For this reason, every honey has a different apitherapic value. The present study researches the biological activities of honey obtained from different regions and plant sources of Turkey.

Materials and Methods

Sampling: The study was conducted with a total of 65 honey samples from different regions of Turkey (11 sample meadow honey from the provinces of Erzurum and Sivas, 11 sample pine honey from Muğla, 11 sample chestnut honey from Sinop, 11 sample Acacia honey from Trabzon, 10 sample from citrus tree honey from Antalya and 11 sample sunflower honey from Samsun). Pollen analyses of all samples were made in the laboratory and purity degree of plant sources was found (>65-70%).

Biochemical analysis: Calculation of the total antioxidant effect in the samples was made according to the free radical scavenging effects of the prepared extracts on DPPH (1,1-diphenyl-2-picrylhydrazyl) in honey by using an indirect method. As a result of DPPH radical scavenging activity, IC50 values were assessed as mg/ml. The measurement was made according to Meda and Dimins method modified at 520 nm with a spectrophotometer (9, 21). AA [%] = (Abs cont-Abs sample)/ Abs cont.X100. Total phenolic concentration level was read at 750 nm in spectrophotometer with Folin-Ciocalteau method and the total phenol amount was calculated as equivalent to mg gallic acid in 100 gram extract (4, 9). The Dowd method was used in total flavonoid analysis. In this method, honey solutions prepared based on quercetin (mgQE/100gr) concentration in mg in a kilogram of honey were read at 415 nm with a spectrophotometer (9, 21).

Statistical analysis: The data obtained from the study were evaluated with the variance analysis (ANOVA) technique in factorial order and the differences between the means were determined by Duncan multiple comparison test. Statistical evaluations were made using the SPSS statistical program (26).

Results

Average values of total phenolic, flavonoid levels and antioxidant activity found in honey samples of groups are given in (Table 1, Figure1).

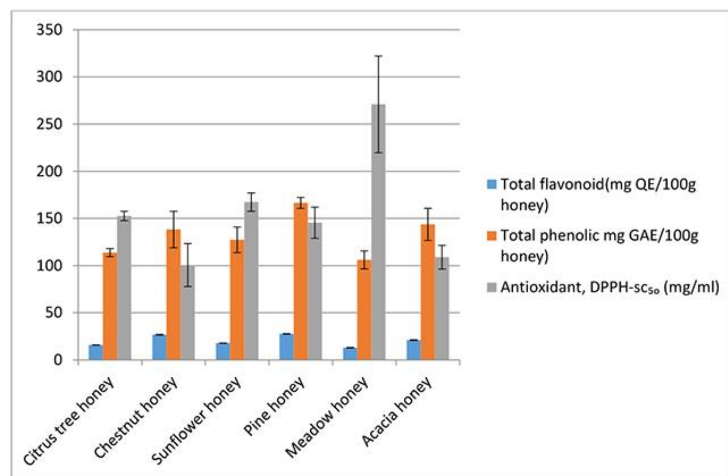

Figure 1. Means of total phenolic, flavonoid and antioxidant analysis results in honey samples

Table 1. Means and standard errors of total phenolic (mg GAE/100 g), flavonoid QE/100 g) and antioxidant (mg/ml) in honeys samples produced from different botanical origins.

Samples	Total flavonoid	(Min-Max)	Total phenolic	(Min-Max)	Antioxidant, DPPH-sc_{50}	(Min-Max)
Citrus tree honey	1.6 ± 0.1	1.2 -1.9	113.77 ± 4.27	100.21-120.62	152.65 ± 4.96	140.40 -163.91
Chestnut honey	2.7 ± 0.4	1.4 -4.0	138.27 ± 19.31	97.64 -206.22	100.54 ± 22.72	41.05 -165.03
Sunflower honey	1.8 ± 0.5	1.7 -2.0	127.27 ± 13.54	88.03 -127.33	167.33 ± 9.69	135.40 -198.81
Pine honey	2.8 ± 0.2	2.1 -3.6	166.46 ± 5.80	149.37-186.11	145.48±16.49	93.61 -206.38
Meadow honey	1.3 ± 0.2	0.5 -1.9	106.04 ± 9.55	68.85 -130.94	270.86 ±51.22	136.17 -506.38
Acacia honey	2.1± 0.4	1.3 -3.5	143.71 ± 16.99	71.16 -183.06	108.91 ± 54.00	69.34 -142.55

The highest total phenolic composition was found in pine honey with 166.46 ± 5.80 (mgGAE/100g honey), while the lowest value was found in flower honey with 106.04 ± 9.55. While the intergroup flavonoid composition lowest value was found in flower and citrus honey (1.30 ± 0.2 and 1.6 ± 0.1), the highest value was found in chestnut and pine honey (2.7 ± 0.4 and 2.8 ± 0.2). Radical scavenging activity comparison of honey groups showed that chestnut honey had the highest activity (100.54 ± 22.72) As a result of the analysis of variance, there was a significant difference between the honey samples (P <0.01).

Discussion and Conclusion

Phenolic acids and flavonoids, which are components of honey, are important due to their antimicrobial, antioxidant, anticancer and antioxidative effects and their being associated with human and animal health and on the other hand due to being a criterion for honey's being refined (1, 5, 6, 22). The concentration of these compounds which significantly affects honey's therapeutic activity differs according to plant flora which constitutes the sources of honey (11).

In the present study, it was found that total phenolic composition differed between 106 - 166 mgGAE/100 g in all honey samples. While the lowest concentration was found in flower honey, the highest concentration was found in pine honey (Figure1). Similarities and differences are seen between our study results and the results of the studies previously conducted. While total phenolic acid concentration was reported as 32.59-114.75 mgGAE/100 gr and an average of 74.38 ± 20.54 in South African honey samples (21), phenolic acid concentration was reported as 36.26-102.80 mgGAE/100g and an average of 65.31±19.50 in a study conducted with Nigeria honey (5). Vit et al., (28) reported in their study that phenolic matter amount differed between 38.15 and 182.10 mgGAE/100 g in Venezuela honey. Total phenolic amount in Yemeni honey obtained from different regions and different plants differed between 56.32 and 246.21 mg/100g (1). In a study conducted on the honey in the Indian Bengal region, it was reported that gallic acid rates per 100 g honey differed between 9.9 ± 0.6 and 44.7 ± 2 mg (7). In a study conducted with different honey from Slovenia, the total phenolic composition was found as 241.4 mg/kg in fir tree honey, as 233.9 mg/kg in chestnut honey and as 44.8 mg/kg in acacia honey (4). In studies conducted on acacia honey, total phenolic compositions differ significantly among countries. While Iranian acacia honey total phenolic content was found as 22.9-65.5 mg gallic acid/g (15), it was found as 627 ± 44.03 in Germany acacia honey (2). In a study conducted on the honey of Black Sea flora, average phenolic content was found as 0.224 mg as gallic acid equivalent for 1 g honey sample (11).

In the study, while the lowest average total flavonoid (mgQE/100g honey) value of honey was found in flower honey with an average of 1.3 ± 0.2 the highest value was found in chestnut honey with an average of 2.7 ± 0.4. In a study conducted on Indian honey, quercetin (mg/100g) amount was found to be between 5.12 ± 0.23 and 19.4 ± 1.38 (Das et al., 2013). In Brazilian multiflora and citrus honey, quercetin (mg/100g) composition was reported as 1.96 ± 1.53 and 0.17 ± 0.15, respectively (20). In a study conducted on Malesian honey, flavonoid average value was reported as 5.42 ± 0.62 (19). In China unifloral honey, flavonoid average value was reported to differ between 9.41 and 102.1 (10). In a study conducted in South Africa, the total flavonoid composition average was reported as 2.57 ± 2.09 in 27 honey samples (21). In a study conducted on pine honey in our country, total flavonoid level was found as 22.80 ± 2.45 on average in QE/kg honey equivalent (25). In the present study, this value was found as 2.8 ± 0.2 in the same honey type.

In the study, total antioxidant activity was found as 100.54 ± 22.72 in chestnut honey and as 270.86 ± 51.22 in multiflora flower honey. When studies conducted in many countries were examined in terms of their similarities and differences of these values, it was found that radical scavenger DPPH average IC50 = 23.92 ± 1.12 mg/mL in Indian honey (7), the same value was found as 10.0 ± 1.8 and 10.7 ± 2.2, respectively in Slovenian chestnut and flower honey (4); average antioxidant activity was found as 31.96 ± 18.07 in chestnut honey in Brazilian multifloral honey and as 15.22 ± 10.75 in citrus honey (20), while antioxidant activity was reported as 35-122 in China unifloral honey (10). Ertürk et al. (11) found IC50 values as between 29.388 and 458.450 mg/mL as a result of the DPPH radical scavenging activity test in Black Sea flora honey in our country.

Some studies show a correlation between the phenolic compound level and antioxidant activity in some types of honey; however, since flavonoids have hydroxyl, they are oxidized very quickly. For this reason, despite structural similarities, the difference between antioxidant activities depends on hydroxylation and methylation degree (1). In our study, no correlation was found between groups. It is possible to say that this is because in addition to flavonoid and phenolic acid, the presence of Vitamin E and C and carotenoids may have influenced total antioxidant activity (27).

Consequently, our observations that phytochemical structure and biological activity of honey differ completely depending on the origins of the plant.

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Conflict of Interest

The authors declared that there is no conflict of interest.

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Investigation of enrofloxacin and ciprofloxacin residues by LC-MS/MS in chicken liver marketed in Afyonkarahisar

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Abstract: Antibiotic residues can remain in animal source foods, mainly in the liver. Enrofloxacin (ENR) is a synthetic fluoroquinolone antimicrobial agent which is extensively used to treat bacterial infections in poultry. This study aimed to evaluate ENR and its main metabolite ciprofloxacin (CIP) occurrence in 100 chicken livers marketed Afyonkarahisar, Turkey. Chicken liver samples were analyzed by LC-MS/MS method with limits of detection for 1.17 µg/kg (ENR) and 1.24 µg/kg (CIP). ENR and CIP were detected in 39% and in 31% of samples, respectively. However, the total sum of both antibiotic residue levels was found to be far below the established maximum residue level (200 µg/kg) in all of chicken liver samples. In conclusion, according to the results of this study, chicken livers marketed in Afyonkarahisar city are safe for public health regarding ENR and CIP residues. Nonetheless, periodical control of chicken liver is advised due to the high incidence of these antibiotics.
Keywords: Antibiotic residue, chicken liver, ciprofloxacin, enrofloxacin, LC-MS/MS.

Afyonkarahisar'da satışa sunulan tavuk karaciğerlerinde enrofloksasin ve siprofloksasin kalıntılarının LC-MS/MS ile araştırılması

Özet: Antibiyotik kalıntıları hayvansal orjinli gıdalarda özellikle karaciğerde bulunabilir. Enrofloksasin (ENR) sentetik bir florokinolon antimikrobiyal ajan olup kanatlılarda bakteriyel enfeksiyonların tedavisinde oldukça yaygın olarak kullanılmaktadır. Bu çalışmada Afyonkarahisar, Türkiye'de satışa sunulan 100 tavuk karaciğerinde ENR ve onun ana metaboliti olan siprofloksasin (CIP) varlığının araştırılması amaçlanmıştır. Tavuk karaciğer örnekleri LC-MS/MS metodu ile analiz edilerek, saptama limitleri ENR için 1,17 µg/kg ve CIP için 1,24 µg/kg olarak belirlenmiştir. ENR ve CIP sırasıyla örneklerin %39 ve %31'inde saptanmıştır. Ancak, tavuk karaciğer numunelerinde her iki antibiyotik kalıntısının toplamı belirlenen maksimum kalıntı düzeyinin (200 µg/kg) oldukça altındadır. Sonuç olarak, Afyonkarahisar ilinde satışa sunulan tavuk karaciğerleri ENR ve CIP kalıntısı bakımından değerlendirildiğinde halk sağlığına risk oluşturmamaktadır. Bununla birlikte, bu antibiyotiklerin yüksek insidensi nedeniyle tavuk karaciğerinin periyodik olarak kontrol edilmesi tavsiye edilmektedir.
Anahtar sözcükler: Antibiyotik kalıntısı, enrofloksasin, LC-MS/MS, siprofloksasin, tavuk karaciğeri.

Introduction

Fluoroquinolones, essential drugs for human and animal health, are widely used for the treatment of several diseases in veterinary medicine (21, 24). Enrofloxacin (ENR), a common fluoroquinolone, has a broad spectrum of action and exhibits high efficacy against infectious diseases that are caused by mycoplasma and gram-positive and gram-negative bacteria (14, 21). Furthermore, this antibiotic is used in the treatment of common poultry infections, such as mycoplasmal infections, colibacillosis and pasteurellosis, due to its rapid oral absorption and long elimination half-life (10, 22). ENR shows its effect by interfering in the activities of bacterial DNA-gyrase and topoisomerase IV enzymes and, consequently, it inhibits bacterial DNA replication and transcription (5). Ciprofloxacin (CIP) is the main metabolite of ENR and occurs at different levels in foods of animal origin after the administration of ENR (12). Currently, the presence of contaminants in animal origin products is a major concern regarding food safety (16). Health problems can occur as a result of the excessive use of veterinary drugs in food-producing animals because most of these substances may produce important toxic effects such as genotoxicity, carcinogenicity and immunotoxicity, as well as endocrine effects, on humans (1, 28). In addition, the improper use of ENR in food-producing animals and not respecting the

necessary withdrawal time of this antibiotic may lead to residues in edible animal tissues. The presence of ENR residues in foodstuffs may cause allergic reactions in hypersensitive individuals and could lead to an increased pathogen resistance to clinical drugs in humans; therefore, they may represent important consequences for public health (8,18). To protect food safety and consumer health, Maximum Residue Limits (MRL) have been established by the European Union for pharmacologically active compounds, including ENR. In chicken liver, the MRL is the total sum of ENR and CIP (200 µg/kg) (7). Therefore, if the residue levels in the target tissues are lower than the established MRL, the related food is considered safe regarding the presence of these antimicrobials (6).

The liver takes its place in the human diet owing to its fundamental nutritive value in providing dietary iron. However, it should be considered that the liver is a very complex organ due to its role in many drug metabolisms. Therefore, edible animal liver tissue must be monitored regularly regarding the presence of residues (11,16). Chicken liver is one of the favorite foods of animal origin due to its high nutritional value and cheap price.

This study aimed to determine the levels of ENR and its metabolite CIP in chicken liver based on LC-MS/MS, with respect to the European Commission MRL.

Material and Methods

Chemicals and reagents: Methanol, formic acid, ENR and CIP were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents and chemicals were analytical grade and obtained from commercial sources.

Sample collection: A total of 100 chicken liver samples were obtained from Afyonkarahisar city, Turkey from September 2017-January 2018. Liver samples were purchased by supermarkets and local markets. The transportation of samples to the laboratory was performed under cold conditions (+4°C) immediately after sampling and the collected samples were stored at -20ºC in a deep freezer until the analysis.

Standard solutions: ENR and CIP stock solutions were prepared at a concentration of 1 mg/ml in methanol. The working solutions were prepared from stock solutions to generate a calibration curve and to perform recovery studies of the chicken liver.

Sample preparation: Each liver sample was homogenized by a food blender and 5 g of sample was transferred into a polypropylene centrifuge tube (50 ml) followed by addition of 10 ml methanol and then the mixture was vigorously shaken for 5 min. After the addition of 1% formic acid (4ml) and 0.01M EDTA (400 µl), the centrifuge tube was vortexed for 3 and 2 min, respectively. Subsequently, centrifugation of the mixture was performed at 2000g for 10 min at 4ºC. After centrifugation, the supernatant of each sample was filtered through a membrane filter (0.45 µm) and the obtained filtrate was transferred to a High Performance Liquid Chromatography (HPLC) vial for injection.

Instrumental conditions: The LC method described here was developed using an LC system of Agilent Technologies 1200 series (Waldbronn, Germany), combined to a binary high-pressure gradient pump. LC separation was performed by using Agilent Zorbax Eclipse XDB C18 column (4.6 x 30 mm x 3.5 µm). Mobile phase A consisted of water containing 0.1% formic acid while mobile phase B consisted of methanol containing 0.1% formic acid. The flow rate and injection volume of the mobile phases, and column temperature were 0.4 ml/min, 10µl, and 40˚C, respectively. LC separation gradient was set to A/B (80/20) at 0.0 min; it was changed to A/B (10/90) and kept unchanged until 4.10 min, A/B (80/20). MS analysis was conducted on Agilent 6460 LC/MS Triple Quadrupole instrument equipped with an ESI (Waldbronn, Germany) in positive ion mode. MS parameters of drying gas temperature, nebulizer gas, sheath gas flow, capillary voltage and sheath gas temperature were set to 350°C, 40 p.s.i., 10 l/min, 4000 V, and 400 °C, respectively. Precursor ion and productions of ENR were 360, 342, and 316, respectively while precursor ion and productions of CIP were 332, 231 and 314, respectively.

Quality parameters of method: The validation of this method was performed by spiking chicken liver samples and limit of detection (LOD), the limit of quantification (LOQ), linearity range, intra- and inter-day precision and recovery were used as quality parameters. The concentrations of the samples were calculated according to the calibration curve method for which a series of external standard solutions are prepared and measured. For the calibration equation, a line is fitted to these data and the obtained equation is employed to calculate the concentration unknown samples. In addition, the strength of the linear regression is expressed with a correlation coefficient (r^2). These parameters were calculated by the software (Agilent MassHunter Workstation Software Version 7) of the analytical instrument. The lowest concentration of ENR or CIP that the analytical process can reliably differentiate from background levels (signal-to-noise ratio≥3) and lowest concentration of ENR or CIP that can be quantified (signal-to-noise ratio ≥10) were defined as LOD and LOQ, respectively.

Results

ENR and CIP in chicken liver samples were quantified by LC-MS/MS. The typical chromatogram and standard calibration curves of ENR and CIP are shown in Figure 1 and Figure 2. LOD, LOQ, calibration curve parameters and retention times are shown in Table 1. Relative standard deviations used for precision and intra-

and inter-day recoveries used for the accuracy of the method are given in Tables 2 and 3. The results of the present study show that 39% of all chicken liver samples (n=100) were contaminated with ENR while 31% of chicken liver samples contained CIP (Table 4). Additionally, the highest concentrations of ENR and CIP were determined to be 30.23 and 6.92 µg/kg, respectively.

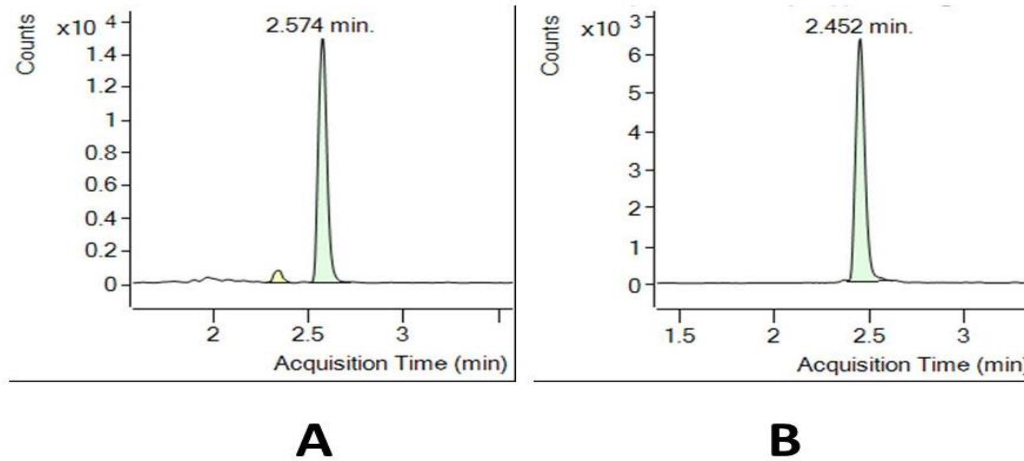

Figure 1. Chromatograms of enrofloxacin (A) and ciprofloxacin (B).

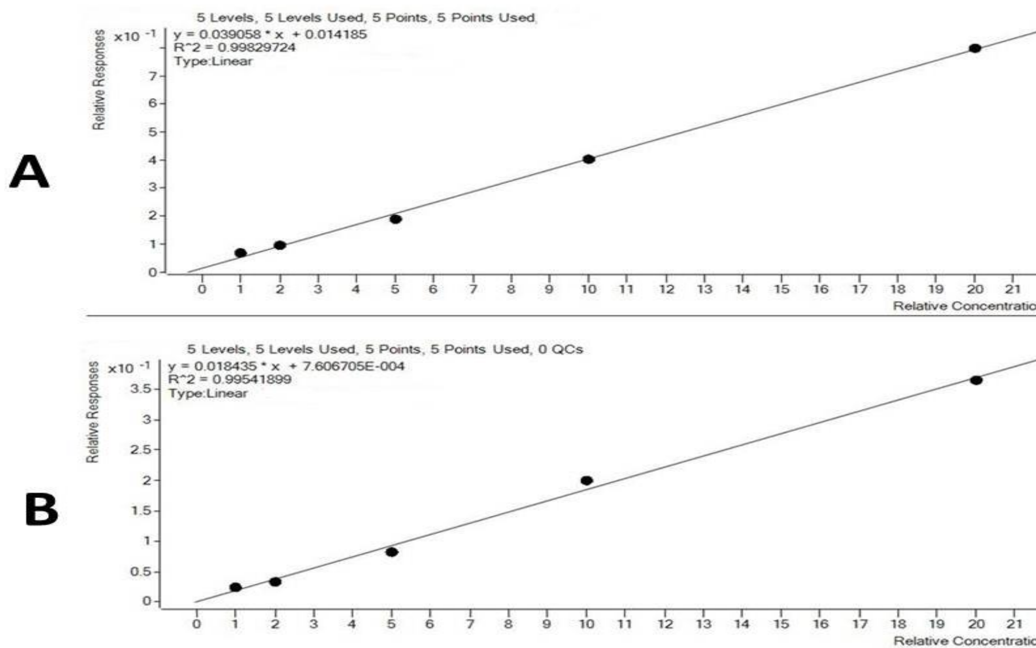

Figure 2. Standard calibration curves of enrofloxacin (A) and ciprofloxacin (B).

Table 1. Analytical parameters of enrofloxacin and ciprofloxacin in chicken livers.

Antibiotic	LOD (µg/kg)	LOQ (µg/kg)	Correlation coefficient (r^2)	Calibration equation	Data point	Linear range (µg/kg)	RT (min)
Enrofloxacin	1.17	3.90	0.995	y=0.039058x +0.014185	5	1-20	2.57
Ciprofloxacin	1.24	4.14	0.998	y=0.018435x +7.606705	5	1-20	2.45

Table 2. Intra- and inter-day precisions for enrofloxacin in chicken liver samples.

Enrofloxacin Spiked (µg/kg)	Intra-day assays (n=10)		Inter-day assays (n=14)	
	Percentage Recovery±CV	RSD (%)	Percentage Recovery±CV	RSD (%)
40	97.86±1.87	1.91	99.30±5.23	5.27
80	90.70±2.86	3.15	90.21±4.51	4.51
160	92.87±2.55	2.75	90.71±2.38	2.62

Table 3. Intra- and inter-day precisions for ciprofloxacin in chicken liver samples.

Ciprofloxacin Spiked (µg/kg)	Intra-day assays (n=10)		Inter-day assays (n=14)	
	Percentage Recovery±CV	RSD (%)	Percentage Recovery±CV	RSD (%)
40	93.78±3.07	3.28	94.48±4.66	4.94
80	89.19±4.49	5.03	88.13±4.87	4.87
160	94.00±2.44	2.6	91.47±1.63	1.78

Table 4. The enrofloxacin and ciprofloxacin levels of chicken liver samples (n=100).

Enrofloxacin	**Range (µg/kg)**	<1.17	1.17-9.99	10-19.99	20-35
	Number of samples	61	30	6	3
Ciprofloxacin	**Range (µg/kg)**	<1.24	1.24-1.99	2-3.99	4-7
	Number of samples	69	13	11	7

Table 5. Previous studies investigating the presence of quinolone-residues in chicken livers.

References	Country	Analyze	Samples size	Positive (%)	Range (µg/kg)	Method
Attari et al. (5)	Iran	ENR	20	17 (85%)	5.30-90.7	ELISA
Salehzadeh et al. (23)	Iran	ENR	90	90 (100%)	4.3-66.2	HPLC
Rezaee et al. (22)	Iran	ENR	50	26 (52%)	16.7-296.7	HPLC
		CIP		15 (30%)	9.8-93.3	
Aslam et al. (4)	Pakistan	ENR	75	59 (78.7%)	527[b]	HPLC
Naeem et al. (19)	Pakistan	ENR	40	37 (92%)	3.02-364	HPLC
		CIP		34 (85%)	2.08-245	
Younus et al. (30)	Pakistan	ENR	45	40 (88.8%)	1.409[a]	ELISA
Sattar et al. (26)	Bangladesh	ENR	50	20 (40%)	-	TLC
		CIP		22 (44%)	-	
Sarker et al. (25)	Bangladesh	ENR	160	57 (36%)	-	TLC
Faten et al. (9)	Egypt	CIP	15	13 (86.7%)	96.33-300.27	HPLC
Sultan (27)	Iraq	ENR	30	10 (33.31%)	0.01-10.69	ELISA
Nizamlıoğlu and Aydın (20)	Turkey	CIP, DAN, DIF, ENR, FLU, MAR, OA, SAR	50	17 (34%)	18.5-147.88	ELISA
Metli et al. (17)	Turkey	CIP, DAN, DIF, ENR, FLU, MAR, NA, NOR, OA, SAR	34	-	-	LC-MS/MS
This Study	Turkey	ENR	100	39 (39%)	1.42-30.23	LC-MS/MS
		CIP		31 (31%)	1.25-6.92	

[a]Mean concentration, Ciprofloxacin (CIP), Danofloxacin (DAN), Difloxacin (DIF), Enrofloxacin (ENR), Flumequine (FLU), Marbofloxacin (MAR), Nalidixic Acid (NA), Norfloxacin (NOR), Oxolinic Acid (OA), Sarafloxacin (SAR)

Discussion and Conclusion

Poultry meat and giblets, including liver, are widely consumed throughout the world. However, the liver is considered a highly risky poultry product due to its role in the detoxification of toxic substances, including antibiotics (2, 13, 15). Quinolones are powerful options among the antimicrobial agents employed in the treatment of various bacterial-caused poultry diseases (29). In addition, the presence of quinolones in poultry products causes public health concerns (21). Local and international regulatory and public health agencies give attention to screening of antibiotic residues in food-producing animals. In Turkey, National Residue Monitoring Plan is established to determine the presence of these substances including quinolone residues in foods of animal origin (3). Several monitoring studies conducted in different countries to investigate the presence of quinolone residues in chicken liver based on various methods (Table 5). Some of them reported very high incidence for quinolone residues in Pakistan (4, 19, 30), Iran (5, 23) and Egypt (9). Attari et al. (5) reported that in 85 % of 20 chicken liver samples collected from the Northwestern part of Iran, the ENR residue level ranged from 5.30 to 90.70 µg/kg, with a mean concentration of 39.54 µg/kg. In another study conducted in Iran using HPLC, a high contamination level (100%) was reported for 90 chicken liver samples in a range from 4.3 to 66.2 µg/kg (23). Similarly, Faten et al. (9) found, using HPLC, that 86.7% of 15 chicken liver samples marketed in Egypt tested positive for CIP at concentrations between 96.33 and 300.27 µg/kg. Our results are lower than these studies. However, the findings of our study are in consistent with other studies for those incidence of quinolone-residues ranged from 33.31% to 44% (20, 26, 27). Sattar et al. (26) performed a monitoring study in Bangladesh based on thin-layer chromatography (TLC) and found contamination levels in chicken liver samples of 40% and 44% for ENR and CIP, respectively. In another study conducted in Iraq, ENR incidence reported as 33.31 % while ENR level ranged from 0.01 µg/kg to 10.69 µg/kg. Nizamlıoğlu and Aydın (20) evaluated a total of 50 chicken liver samples regarding the presence of quinolone-residues based on ELISA method and reported that 34 % samples contained quinolones. Additionally, in a study conducted in Antakya province of Turkey, chicken liver samples were collected from 34 different markets and analyzed regarding 38 antibiotic residues and none of the samples have quinolone residues (17). Our results are not compatible with this study. In the present study, the ENR contamination level was found to be between 1.42 and 30.23µg/kg (39%), and the CIP contamination level was found to be between 1.25-6.92 µg/kg (31%). According to the results of the present study, the total sum of ENR and CIP did not exceed the established MRL in any of the chicken samples. Taken together, different incidences of quinolone-residues may be due to misuse or overuse of these antibiotics or disrespect of withdrawal time during poultry breeding.

In conclusion, poultry meat and meat products are extensively consumed throughout the world owing to their nutritive value and suitable price. However, antibiotic residues in foods of animal origin are an important public health issue. Therefore, monitoring programs for veterinary drug residues in these types of foods have been performed by national authorities. This study contributes to those residue monitoring programs by providing a highly sensitive and accurate LC-MS/MS method for chicken liver samples.

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Conflict of Interest

The authors declared that there is no conflict of interest.

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Honey price estimation for the future in Turkey; example of 2019-2020

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Abstract: The purpose of the present study is to develop models for the time series data of honey prices in Turkey between 1998 and 2018 using Box-Jenkins and Winter's Exponential Smoothing methods and to evaluate the TRY- and US$-denominated forecasts made using such models for 2019-2020. The method employed to analyse the time series data is the Seasonal Auto Regressive Integrated Moving Average (SARIMA). The most suitable model to forecast the honey prices was found to be SARIMA (2,1,2)(1,0,1) for TRY-denominated prices and SARIMA (0,1,3)(0,0,0) for US$-denominated prices. It was forecast that the average price of 1 kg of honey would be 49.08 TRY/6.15 US$ at the end of 2020. Since the study will provide beekeepers with information on future prices, it will help them better plan their production activities. The price forecasts in this study will be useful for producers and those involved in sale of honey with respect to prediction of how the honey prices will change in the years to come.
Keywords: Forecasting, honey, time series, Turkey.

Türkiye'de bal fiyatlarının geleceğe yönelik kestirimi; 2019-2020 örneği

Özet: Bu çalışmada, Türkiye'de 1998-2018 yıllarına ait aylık bal fiyatları zaman serisinin Box-Jenkins ve Winter's Üstel Düzgünleştirme yöntemleri ile modellenerek ve bu modellerin 2019-2020 yılları arasında TL ve dolar bazında öngörülerinin değerlendirilmesi amaçlanmıştır. Zaman serilerinin analizlerinde mevsimsel otoregresif hareketli ortalama (SARIMA- Seasonal Autoregressive Integrated Moving Average) yöntemi kullanılmıştır. Bal fiyatları için belirlenen en uygun tahmin modeli TL bazında SARIMA(2,1,2)(1,0,1) modeli ve dolar bazında SARIMA(0,1,3)(0,0,0) modeli olduğu tespit edilmiştir. Çalışma sonucunda 2020 yılı sonunda bal fiyatlarının doğrusal artış ile ortalama 49.08 TL/kg ve 6.15 US$ olacağı tahmin edilmiştir. Sonuç olarak arıcılıkla uğraşanların gelecekteki fiyatlar hakkında bilgi sahibi olmaları onlara üretim faaliyetinin planlanmasında faydalı bilgiler sağlayacaktır. Çalışmada yapılan fiyat tahminleri balın satış aşamasında faaliyet gösteren kişiler ve üreticiler için ürün fiyatlarının nasıl şekilleneceği konusunda öngörü sahibi olmalarını sağlayacaktır.
Anahtar sözcükler: Bal, tahminleme, Türkiye, zaman serisi.

Introduction

Today, the beekeeping industry has become a significant rural production sector with its increasing economic returns and product diversity (11). Turkey ranks second following China in both the total number of hives and total honey production (12). In 2018, Turkey produced 107 thousand tons of honey with a total of 8.1 million honey-producing hives (35).

While the marketing infrastructure of honey in Turkey is conventional, the marketing channels are divided into three groups, namely, producers, wholesalers and retailers (10). In addition to the marketing channels, the beekeeping enterprises are able to find markets for their products through direct sales to processing companies and via cooperatives. However, marketing via cooperatives is a last resort for producers due to its low margin of profit (25). The major marketing-related problems of beekeeping enterprises are that they cannot sell their products in a timely manner and cannot charge the price they prefer, and that a quality-price relationship does not exist for honey in the domestic market (11, 29).

Previous studies found that honey production costs varied depending on the total number of hives belonging to the enterprises (7, 22), and that the enterprises could reduce their production costs through sale of by-products and migratory beekeeping (11). In 2018, the average retail

price of strained honey in Turkey was 30.48 TRY (Turkish Lira)/kg (minimum-maximum: 28.22-33.72, standard deviation: 2.10) or 6.43 US$/kg (minimum-maximum: 5.0-7.5, standard deviation: 0.82) (36).

The future of honey production and prices is important for both producers and consumers. Honey consumption per capita in Turkey was 0.84 kg in 2003 and rose to 1.22 kg as of 2013, the most recent year for which latest data are available (13). It is noted that honey price is the most important factor in the countries' honey export and competitiveness in the international market. Taking this into account, estimations and inferences are made regarding the competitiveness of countries in the international market using the past price data (20). Today, honey price affects the consumer demand for honey, along with a number of factors such as quality, production technique and origin of the honey (19, 21).

Time series is a series of observations made at certain time intervals, which allows development of an appropriate model using statistical methods to make forecasts (2). Time series analysis has been recently found wide use in studies focusing on honey production, specifically to predict the number of colonies and honey export and import (23, 28), to determine the competitive power in honey export (34), to forecast honey production using Autoregressive integrated moving average (ARIMA) models (6, 9, 18) and to determine the supply of and demand for honey (26).

The main objective of our study is to examine the fluctuations in TRY- and USD-denominated honey prices. Furthermore, we aim to develop models for the time series data of honey prices without taking into account the exchange rate parity and to make price forecasts. What we intend to do is to determine and predict the fluctuations in TRY- and USD-denominated prices.

The purpose of the present study is to use known models for the time series data of honey prices in Turkey between 1998 and 2018 using Box-Jenkins and Winter's Exponential Smoothing methods and to evaluate the TRY- and US$-denominated forecasts made using such models for 2019-2020.

Material and Methods

Data set: The study consisted of data sets retail honey prices on a monthly basis belonging to the 1998-2018 year in Turkey (36). In the analysis of the time series, the predictions obtained by using the seasonal autoregressive moving average (SARIMA) method were evaluated. For analysis of time series, the SPSS program version 25.0 was used (15).

The Box-Jenkins method (ARIMA): Autoregressive Integrated Moving Average (ARIMA) method used in forecasting time series events was developed by Box and Jenkins (5). ARIMA modeling approach is limited to the assumption that there is linearity between the variables. Apart from that, the researchers developed alternative modeling perspectives for forecasting the time series events not fulfilling the linearity assumption.

ARIMA or Box-Jenkins models are the combinations of AR and MA models administered to the series differenced at degree d. The essence of the Box-Jenkins method is the choice of an ARIMA model that is the most suitable one among various models based on the structure of the current data but contains limited number of parameters. As a whole, these models are represented as ARIMA (p, d, q).

In the models (38),

p: degree of autoregressive model, q: order of moving average model, d: degree of non-seasonal differencing.

The expression of ARIMA (p, d, q) model can be defined as indicated in equation 1

$$Z_t = \phi_1 Z_{t-1} + \phi_2 Z_{t-2} + \dots + \phi_P Z_{t-P} + a_t - \theta_1 a_{t-1} - a_t - \theta_2 a_{t-2} - \dots - a_q - \theta_q a_{t-q} \quad [1]$$

Here:

ϕ_P : parameter values for autoregressive operator, a_t : error term coefficients, θ_q : parameter values for moving average operator, Z_t: time series of the original series differenced at degree d. In other words,

$$\phi_P \quad W_t = Y_t - Y_{t-1}, t = 1,2,\dots,t \quad [2]$$

The first differences series is defined as given in equation 2. Here:

W_t = The first differences series, Y_t = the random variables subset of the original time series. If the first differences series is not stationary, stationary is checked by differencing the first time series again. This is modeled as given in equation 3.

$$Z_t = W_t W_{t-1} \quad t = 1,2,\dots,t \quad [3]$$

When the degree of difference is d = 0 (that means that the original series is stationary), the ARIMA model will be AR, MA, or ARMA model. Due to this feature, it can be said that ARIMA models incorporate all of the Box Jenkins models.

As done by ARIMA (p, d, q) model, seasonal ARIMA(P,D,Q)s models only distinguish the interval between the observations that effect one another. These are (seasonal) periods. They do not indicate period involving a single interval.

For example: ARIMA(1,0,0)12 model is [monthly AR(1)]].

$$Z_t = \alpha + \phi_1 Z_t\text{-}12 + A_t \quad [4]$$

Thus, autocorrelation decrease exponential lags may be at 12, 24, 36, etc.

Partial autocorrelation lag is 12 = ϕ_1

If lag is after 1, it becomes equal to 0.

Seasonal ARIMA(P,D,Q)s Models ARIMA(p, d, q) Models relationship can be expressed as SARIMA models:

$$\Phi P(B)\ \phi p(B)\ [1-B^{s}]^{D}\ [1-B]^{d} Z_t = \alpha + \Theta Q(B)\ \theta q(B)\ A_t \quad [5]$$

This model is used if we want to understand what is meant by autocorrelation design through two autoregressive polynomials and two moving average polynomials, and this can be referred to as difference operator as well (27). The model establishment process involves certain repetitive steps (5). These steps are indicated in the flow chart given in Figure 1.

The establishment of Box-Jenkins ARIMA models involves four main steps. In the first step, the class of the general model is determined. In selecting the general model, the graphs of autocorrelation and partial autocorrelation functions are used. The features of theoretical functions concerning ARIMA models are used based on autocorrelation and partial autocorrelation functions in Figure 1 (5). In the second step, a transient model compliant with the structure of the data is determined. To this end, autocorrelation and partial correlation functions are used. In determining the model, a model is selected from model classes such as AR, MA, ARMA, ARIMA, and SARIMA (32). In the third step, the parameters of the transient model are forecasted by use of efficient statistical techniques, and the standard errors of coefficients are calculated to test whether or not they are significant. In the last stage, compliance of the model is checked for forecasting. To this end, the autocorrelation function of the model is examined by drawing the graph of the autocorrelation coefficients of the errors of the transient model that is assumed to be compliant. If this function displays a particular shape, it is concluded that errors are not random. This kind of finding means that the determined transient model is not compliant. Therefore, one turns to the second step again, and this process is repeated until the compliant model is determined through a new transient model. The model passing the compliance check is now ready to be used for forecasting (3, 17, 31, 39).

Moving averages and exponential smoothing techniques were used in forecasting. Simple exponential smoothing is a method derived from the moving averages technique, and its equation is as follows:

$$Z_t^{'} = \alpha Z_t + (1-\alpha) Z_{t-1}^{'}$$

$$\hat{Z}_{t+1} = Z_t^{'} \quad [6]$$

where $\hat{Z}_{t+1}$ is the forecast value for the next period, α is the smoothing factor in the range of 0< α <1, Z_t is the actual value of the new observation at time t, and $Z_t^{'}$ is the previous smoothed value. Note that α is set such that it minimises the mean squared errors.

t is the value observed at Y_t. Then, t is a seasonal component, b_t is the smoothing components of the trend t, L is the number of periods in a season, F_{t+m} is one forecast ahead of m periods, m is the number of forecast periods, α (Alpha) smoothes the parameter, β (Beta) is the seasonal smoothing parameter, and γ (Gamma) is the smoothing parameter of trend (8).

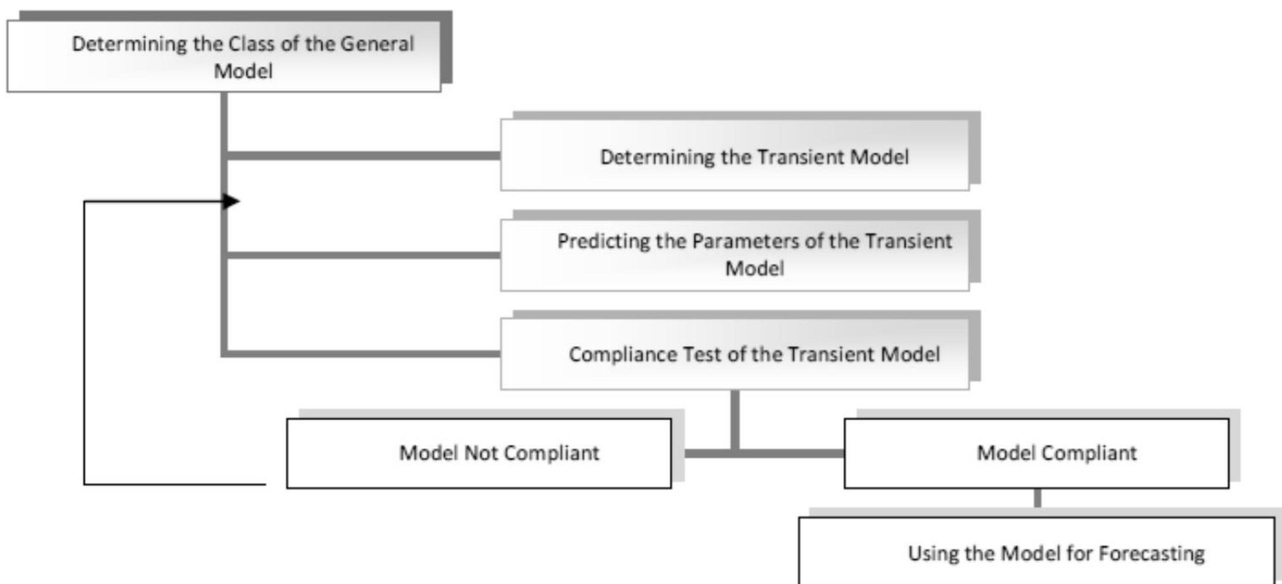

Figure 1. Model establishment process.

Valid criteria to test model validity:

- Forecast Error (e): $\mathbf{e}_t = \mathbf{A}_t - \mathbf{F}_t$ [7]
- Mean Forecast Error (MFE or Bias): $MFE = \frac{\sum_{i=1}^{n}(e_i)}{n}$ [8]
- Mean Absolute Deviation (MAD): $MAD = \frac{\sum_{i=1}^{n}|e_i|}{n}$ [9]
- Mean Absolute Percentage Error (MAPE): $M = \frac{1}{n}\sum_{t=1}^{n}\left|\frac{A_t - F_t}{A_t}\right|$ [10]
- Mean Square Error (MSE): $MSE = \frac{\sum_{t=1}^{N} E_t^2}{n}$ [11]

Results

A time series analysis of the honey prices (in TRY and US$) between 1998 and 2018 was conducted to forecast the future prices of honey. Time series graph of honey prices is given in Figure 2.

Figure 2 shows that the series of TRY prices has an increasing trend, along with some fluctuations. Seasonal effects and trends in the time series prevent the series from being stationary. Autocorrelation function (ACF) and partial autocorrelation function (PACF) graphs are presented in Figure-3 for understanding the stability in the series. It is understood that the series is not stationary because there are more than one delays outside the confidence limits. In order for the series to be stationary and to decrease the difference between the values, the logarithm of the series was taken and the difference was continued until the series was stationary. It is concluded that after the difference for the trend, the series becomes stationary. The time series graph of honey prices obtained after taking its first difference is given in Figure 4, and the ACF and PACF graphs are given in Figure 5.

When the ACF and PACF graphs are examined in Figure 5, it is seen that the two delays exceed the confidence limit and the other two delays are close to the confidence limit. It can be said that the series has become static. In addition, the stability analysis of the series was performed by Augmented Dickey Fuller (ADF) unit root test (14). According to the ADF test result, the series was not stationary (t = -0,258; P = 0.111) before the difference was obtained, whereas after the difference-taking process the series became stable (t = -14,789; P = 0.001).

Several different models were tested and the most suitable model was found to be SARIMA (2,1,2) (1,0,1) for TRY-denominated prices and SARIMA (0,1,3) (0,0,0) for US$-denominated prices.

Figure 2. Time series graph of honey prices (TRY-US$).

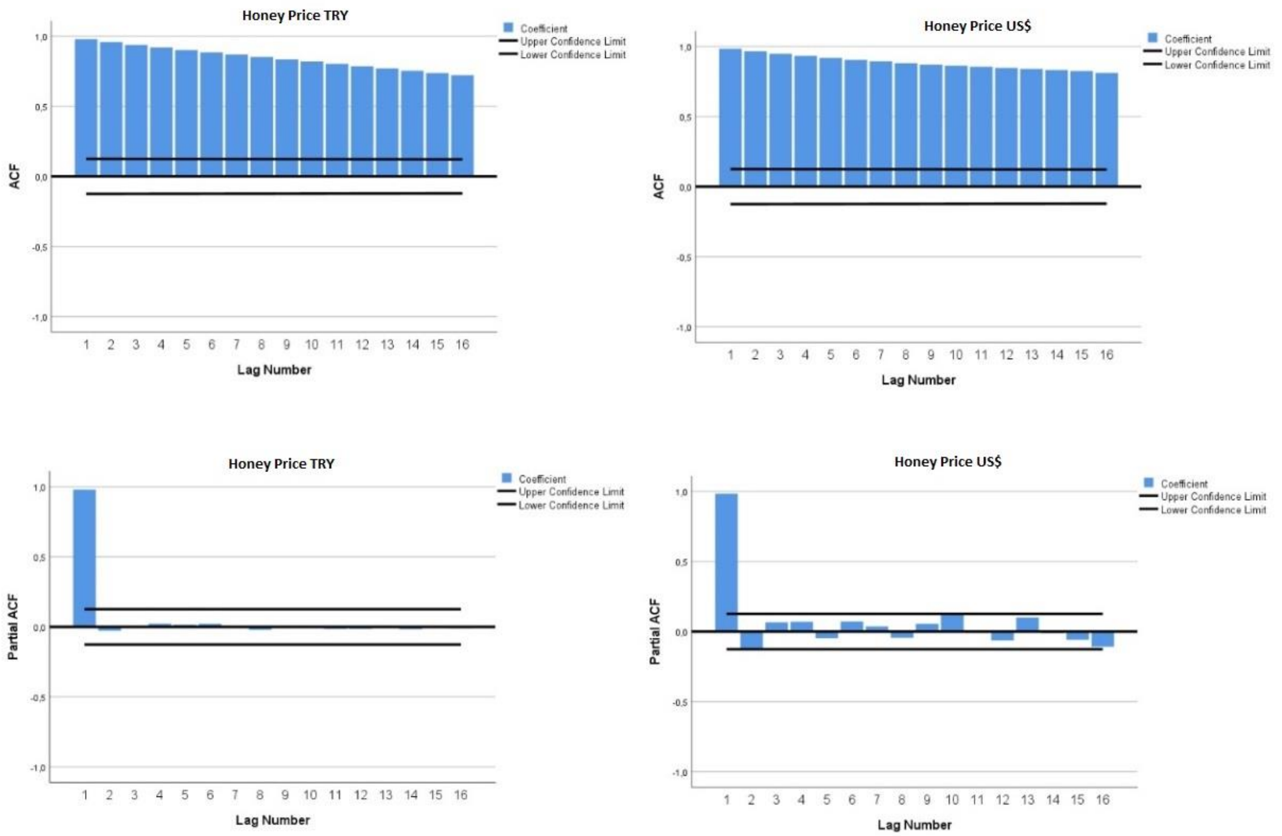

Figure 3. Autocorrelation function (ACF) and partial autocorrelation function (PACF) graph of honey series.

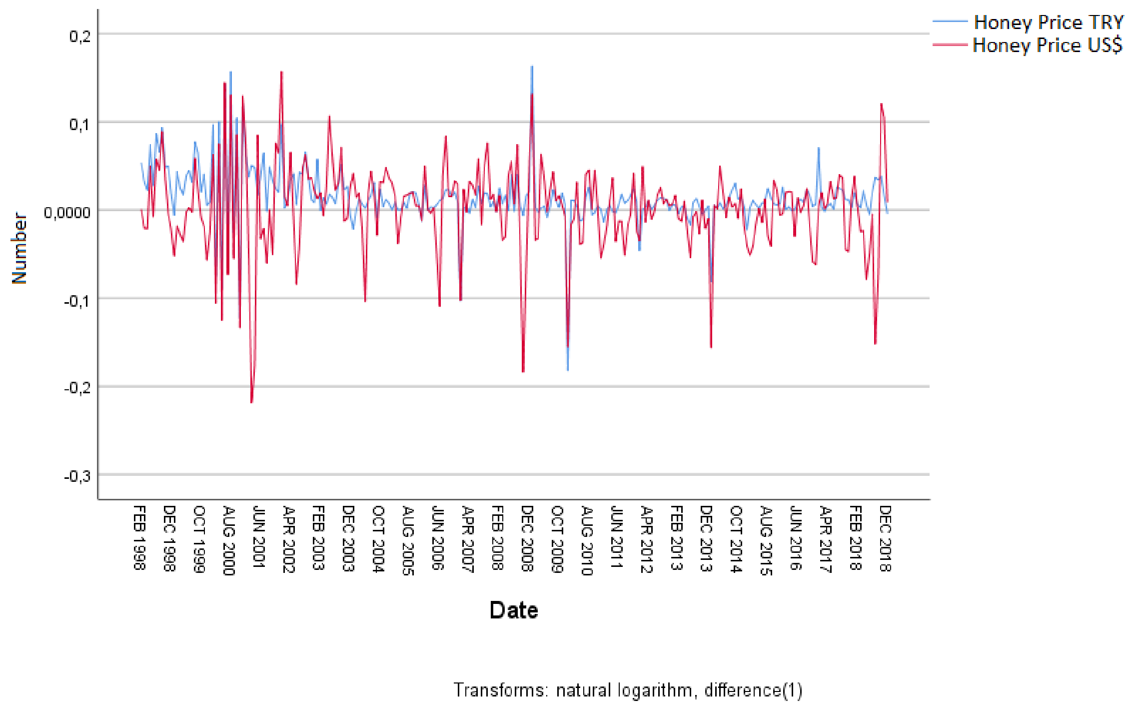

Figure 4. Graph of time series taken from the difference of honey prices.

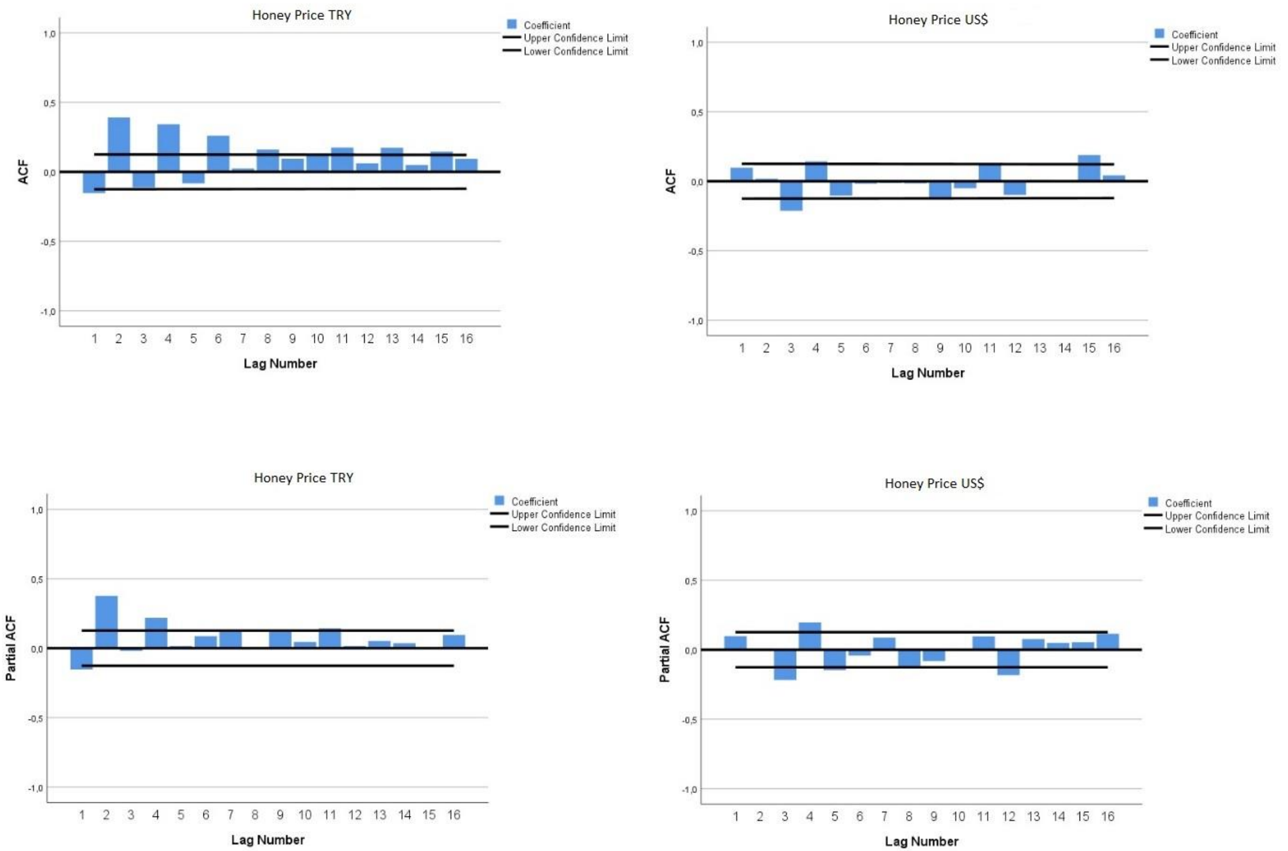

Figure 5. Autocorrelation function (ACF) and partial autocorrelation function (PACF) graph of the difference series of honey prices.

Table 1 illustrates the forecast values of honey prices until 2020. Honey prices (TRY/kg) are expected to follow an increasing trend between 2019 and 2020, similarly to that in the previous years. Accordingly, honey price is forecast to range between 30.07 and 75.81 TRY/kg as of December 2020 with an increase of 44% (average forecast value 49.08 TRY/kg). As is seen in Figure 6 where the results of the model SARIMA(2,1,2)(1,0,1) are shown, honey prices have been in a drastic increasing trend after 2014. The graph of honey prices in US$ indicates that the prices have a constant trend similar to the previous years. Results of the model SARIMA(0,1,3)(0,0,0) indicate that honey prices peaked in 2009 after which they followed a decreasing trend (Figure 6). The average value of honey prices as of December 2020 was forecast to be 6.15 US$/kg (3.87-8.79 US$).

The goodness of fit of the obtained models is examined in comparison with a criterion known as R^2 (determination coefficient). The values are between 0-1 and the values are close to 1. The stationary R^2 is a measure that compares the stationary part of the model with the basic model. It is preferred in case of trend or seasonal structure in the series. RMSE is the square root of the mean squares error. It refers to how different the model is from the level estimated by the model in the dependent series. Smaller values indicate better model estimates. MAPE is the mean absolute percentage error. It is independent of the units of the series and can therefore be used to compare different series. MAE stands for mean absolute error and is expressed in the original unit of the series. MaxAPE is maximum absolute percentage error. It shows the maximum error among the forecast values and is expressed as a percentage. Therefore, it is independent of the unit used. It is useful for imagining a worst-case scenario for the forecasts. MaxAE measures maximum absolute error, expressed in the same unit as that of the dependent series. Normalized BIC (Bayesian information criteria) is a general measure of the overall fit of a model. It is used to make a comparison between different models for a series, with the low values suggesting a better model (1).

From Table 2, it is clear that the Box-Jenkins models developed for honey prices in TRY and US$ are statistically significant (P=0.045 and P=0.001). MAPE value indicates that the series have quite usable forecasts.

Table 1. Monthly honey prices forecast values according to Box Jenkins models

Date month/year	Predicted honey price (TRY/kg) SARIMA (2,1,2)(1,0,1)	Lower confidence limit	Upper confidence limit	Predicted honey price (US$/kg) SARIMA (0,1,3)(0,0,0)	Lower confidence limit	Upper confidence limit
Jan 2019	34.07	32.12	36.10	6.17	5.55	6.81
Feb 2019	34.43	31.91	37.10	6.08	5.22	6.99
Mar 2019	34.91	31.60	38.47	6.10	5.04	7.22
Apr 2019	35.35	31.50	39.53	6.10	4.95	7.33
May 2019	35.79	31.28	40.78	6.10	4.87	7.43
Jun 2019	36.29	31.22	41.95	6.10	4.79	7.53
Jul 2019	36.96	31.22	43.46	6.11	4.72	7.62
Aug 2019	37.82	31.43	45.13	6.11	4.65	7.71
Sep 2019	38.64	31.54	46.86	6.11	4.58	7.79
Oct 2019	39.33	31.58	48.42	6.11	4.52	7.87
Nov 2019	39.88	31.44	49.88	6.12	4.46	7.95
Dec 2019	40.42	31.34	51.34	6.12	4.41	8.03
Jan 2020	40.88	31.10	52.78	6.12	4.36	8.10
Feb 2020	41.47	30.98	54.38	6.12	4.30	8.17
Mar 2020	41.97	30.76	55.95	6.13	4.25	8.24
Apr 2020	42.62	30.66	57.73	6.13	4.21	8.3
May 2020	43.12	30.43	59.38	6.13	4.16	8.37
Jun 2020	43.83	30.34	61.33	6.13	4.12	8.43
Jul 2020	44.62	30.28	63.47	6.13	4.07	8.50
Aug 2020	45.74	30.43	66.14	6.14	4.03	8.56
Sep 2020	46.73	30.47	68.70	6.14	3.99	8.62
Oct 2020	47.66	30.44	71.21	6.14	3.95	8.68
Nov 2020	48.34	30.24	73.43	6.14	3.91	8.74
Dec 2020	49.08	30.07	75.81	6.15	3.87	8.79

*The calculations made in Turkish Lira (TRY) were converted into US$ using the exchange rate in the relevant period (Avarage exchange rate for 2018: US$1= ₺4.74).

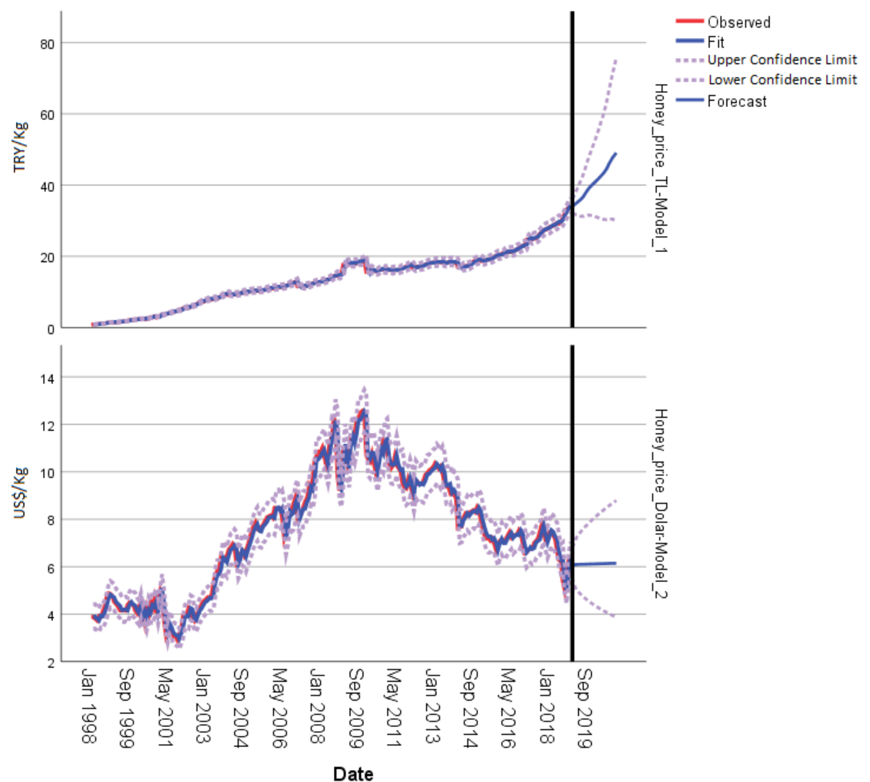

Figure 6. The price of honey Box Jenkins model forecast chart.

Table 2. Box jenkins model fit criteria

Model	Model fit statistics								Ljung-Box Q(18)		
	Stationary R-squared	R-squared	RMSE	MAPE	MAE	MaxAPE	MaxAE	Normalized BIC	Statistics	DF	P
Honey price TRY SARIMA (2,1,2)(1,0,1)	0.244	0.997	0.392	1.751	0.191	22.392	3.518	-1.741	10.582	12	0.045
Honey price US$ SARIMA (0,1,3)(0,0,0)	0.040	0.979	0.357	3.690	0.252	21.128	1.971	-2.041	45.487	17	0.001

Discussion and Conclusion

In Turkey, the production of apicultural products is one of the branches of production that has a high competitiveness among animal products (24). In order for businesses in this sector to plan their production and manage their risks, they need to have knowledge of apicultural practices as well as forecasts for the future of the sector. The models developed by statistical methods enable such forecasts. Since the data are a series of observations made at certain time intervals, they are modelled by time series analysis (16).

A study forecasting the supply of and demand for honey in Turkey using the Box-Jenkins model predicted that the supply of and demand for honey would increase from 1.54 kg/person to 1.40 kg/person in 2020 and 2023, respectively (26).

Using the time series analysis, it was forecast that honey production would increase to 115 thousand tons and the number of colonies would reach 10 million in 2020 (28). Using the ARIMA model, it was forecast that in 2020 the honey production, number of hives and beeswax production would reach 14.6 thousand tons, 1,226 thousand and 552.9 tons, respectively (18).

In a study conducted to forecast honey production in Turkey and to identify the type of model that gives the most successful results, the most suitable forecast model was found to be ARIMA(0,1,1), and using this model it was forecast that honey production in Turkey would constantly increase, reaching 106.410 tons in 2019 and 107.887 tons in 2020 (9). In a study conducted to forecast honey production using the ARIMA model and time series data, the most suitable forecast model was found to be ARIMA(0,1,1), and using this model it was forecast that honey production in Turkey would constantly increase between 2017 and 2023, reaching 121.216 tons in 2023 (6). Another study conducted in the Czech Republic predicted using the ARIMA model that the number of honey-producing hives would be 529.730, the number of beekeepers 47.373 and the price of honey 132 CZK/kg in 2011-2012 (30).

The present study used the honey prices in TRY and US$ between 1998 and 2018. Honey prices until 2020 were forecast using the SARIMA models selected in the study. According to the results of the analysis, average honey prices in Turkey were forecast to reach 37.82 TRY/kg and 6.11 US$/kg in August 2019 and further increase to 49.08 TRY/kg ve 6.15 US$/kg in December 2020 (Table 1).

Among the model fit criteria in Table 2, MAPE value suggests that the forecast model developed for honey prices has a percentage error of 1.751% for prices in TRY and 3.690% for prices in US$. The main reason for the difference between TRY and dollar MAPE value is that TRY is depreciating against the dollar. Forecasts with an MAPE value below 10% are considered good forecasts (33). So, it may be said that the forecast values obtained from both models developed for honey prices in TRY and US$ have a sufficient level of accuracy.

The top three exporters of honey in the international market are China, Argentina and Mexico. The main reason why they are leaders in this market is that they export at a price level lower than the average world price. While average honey export price in the world was $1.9/kg in 2006, the export prices of China, Argentina and Mexico were $1.3/kg, $1.4/kg and $1.9/kg, respectively (20). In the same year, Turkey exported honey at an average price of $2.3/kg. The price of honey exported by Turkey in 2016 is reported to be $4.11/kg (13).

Although the major factor affecting honey export is reported to be its price (20), increasing concerns, including over food reliability and residues, currently cause trade restrictions to be imposed on many countries in international trade. China is one of the major exporters on which restrictions are imposed (37). The restrictions on China are mostly imposed by European Union countries (20). Additionally, it is noted that consumers are willing to pay higher prices for quality honey (19). Particularly in EU countries, the origin, value and production technique of honey are reported to be the major factors affecting the consumers' decision to purchase honey (4, 21). Although Turkey seems to have disadvantages in honey export from

the perspective of honey price compared to China, Argentina and Mexico, it may gain an advantageous position, particularly in the EU market, as the honey produced in Turkey is of higher quality and the price forecasts in this study suggest that the USD-denominated price of Turkish honey may remain constant.

In conclusion, the forward forecasting of honey prices using time series data and the sharing of the up-to-date data with producers and producer associations at certain intervals will make a significant contribution to the development of a production and marketing strategy in Turkey, which is a leading country in the beekeeping sector. With timely and accurate price forecasts, producers can have the opportunity to develop their migratory beekeeping plans more precisely one year in advance. Additionally, they can increase their total sale revenues by developing a strategy regarding how much of the honey produced they will market via which marketing channel. If the beekeeping registration system is improved, price forecasts for various types of honey as well as other beekeeping products such as pollen and propolis can provide producers with motivating information for the next year with regard to which nectar flow they will go for and what kind of a migratory beekeeping route they will follow. Using the forecasts made with producer associations and public authorities, producers can be prompted to develop a production pattern and plan that is aimed at first meeting the domestic demand and then increasing export of high-quality beekeeping products.

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Conflict of Interest

The authors declared that there is no conflict of interest.

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Knowledge and attitudes in food safety and the occurrence of indicator bacteria on hands of food handlers at the point of pastrami sale

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Abstract: The aim this study was to evaluate food safety knowledge and attitudes of food handlers and to determine the presence of indicator bacteria on the hands of food handlers in the pastrami retail points in Kayseri, Turkey. Food handlers were interviewed and the samples were taken from their hands by touching the petri dishes. Food handlers' knowledge and attitudes were evaluated by questionnaires and checklists. Conventional methods were followed for microbiological analyses. The presence of total coliforms, *E. coli*, members of the family Enterobacteriaceae and *S. aureus* on the hands of food handlers were analyzed. Results indicated that coliforms were present in 37.3% of food handlers hands and 32% exceeded the limit when compared to the literature suggesting a target value of <2.5 cfu/cm^2. *E. coli,* Enterobacteriaceae and *S. aureus* were present in 10.6%, 44.6% and 34% of handler's hands, respectively. Food safety knowledge evaluation received high scores with mean score of 69.06%. Only one handler was below the score of 50%. Despite the high scores on food safety knowledge, high numbers of hand samples were found to be contaminated with pathogens. Data obtained from this study reveal that despite their adequate food safety knowledge, food handlers on pastrami retail points were found insufficient for implementation. Therefore, it is not only sufficient to provide food safety trainings but also the sources of problems, such as lack of hygiene infrastructure, in the practice should be identified and resolved in retail points.
Keywords: Food handler, food safety knowledge, indicator bacteria, pastrami.

Pastırma satış noktalarındaki gıda çalışanlarının gıda güvenliği bilgi ve tutumları ve ellerindeki indikatör bakteri varlığının belirlenmesi

Özet: Bu çalışmanın amacı, Kayseri'de bulunan pastırma satış noktalarındaki gıda çalışanlarının gıda güvenliği bilgi ve tutumlarını değerlendirmek ve ellerindeki indikatör bakterileri varlığını tespit etmektir. Gıda çalışanlarıyla görüşülmüş ve çalışanların ellerinden petrilere dokundurularak örnek alınmıştır. Gıda çalışanlarının gıda güvenliği bilgi ve tutumları, anketler ve kontrol listeleri ile değerlendirilmiştir. Mikrobiyolojik analizler için geleneksel yöntemler takip edilmiştir. Gıda çalışanlarının ellerinde total koliform, *E. coli*, Enterobacteriaceae türleri ve *S. aureus* analiz edilmiştir. Elde edilen sonuçlara göre gıda çalışanlarının %37,3'ünün el örneklerinde koliform bulunmuş ve %32'sinin literatürde koliformlar için limit değer olarak belirtilen <2,5 cfu/cm^2'yi aştığı belirlenmiştir. *E. coli*, Enterobacteriaceae türleri ve *S. aureus*, gıda çalışanlarına ait el örneklerinin sırasıyla % 10,6, % 44,6 ve % 34'ünde bulunmuştur. Gıda güvenliği bilgi değerlendirme anket çalışması, ortalama % 69,06 skoru ve % 50 puanın altında tek gıda çalışanı ile yüksek puanlar almıştır. Gıda güvenliği bilgisine dair yüksek puanlara rağmen, gıda çalışanlarına ait el örneklerinin patojenlerle kontaminasyon düzeyleri oldukça yüksek bulunmuştur. Bu çalışmadan elde edilen veriler, Türkiye'de pastırma satış noktalarında çalışanların gıda güvenliği hakkında yeterli bilgiye sahip olmalarına rağmen uygulama noktasında yetersiz kaldıklarını ortaya koymaktadır. Dolayısıyla sadece gıda güvenliği eğitimi vermek yeterli olmamakta, satış yerlerindeki hijyen altyapı yetersizliği gibi uygulamadaki aksaklık nedenlerinin da belirlenerek çözülmesi de gerekmektedir.
Anahtar sözcükler: Gıda çalışanı, gıda güvenliği bilgisi, indikatör bakteri, pastırma.

Introduction

Pastrami is a raw, cured, edible paste coated and commonly consumed ready-to-eat (RTE) meat product in Turkey. It is often sliced now of sale by food handlers. RTE products that have been associated with several outbreaks are recognized to be contaminated during slicing (32).

Food borne diseases remain the significant public health problem although improvements in health systems and the increase in food hygiene training programs in developed countries. A report from the USA indicates annually 76 million food borne diseases with 5200 cases of mortality (6).

Food handlers should have the necessary knowledge and skills to enable them to apply hygiene practices that are obligatory in EU (27). According to EU regulation no: 852/2004 on the hygiene of food staff, it is required for food handlers to be trained on food hygiene according to their work activities. Besides, food business operators are required to ensure the hygiene requirements at all stages of food production (34). Hence, great emphasis on managers was underlined to provide food hygiene training program for food handlers and to monitor their performance at work place (19). Previous data on risk factors for food borne disease suggest that, most of the outbreaks occur through undesirable food handling practices of food handlers that cannot be easily overturned, even by the training programs (9,16,37,38).

Presences of indicator organisms are commonly used to evaluate the effectiveness of the sanitation programs (17,30). Some pathogens including *S. aureus* and *E. coli* have shown to survive on hands and surfaces even for days after the contact with the organism (20,22).

The safety of pastrami should be guaranteed in every stage of food chain to protect public health. Lack of knowledge or negligence of food handlers are predisposing factors in increasing prevalence of food borne disease. Assessment of food handler's knowledge and practices are essential to provide required data for further action that are previously studied by some other reporters (4,19,21,27,28,40). There is need to understand all factors contributing to poor hygiene practices at the point of sale. Therefore, this study is designed to evaluate the food handling attitudes and food safety knowledge of pastrami handlers and to highlight the importance of hand hygiene in pastrami contamination in Kayseri, Turkey.

Materials and Methods

This study was designed among pastrami handlers at retail points where pastrami is often sliced at moment of sale in Kayseri, Turkey. Total of 150 retail points located in city center were visited. Samples were collected from 150 food handler's forefingers and thumps during working hours without previous notification and were transported to laboratory in a cool box.

Rodac plates containing Baird Parker Agar (Merck 1.05406) with Egg-yolk Tellurite emulsion were used to sample forefingers for *S. aureus* enumeration. Agar plates were incubated at 35°C for 24-48 h and black colonies with clear zones around were evaluated after being confirmed with coagulase test (3). *S. aureus* ATCC 25923 was used as positive control.

Rodac plates containing Chromocult Coliform Agar (Merck 1.10426) were used to sample thumbs; to enumerate total coliforms, *E. coli* and members of Enterobacteriaceae family. Plates were incubated at 35-37 °C for 24 h and colonies were differentiated presumptively on the basis of their appearance and color. In this context, colonies salmon to red in color were reported as total coliforms, dark blue to violet as *E. coli* and colorless ones as members of Enterobacteriaceae family (5,11).

A checklist was formed to evaluate food hygiene level in the establishments. Fourteen food construct items were selected including food handlers attitudes (4 items) and retail store conditions (10 items). The checklist was aiming to determine the food safety attitude of food handlers depending on the observations of the interviewer. Additionally, two questionnaires, modified from previous studies, were designed to determine the food safety knowledge and demographic characteristics of food handlers (14,28,40). The emphasis was put on hygiene practices, personal hygiene, food borne disease and temperature control. Handlers with the correct answers lower than 10 for 16 questions (≥62.5% accuracy) were considered to have "insufficient" knowledge and those with scores greater than 10 points were reported to have "Good" knowledge.

The population of the study consisted of 468 active markets and delicatessens operating in the central districts of Kayseri. Previsit observations revealed that almost half of the markets were retailing pastrami (N = 234). In the study, it was calculated that at least 145 retail points should be taken by using stratified sampling method according to districts with 95% confidence interval. The sample size of the study was determined as 150 pastrami retail points throughout Kayseri (41).

Calculation of sample size (n0) is given in the following equation;

$$n_0 = \frac{Nt^2pq}{d^2(N-1) + t^2pq} = \frac{234(1{,}96)^2 0.5 * 0.5}{0.05^2(234-1) + (1.96)^2 * 0.5 * 0.5} \cong 145$$

Representation power: 150/234=0,64

N = Population size; t-table value for t = 95% confidence interval = 1.96; p, q = The frequency of occurrence of the event of concern, agent being (+) and (-) p = 0.5, q = 0.5; d = Deviation according to the frequency of occurrence.

Table 1. Hand hygiene profiles of food handlers.

Microorganisms	n	No. of positive samples (%)	No. of positive samples exceeding the target value (cfu/cm^2)
*Enterobacteriaceae**	150	67 (44.6)	-
Coliforms		56 (37.3)	48 (32) ≥2.5
E. coli		16 (10.6)	16 (10.6) >1
*S. aureus**		51 (34.0)	-

n: Total number of hand samples from different pastrami store.
*: No target value

Results

No target values were found in Turkish Food Codex for bacteriological counts on food handlers hands. Therefore, only coliforms and *E. coli* positive samples were compared to the literature target values of ≥2.5 cfu/cm^2 and ≥1 cfu/cm^2, respectively. Table 1 summarizes the microbial contaminations on hands of pastrami food handlers.

When demographic characteristics of food handlers were considered, all food handlers (100%) analyzed were male. Approximately two third of the participants (65.2%) were aged between 25 and 45 years. Most of them were married (82.6%) and the education level of largest group (47.3%) was primary school. Two third of the participants had the experience more than 10 years on their work. The demographic characteristics of pastrami food handlers were presented in Table 2.

Table 2. Demographic characteristics of pastrami food handlers.

Characteristics	N	%
Gender		
Male	150	100
Age		
17-25	14	9.3
25-35	46	30.6
35-45	52	34.6
45-55	24	16
>55	14	9.3
Marital status		
Married	124	82.6
Education		
Primary school	71	47.3
12th year	63	42
University	16	10.6
Experience (years)		
0-10	50	33.3
10-25	58	38.6
>25	42	28
Position		
Owner	60	40.1
Staff	90	59.9

N: Number of retail points.

Table 3. Food safety construct items.

	n	%
Food Handler Attitude		
1. Glove use	14	9.3
2. Apron use	72	48
3. Nail length		
Trimmed short	134	89.3
Long	16	10.6
4. Wearing jewelry	22	14.6
Retail store conditions		
1. Type of retail store		
Producer	4	2.6
Market	20	13.3
Franchise	126	84
2. Retail store age		
≥10	56	37.3
<10	94	62.6
3. Number of Employees		
>5	12	8
2-5	90	60
1	48	32
4. Floor material		
Tile	126	84
Other	24	16
5. Wall material		
Tile	80	53.3
Other	70	46.6
6. Product variety to be sailed		
Only pastrami	124	82,6
Market (All meat and meat products)	26	17,3
7. Cutting board material		
Wood	116	77.3
Acrylic	32	21.3
Other	2	1.3
8. Knife haft material		
Wood	136	90.6
Acrylic	12	8
Other	2	1.3
9. Cleaning agent		
Detergent	118	78.6
Bleach	4	2.6
Detergent + Bleach	20	13.3
Other	8	5.3
10.Pastrami storage conditions		
Refrigerator temperature	96	64
Room temperature	12	8
Both	42	28

n: Number of retail points.

Table 4. Food safety knowledge of food handlers in pastrami retail points.

Statement	Correct Answer (%)
1. Hand washing before handling reduces food contamination risk	100
2. Food borne infections could be reduced if the food handlers properly wash their hands where necessary	88
3. Disposable gloves should be worn before handling pastrami	96
4. Proper cleaning and sanitation of utensils reduces the food contamination risk	96
5. Food and beverage consumption by handlers in the work place increases the food contamination risk	44
6. Previous preparing of food prevents food contamination	36
7. Repetitive heating of cooked food can increase contamination risk	80
8. Foods must be stored at 5 °C	80
9. Periodical check of refrigerator temperatures could reduce the microbial growth in food	85
10. Freezing could kill all the bacteria in food	40
11. Contaminated food always changes in color, odor and taste	8
12. In case of infectious wounds on skin, take a break from work is necessary	92
13. Is it possible that your diarrheal infection threatens consumers health	44
14. Healthy people could also carry pathogen microorganisms on their skin, nose and mouth	84
15. Healthy adults and the people at risk group (children, pregnant woman, elderly) can be considered at equal risk for food borne disease	64
16. Food-borne diseases could be the cause of abortion in pregnant woman	68

Mean: 69.06%

The majority of food handlers were recorded to not to use gloves (90.7%) and not to wear (52%) aprons during handling pastrami. In addition, 10.6% of them had long nails and 14.6% wore jewelry. Checklist of food safety construct items (food handler attitude and retail store conditions) and their corresponding scores were summarized in Table 3. Within the scope of the current study, 60% of attendants declared that they received formal food hygiene training (Data not shown).

The average of correct answers for the questionnaire was 11, corresponding to 69.06% of questions answered correctly. The maximum score was 15 (93.75%) and the minimum was 6 (37.5%). Table 4 shows the percentage of correct answers obtained for each question. Except for questions 5, 6, 10, 11 and 13 (given bold in Table 4), all other questions received high percentage of correct answers (above 50%). The highest scores achieved by food handlers in this study focused on general cleaning procedures.

Almost all of the food handlers (92%) in our study believed that contaminated food could be distinguished by sensory properties. Half of them (56%) did not believe that food and beverage consumption in work place and their diarrheal infections threaten consumer's health. The lowest level of knowledge was obtained from the topics closely related to food safety issues especially source of food contamination (questions 5, 13) and sensory properties of contaminated food (question 11).

Questions, emphasizing hand washing before handling had highest percentage of correct answers followed by questions related to wearing gloves and cleaning of utensils, respectively.

Discussion and Conclusion

RTE food handlers has critical role to ensure food safety and to prevent food borne diseases. As stated by Codex Alimentarius (10), all individuals in contact with food must be qualified and must recognize their role and responsibility in not to contaminate food. Likewise, every food producing point should provide a training program.

S. aureus is the very common pathogen reaching RTE food by food handlers who carry this agent on their nasal cavity or skin (1,33). Nose flora often contaminates fingers and back side of hands (15). Being the permanent flora of human skin, *S. aureus* could not be removed even after proper hand washing practices therefore no fixed acceptable contamination level is available for this agent (2). As shown in Table 1, *S. aureus* were isolated from 34% of the sampled population's hands ranging from 1 to 4.0×10^1 cfu/cm^2. Higher results were obtained by Lues et al. (24) and Soares et al. (40) who reported 88% and 53.3% of food handler's hands to be contaminated with *S. aureus* and coagulase-positive staphylococci, respectively.

In contrast to *S. aureus*, Enterobacteriaceae numbers could be reduced by proper hand washing. Therefore, its presence points out to the possible presence of *Salmonella*, *Shigella*, *Yersinia*, *Proteus*, *Klebsiella* spp. and others (31). In this study, Enterobacteriaceae was detected from 44.6% of hands ranging from 5 to 20 cfu/cm^2. Higher results were obtained in a comparable study, reporting

Enterobacteriaceae to be isolated from 56% of food handler's hands in South Africa (24). Enterobacteriaceae presence on hands is reported to not to be a good indicator of personal and toilet hygiene as hands might be contaminated with Enterobacteriaceae irrelevant to toilet use and could be reduced by hand washing (12).

Coliforms are often used to evaluate the efficiency of sanitation programs as their presence indicates the increased risk of pathogen existence. For different kind of surfaces, target value was suggested as <2.5 cfu/cm^2 (30). In this study, 32% of hands were found to exceed the target value for coliforms, which reveals the improper personal hygiene. Lues et al. (24) and Campos et al. (8) also reported 40% and 55.6% of retail food handler's hands to be contaminated with coliforms which are higher than our results.

Being normally absent on hands, *E. coli* is regarded as the better indicator of the fecal contamination and enteric pathogens compared to all Enterobacteriaceae family members (12). *E. coli* was detected from 16% of the food handler's thumbs in this study. *E. coli* presence on the handler's hands is worthy of consideration as these isolates could involve Enterohaemorrhagic *E. coli* O111 or *E. coli* O157:H7. In the view of the literature, the specified limit for *E. coli* on surfaces is 1 cfu/cm^2 (23). High prevalence rate of the indicator bacteria in hands and some inappropriate attitudes observed in this study could be sourced from poor working conditions, and lack of periodical training. Most of the retail points were observed to have no hand washing sink to be used where needed.

All of the participants were male in this study. On the contrary Santos et al. (35) and Martins et al. (27) report 85% and 96% of participants to be female in Portugal, respectively. In the present study, about two third of the participants (65.2%) were aged between 25 and 45 years. Most of them were married (82.6%) and the education level of largest group (47.3%) was primary school. Two third of the participants had the experience more than 10 years on their work. Similarly, Marais et al. (25) reported more than 60% of managers to have >5 years of experience in food industry. Hand hygiene profiles of experienced food handlers were reported to be more satisfactory than the hands of inexperienced staff (23). It is indicated that experienced staff are less likely to participate training activities (43).

Most of the food handlers were observed to not to use gloves (90.7%) and more than half of the participants were not wearing aprons during handling pastrami. Although food handlers knew when gloves needed to be worn, in practice this remains a problem, as they state that wearing gloves during pastrami slicing does not allow to slice thinner as preferred by consumers. In this regard hand hygiene and proper glove and apron use by food handlers need to be emphasized within the frame of the training program. On the contrary, Eren et al. (13) indicated that most of the food handlers had proper attitudes during food preparation including wearing aprons and gloves, using different cutting boards and knifes for raw meat and salads.

In this study, 10.6% of them had long nails and 14.6% wore jewelry. These results on long nails are lower than that reported by Miranda et al. (29) and Campos et al. (8) reporting 41.7% and 81.5% of long nailed food handlers respectively.

In this study, 60% of attendants declared that they received formal food hygiene training (Data not shown). Such training was done as once-off activity without refreshing training. Similarly, Baş et al. (4) reported that almost 52% of participants received food safety training in Turkey and the food safety knowledge score was 43.4. Campos et al. (8) in Brazil and Silva et al. (39) in Spain stated that only 35.9% and 43.3% of food handlers received periodical training, respectively. In this study, 79.3% (119/150) of the participants had the score above 62.5% and were reported to have "good knowledge" on food safety. Only one food handler had the score below 50% (Data not shown).

Food handlers who participated in the study answered 69.06% of questions correctly. This is higher than that obtained by Gomes-Neves et al. (14) and Martins et al. (27) who reported 62% and 56.5% of handlers had correct answers in Portugal respectively. Similarly, Marais et al. (25) obtained 46% of correct answers from their food hygiene knowledge study on food handlers in South Africa which is similar to Baş et al. (4) with the mean score of 43.4% in Turkey. On the other hand, Yardımcı et al. (44) reported that food staff achieved 76.5% of the total score from personal hygiene, 73.3% of food hygiene, and 76.6% of kitchen and equipment hygiene knowledge tests.

In this study the lowest level of knowledge was obtained from the topics closely related to food safety issues especially source of food contamination (questions 5, 13) and sensory properties of contaminated food (question 11) which is different from those obtained by Jevsnik et al. (19), Santos et al. (35), Marais et al. (25) and Martins et al. (27) reporting that food handlers have significantly lower level of knowledge on the role of temperature in maintaining food safety. These results suggest significantly lower level of knowledge and understanding probably due to lack of appropriate hygiene training on these topics. Low level of knowledge may result in decreased level of concern during food handling and promote wrong practices (27).

In our study, 92% of attendants believe that contaminated food could be distinguished by visual, olfactory or taste properties. These results are in same line with Gomes-Neves et al. (14), Walker et al. (42) and

Jevsnik et al. (19) with 55.7%, 57% and 52.5% of incorrect results for the similar questions respectively.

In this study, 56% of food handlers did not believe that food and beverage consumption in work place and their diarrheal infections threaten consumer's health which implies that they do not have perceptions on the sources of food contaminations. According to Codex Alimentarius (10), food handlers should not eat, handle money or engage in any act that could contaminate the food during performance of their activities. Lack of knowledge on contamination sources might increase improper implementations in work place which in turn could promote microbial hazards and food borne diseases.

Other topics having low scores from food handlers were; freezing and in advance preparing of food. More than half of respondents (60%) believed that freezing process could kill all the bacteria in the food. Baş et al. (4) also reported deficit knowledge on the critical temperature of food preservation among food handlers.

In our study, questions emphasizing hand washing had highest score followed by questions related to wearing gloves and cleaning of utensils, respectively. Our results are compatible to the works of Martins et al. (27) and Marais et al. (25) reporting the best results from the issues of cleaning and sanitation. Although 96% of participants had the correct answers on glove use, their glove use practice does not reach this score. Seaman and Eves (36) underline that training could only be effective if the knowledge leads to required changes in behavior during food handling.

In this study, food handler's awareness on not handling food in case of infectious wounds on their skin was evident (92%). Most of food handlers (64-68%) gave correct answers to the questions referring to high risk population group.

Result of this study revealed that although the food handlers have the satisfactory knowledge on food safety, hand hygiene profiles do not seem to be parallel to their questionnaire results. A possible explanation for these findings may be related to low educational level of food handlers. This finding corroborates with the results of Baş et al. (4) who indicate that training may cause an increase in knowledge on food safety however may not always give rise to a positive change in behavior. Similarly, no significant relation was reported between the hygiene training of food handlers and hygiene standards of retail points (42). It is a great concern in food industry to encourage food handlers implement the principles they learnt on food safety (26). However, Cakiroglu and Ucar (7) reported inadequate knowledge of hygiene perception among food handlers and they underlined the immediate need for training on the subject.

The reality of the retail points exhibited that despite the fact of high level of food safety knowledge, food handler's hand hygiene profiles were not reflecting the same results. The main problems identified within the frame of this study were poor working conditions, lack of hand washing infrastructure in the retail points and lack of specific, periodic and formal training among food handlers although it is legal requirement in Turkey. Evaluation of the effectiveness of training is an important part of food safety, as stated by ISO 22000:2005 (18).

Data obtained from this study reveal that despite their adequate food safety knowledge, food handlers on pastrami retail points were lack of food safety practices. Therefore, it is not only sufficient to provide food safety trainings, but also the sources of failures in the practice should be identified and resolved.

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Conflict of Interest

All authors declare that they have no conflicts of interest.

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Three-dimensional tomographic reconstruction and morphometric analysis of skull in gazelles (*Gazella subgutturosa*)

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Abstract: This study was carried out to determine the osteometric features of the skull by using three dimensional computed tomography images in gazelles (*Gazelle subgutturosa*). In the study, nine skull samples of adult gazelles (*Gazella subgutturosa*) were used. Images of the skull sections of 0.625 mm thickness were acquired by using a computer tomography device with 64 detectors applying 80 kV, 200 mA and 639 mGY. Three-dimensional images of the skull samples were reconstructed and morphometric measurements (39 linear, 1 volumetric and 1 surface area) were performed by using the software program MIMICS 12.1. Mean skull volumes in males and females were found to be 115.74±2.43 cm^3 and 87.69±1.09 cm^3 while the mean skull surface areas in males and females were 79.62±8.56 cm^2 and 77.34±1.18 cm^2, respectively. Significant differences between males and females for median frontal length (MFL), frontal length (FRL), upper neurocranium length (UNCL), greatest length of the lacrimal bone (GLLB), oral palatal length (OPL), length of the upper molar row (LUMR) and the greatest neurocranium breadth (GNCB) were observed. The difference in the cranial index between males and females was statistically significant (P<0.01). The data obtained in this study will contribute to detect differences between the gazelles and other species with respect to skull morphometry.
Keywords: Computed tomography, gazelle, morphometry, reconstruction, skull.

Ceylanlarda (*Gazella subgutturosa*) kafatasının üç boyutlu tomografik rekonstruksiyonu ve morfometrik analizi

Özet: Bu çalışma; ceylan kafatasının bilgisayarlı tomografi görüntülerini kullanılarak kafatası kemiklerinin osteometrik özelliklerini belirlemek amacıyla yapılmıştır. Çalışmada 9 adet erişkin ceylan (*Gazella subgutturosa*) kafatası kullanıldı. Kafataslarının 64 dedektörlü CT cihazı ile 80 kv, 200 MA, 639 mGY ve 0,625 mm kesit kalınlığında görüntüleri alındı. Bu görüntüler MIMICS 12.1 programı yardımıyla üç boyutlu yapıya dönüştürülerek morfometrik ölçümleri (39 linear, 1 hacim ve 1 yüzey alanı) yapıldı. Erkeklerde kafatasının ortalama hacim değeri 115,74±2,43 cm^3, dişilerde 87,69±1,09 cm^3 olarak tespit edilirken cranium'un ortalama yüzey alanı erkeklerde 79,62±8,56 cm^2, dişilerde 77,34±1,18 cm^2 olarak bulundu. Çalışmada, median frontal uzunluk (MFL), frontal uzunluk (FRL), üst neurocranium uzunluğu (UNCL), lacrimal kemiğin maximum uzunluğu (GLLB), oral palatal uzunluk (OPL), üst molar diş sırası uzunluğu (LUMR) ve en büyük neurocranium genişliği (GNCB) parametrelerinde dişi ve erkekler arasındaki farklar istatistiksel olarak anlamlı bulundu. Cranial index değeri açısından dişi ve erkekler arasındaki fark istatistiksel olarak anlamlıydı (P<0,01). Çalışmada elde edilen bilgilerin ceylan türlerinin tipolojisi ile diğer türlerle arasındaki farklılıkların tespitine katkı sunacağı düşünülmektedir.
Anahtar sözcükler: Bilgisayarlı tomografi, ceylan, kafatası, morfometri, rekonstruksiyon.

Introduction

Even among the closely related species, there are apparent differences in the skeletal systems. These differences are crucial for taxonomic classification of species and for evaluation of the archaeological or forensic findings (26). Skull is the most studied bone for reconstructing the evolutional taxonomy. However, the assignment of the species based on skull characteristics is difficult due to variation within species (1). Knowledge of cranial morphometry is also important for the diagnosis of cranial or dental deformities for designing implants or dental instruments (26, 27).

Three different techniques have been used for obtaining osteometric parameters. The first is the measurement of bones obtained from archaeological excavations or after maceration by using a compass (23). The second is the evaluation of the radiological images from the target region (16). The third one is the measurement of the images obtained by using computer tomography (CT), which is a recently more frequently used technique (27). Images of two-dimensional sections from CT are compiled to reconstruct a three dimensional (3D) image using special software programs (10, 22). The 3D modeling technique is widely employed in plastic surgery, orthopedic surgery, neurosurgery, traumatology and medical education (17).

Gazella is one of the most species-rich genus comprising numerous species within Bovidae (1). Gazelles in Sanliurfa belongs to *Gazella subgutturosa*, which has a wide distribution area ranging from China to North Africa. Since the second half of 20th - century number of the gazelles have rapidly declined due to human activities including habitat destruction, expansion of the agricultural areas, hunting, etc. (19).

Several morphometric studies have been performed for establishing a comprehensive and reliable database in gazelles (1, 9, 31). The objective of this study was to morphometrically analyze the skulls of gazelles by using the CT images in order to provide species specific data that can be used by veterinary clinicians for managing pathological formations on the skull.

Material and Methods

Animal material: In the study nine cadavers (5 females and 4 males) of adult gazelles were used. Body weights of the cadavers were among 11.4 - 18.1 kg. The cadavers were submitted to the clinics of Harran University Animal Hospital in Sanliurfa province of Turkey for treatment yet died for various reasons. The animals had no clinical or pathological skull problems. The use of the cadavers was approved by the General Directorate of Nature Conservation and National Parks-Turkey (Approval no: 2017/209842) and Harran University Animal Experimentation Local Ethics Committee (Approval no: 2018/006-11).

CT-Imaging, reconstruction and morphometric analysis: For obtaining the CT images the gazelle cadavers were placed on a sternal position into a CT device with 64 detectors (GE Company, USA) . Images of the skull sections of 0.625 mm thickness were acquired by applying 80 kV, 200 mA, and 639 mGY. The CT images were stored in DICOM format and the 3D skull images were reconstructed using the basic module of the 3D modeling program MIMICS 20.1 (The Materialise Group, Leuven, Belgium). Osteometric measurements on the digital images were performed for 39 different parameters according to the measurement points reported in the literature (25, 29). Definitions and the abbreviations of the studied parameters were shown in Table 1. After morphometric measurements, volume and surface area of the skulls were estimated by excluding the horns and mandible. Further 6 different indices were calculated based on the craniometric measurements (Table 2). The definitions were based on Nomina Anatomica Veterinaria (20).

Statistical analysis: All morphometric parameters were expressed as Mean ± Standard Error (SE). The presence of significant differences between sexes was examined by using the Mann-Whitney U test. For statistical analyses SPSS, 17.0 was used.

Results

In this study, 39 linear parameters of the skull were measured (Figure 1-4). The mean ± standard error values for each parameter in males and females were shown in Table 3. Statistically significant differences ($P<0.05$) between males and females for MFL (median frontal length), FRL (frontal length), UNCL (upper neurocranium length), GLLB (greatest length of the lacrimal bone), OPL (oral palatal length), LUMR (length of the upper molar row) and GNCB (greatest neuro-cranium breadth) were observed.

Furthermore, cranial volume values in males and females were detected to be 115.74±2.43 cm^3 and 87.69±1.09 cm^3, respectively. The cranial surface area in males and females was 79.62±8.56 cm^2 and 77.34±1.18 cm^2, respectively (Table 4). The difference in mean cranial volume between males and females was significant while there was no difference in cranial surface area between sexes. Data on the skull indices have been shown in (Table 5). A statistically significant difference between males and females was observed only for cranial index values.

Table 1. Studied cranial parameters (according to Von den Driesch (30)).

Parameter	Abbreviation	Definition
1	TLS	Total length of the skull: the distance between akrokranion-prosthion
2	CBL	Condylobasal length: caudal border of occipital condyles-prosthion
3	TLCB	Total length of the cranial base: basion-prosthion
4	SSL	Short skull length: basion-premolare
5	PPL	Premolare-prosthion length
6	NCL	Neurocranium length: basion-nasion
7	ULVC	Upper length of the viscerocranium: nasion-prosthion
8	MFL	Median frontal length: akrokranion-nasion
9	ACBL	Akrokranion-bregma length
10	FRL	Frontal length: bregma-nasion
11	UNCL	Upper neurocranium length: akrokranion-supraorbitale
12	FCL	Facial length: supraorbitale-prosthion
13	ACIO	Akrokranion-infraorbitale length
14	GLLB	Greatest length of the lacrimal bone
15	GLNB	Greatest length of the nasal bone: nasion-rhinion
16	EOPL	Entorbitale-prosthion length
17	DOCI	Distance between the caudal border of occipital condyle and the infraorbitale
18	DTL	Dental length: postdentale-prosthion
19	OPL	Oral palatal length: palatinoorale-prosthion
20	LLPM	Lateral length of the premaxilla: nasointermaxillare-prosthion
21	LMTR	Length of the maxillary tooth row
22	LUMR	Length of the upper molar row
23	LUPR	Length of the upper premolar row
24	GIWO	Greatest inner width of the orbit: ectorbitale-entorbitale
25	GIHO	Greatest inner height of the orbit
26	GMB	Greatest mastoid breadth: otion-otion
27	GBOC	Greatest breadth of the occipital condyles
28	GBPP	Greatest breadth at the bases of the paracondylar processes
29	GBFM	Greatest breadth of the foramen magnum
30	HFM	Heigth of the foramen magnum: basion-opisthion
31	LBP	Least breadth of parietal
32	GBLH	Greatest breadth between the lateral borders of the horncore base
33	GNCB	Greatest neurocranium breadth: euryon-euryon
34	GFB	Greatest frontal breadth: ectorbitale-ectorbitale
35	LBO	Least breadth between the orbits: entorbitale-entorbitale
36	FCB	Facial breadth: between facial tuberosities
37	GBAN	Greatest breadth across the nasal bones
38	GBAP	Greatest breadth across the premaxilla
39	GPB	Greatest palatal breadth

Table 2. Indices and formulas of the skulls (According to Parés-Casanova (26)).

Studied indexes	Formulas
Skull index	greatest frontal breadth (var. 34) / total length of the skull (var. 1) ˣ 100
Cranial index	greatest neurocranium breadth (var. 33) / median frontal length (var. 8) ˣ 100
For. magnum index	height of the for. magnum (var. 30) / greatest breadth of the for. magnum (var. 29) ˣ 100.
Orbital index	orbital inner width (var. 24) / orbital inner height (var. 25) ˣ 100
Facial index	facial width (var. 36) / facial length (var. 12) ˣ 100.
Nasal index	greatest breadth across the nasals (var. 37) / greatest length of the nasals (var. 15) ˣ 100.

Table 3. The mean and standard deviations of the skull measurements (mm).

Parameter		General statistics			Females	Males	P
		Mean±SEM	Min.	Max.	Mean±SEM	Mean±SEM	
1.	TLS	165.59±2.78	155.29	178.92	164.40±4.89	167.08± 2.28	0.730
2.	CBL	163.87±2.85	156.03	180.27	162.70±4.43	165.34± 3.84	0.556
3.	TLCB	153.65±2.34	146.88	169.08	153.29±4.00	154.10± 2.43	0.556
4.	SSL	113.10±2.12	104.46	125.48	111.51±3.75	115.10± 1.04	0.190
5.	PPL	40.02±0.93	34.40	44.49	40.86± 1.15	38.97± 1.53	0.556
6.	NCL	103.56±1.52	100.01	113.93	105.51± 2.41	101.13± 0.80	0.190
7.	ULVC	83.71±1.88	73.97	92.27	84.01± 2.07	83.33± 3.75	0.905
8.	MFL	98.45±2.19	89.29	112.52	102.37± 2.66	93.55± 1.56	0.016
9.	ACBL	31.92±2.07	22.97	40.71	33.32± 3.27	30.17± 2.45	0.556
10.	FRL	82.41±2.91	71.92	97.85	88.27± 3.09	75.08± 1.58	0.032
11.	UNCL	72.83±3.29	55.76	90.14	79.14± 3.26	64.94± 3.15	0.016
12.	FCL	121.28±4.94	93.00	141.39	111.89± 5.02	133.03± 4.68	0.016
13.	ACIO	118.75±1.79	111.49	129.16	118.72± 2.73	118.78± 2.62	0.730
14.	GLLB	21.09±0.79	16.51	23.53	19.80± 1.11	22.71± 0.38	0.05
15.	GLNB	49.95±2.93	36.75	59.93	46.27± 4.49	54.55± 2.26	0.286
16.	EOPL	80.65±1.55	73.64	87.64	79.35± 2.30	82.28± 1.99	0.556
17.	DOCI	118.53±1.89	112.62	130.91	120.05± 3.20	116.63± 1.48	0.730
18.	DTL	94.40±1.95	88.18	105.11	96.98± 2.93	91.18± 1.51	0.111
19.	OPL	77.97±3.60	64.86	94.76	85.47± 3.72	68.60± 1.34	0.016
20.	LLPM	50.02±1.42	43.98	57.90	48.08± 1.48	52.45± 2.22	0.190
21.	LMTR	53.22±1.28	47.67	59.51	51.78± 1.70	55.02± 1.73	0.413
22.	LUMR	30.15±1.42	24.39	36.17	27.83± 1.70	33.04± 1.52	0.05
23.	LUPR	22.32±0.69	18.70	25.06	23.44± 0.60	20.91± 1.03	0.111
24.	GIWO	33.46±0.32	32.19	34.86	33.16± 0.52	33.83± 0.28	0.413
25.	GIHO	34.88±0.57	32.59	37.23	34.10± 0.54	35.84± 0.94	0.111
26.	GMB	51.95±1.04	46.46	55.96	52.76± 1.76	50.94± 0.83	0.413
27.	GBOC	33.92±1.57	27.31	40.84	34.13± 2.31	33.65± 2.41	1.000
28.	GBPP	48.28±1.67	39.79	54.17	48.12± 2.83	48.47± 1.83	1.000
29.	GBFM	16.28±0.36	14.57	18.35	15.87± 0.41	16.80± 0.57	0.190
30.	HFM	15.03±0.32	13.44	16.27	15.53± 0.27	14.42± 0.52	0.190
31.	LBP	35.74±0.96	30.88	40.14	36.42± 1.52	34.89± 1.11	0.413
32.	GBLH	55.75±2.25	49.95	65.15	-	55.75± 3.37	-
33.	GNCB	56.84±0.84	52.98	60.25	55.21± 0.75	58.88± 0.89	0.032
34.	GFB	69.16±2.24	57.12	75.93	68.95± 3.29	69.44± 3.47	0.905
35.	LBO	82.07±1.11	78.56	88.03	80.80± 1.83	83.66± 0.55	0.190
36.	FCB	56.47±1.12	51.96	63.40	56.79± 1.94	56.09± 1.10	1.000
37.	GBAN	24.60±1.19	19.32	28.82	23.30± 1.58	26.24± 1.64	0.286
38.	GBAP	28.96±1.17	21.45	33.91	27.84± 1.64	30.35± 1.61	0.730
39.	GPB	47.39±0.87	44.46	51.77	47.39± 1.24	47.40± 1.40	0.905

S.E.: Standard error of mean.

Table 4. The mean and standard deviations of the skull volume and surface area.

Parameter	General statistics			Females	Males	P
	Mean±SEM	Min.	Max.	Mean±SEM	Mean±SEM	
Volume (cm^3)	101.71±2.31	69.14	151.67	87.69±1.09	115.74±2.43	0.008
Area (cm^2)	78.48±9.80	61.31	91.17	77.34±1.18	79.62±8.56	NS

SEM: Standard error of mean, NS: Non significant.

Table 5. The mean and standard deviations of the craniofacial indices.

Index	General statistics			Females	Males	P
	Mean±SEM	Min.	Max.	Mean±SEM	Mean±SEM	
Skull	41.86±1.50	32.94	46.36	42.12±2.45	41.54±1.86	0.730
Cranial	58.01±1.74	49.87	65.60	54.03±1.08	62.98±1.18	0.016
For. magnum	92.84±3.35	73.24	108.92	98.16±3.53	86.18±4.49	0.111
Orbital	96.09±1.49	90.59	104.02	97.27±1.12	94.61±3.15	0.286
Facial	47.45±2.92	37.97	68.17	51.51±4.36	42.36±2.04	0.063
Nasal	50.30±3.17	38.44	64.27	51.67±4.70	48.60±4.67	0.556

SEM: Standard error of mean.

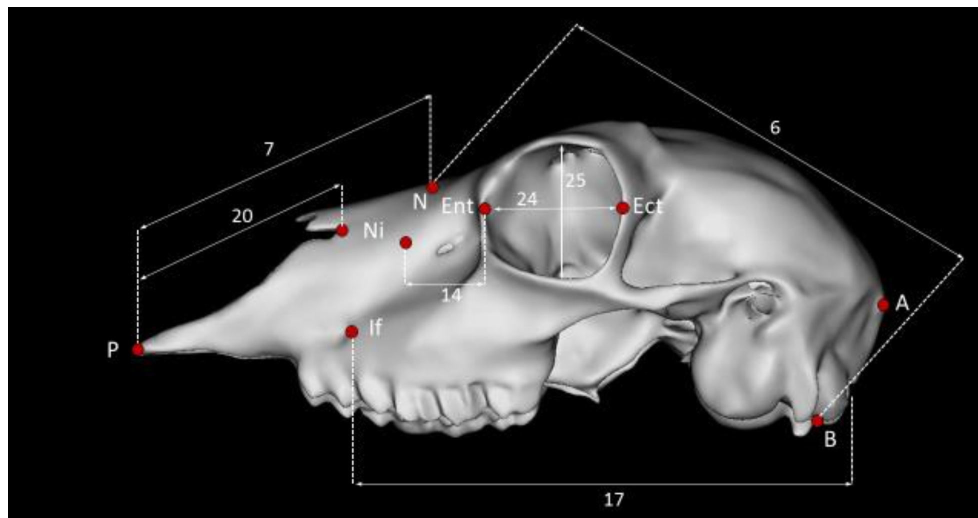

Figure 1. Measurement points of craniometric variables in the gazelle skull (lateral view).
A: Akrokranion, **Br:** Bregma, **Ect:** Ectorbitale, **Ent:** Entorbitale, **Ni:** Nasointermaxillare **If:** Infraorbitale, **N:** Nasion, **P:** Prosthion, **6:** Neurocranium length (NCL), **7:** Upper length of the viscerocranium (ULVC), **14:** Greatest length of the lacrimal bone (GLLB), **17:** Distance between the caudal border of one occipital condyle and the infraorbitale of the same side (DOCI), **20:** Lateral length of the premaxilla (LLPM), **24:** Greatest inner width of the orbit (GIWO), **25:** Greatest inner height of the orbit (GIHO).

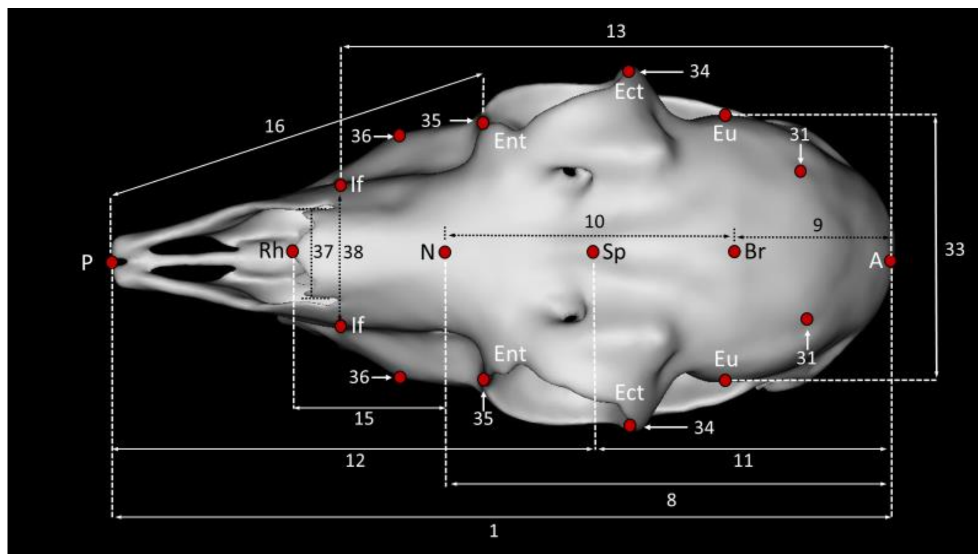

Figure 2. Measurement points of craniometric variables in the gazelle skull (dorsal view).
A: Akrokranion, **Br:** Bregma, **Ect:** Ectorbitale, **Ent:** Entorbitale, **Eu:** Euryon, **If:** Infraorbitale, **N:** Nasion, **P:** Prosthion, **Rh:** Rhinion, **Sp:** Supraorbitale, **1:** Total length of the skull (TLS), **8:** Median frontal length (MFL), **9:** Akrokranion-bregma length (ACBL), **10:** Frontal length (FRL), **11:** Upper neurocranium length (UNCL), **12:** Facial length (FCL), **13:** Akrokranion-infraorbitale length (ACIO), **15:** Greatest length of the nasal bone (GLNB), **16:** Entorbitale-prosthion length (EOPL), **31:** Least breadth of parietal (LBP), **33:** Greatest neurocranium breadth (GNCB), **34:** Greatest frontal breadth (GFB), **35:** Least breadth between the orbits (LBO), **36:** Facial breadth (FCB), **37:** Greatest breadth across the nasal bones (GBAN), **38:** Greatest breadth across the premaxilla (GBAP).

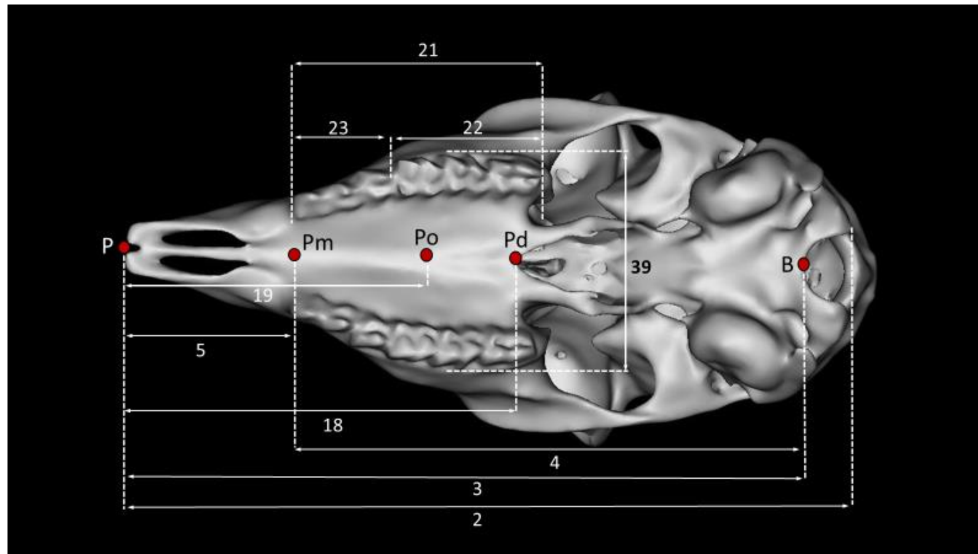

Figure 3. Measurement points of craniometric variables in the gazelle skull (ventral view).
B: Basion, **P:** Prosthion, **Pd:** Postdentale, **Pm:** Premolare, **Po:** Palatinoorale, **2:** Condylobasal length (CBL), **3:** Total length of the cranial base (TLCB), **4:** Short skull length (SSL), **5:** Premolare-prosthion length (PPL), **18:** Dental length (DTL), **19:** Oral palatal length (OPL), **21:** Length of the maxillary tooth row (LMTR), **22:** Length of the upper molar row (LUMR), **23:** Length of the upper premolar row (LUPR), **39:** Greatest palatal breadth (GPB).

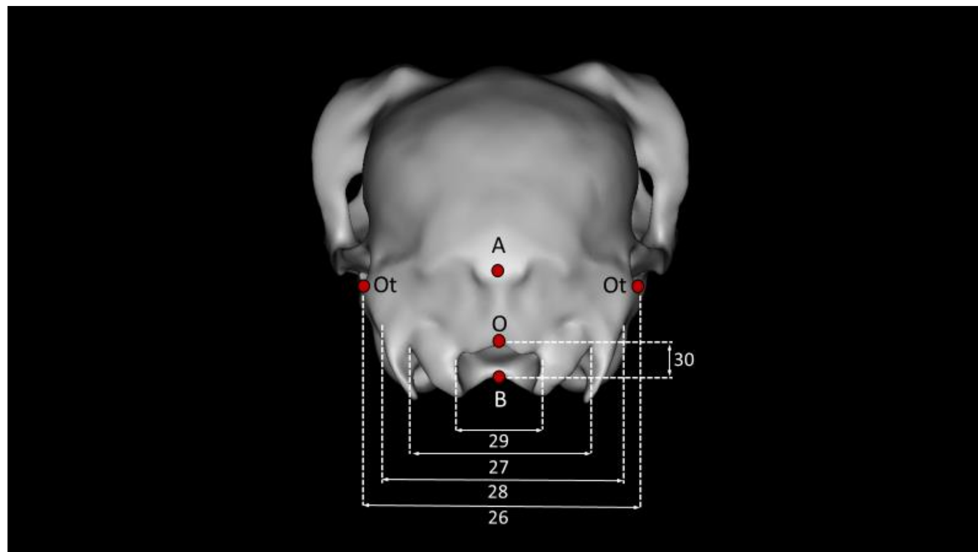

Figure 4. Measurement points of craniometric variables in the gazelle skull (occipital view).
A: Akrokranion, **B:** Basion, **O:** Opisthion, **Ot:** Otion, **26:** Greatest mastoid breadth (GMB), **27:** Greatest breadth of the occipital condyles (GBOC), **28:** Greatest breadth at the bases of the paracondylar processes (GBBPP), **29:** Greatest breadth of the foramen magnum (GBFM), **30:** Heigth of the foramen magnum (HFM).

Discussion and Conclusion

Craniometric analyses have been used to differentiate species within the same genus and to investigate morphological variations within species. Several reports on craniometric measurements using traditional methods (the help of scale and digital calipers) in gazelles are found in the literature (7, 31). This study presents for the first time morphometric and volumetric measurements of the skull in gazelles by using three-dimensional CT images. Due to the lack of data on CT based measurements in gazelles, data obtained from different gazelle species by traditional methods or data obtained from sheep and goats were used for comparison.

Due to remarkable morphological variations both among gazelle species and among individuals within the same species, assigning an individual to a certain species might be difficult (28). Therefore, more data are required for assessing the morphometric variation within the species. On the other hand, craniofacial index parameters are also necessary for examining craniofacial deformities and investigating brain development (13).

Zhu (31) has reported the skull index by examining the craniometrics values of Tibetan gazelle as 43.22±0.44 mm, cranial index as 58.37±0.80 mm and facial index as 116.37±1.24 mm. The facial index value found in the present study (47.45±2.92 mm) was lower than that reported by Zhu (31). The difference might be attributed to the use of different species and methods.

The orbital region plays an important role in craniofacial measurements, forensic processes and differential diagnosis (8). A tubular shape of orbita was observed in gazelles in the present study. The orbita can have a different shapes depending on the species and the breed of the same species. It has been reported that orbita has the shape of almond in Spanish Xisqueta sheep (24) while it has an oval shape in Mehreban sheep of Iran (14). Even a bilateral variation between the right and left orbitas in Kagani goats (*Capra hircus*) has been reported (12). In accordance with the present study Leslie (18), has reported a similar shape of orbita in *Procapra picticaudata*. Similar to our findings Parés-Casanova et al. (24) have reported an orbital index value of 97.27±1.12 mm and 94.61±3.15 mm in female and male Spanish Xisqueta sheep respectively.

Mean breadth and height of foramen magnum in the gazelles were measured as 16.28±0.36 mm and 15.03±0.32 mm respectively and foramen magnum index was 92.84±3.35 mm. These values were lower than those found in sheep (21) and goats (15). Similar to those reported in sheep and goats (15, 21) the horizontal diameter of the foramen magnum was longer than its vertical diameter in the gazelles.

Sexual dimorphism is common among mammals and has been an important evolutionary factor in social ecology (5). The effect of sex on bone morphology has been intensively studied in humans (2) goats (6) and wild sheep (11). However, the limited number of studies on the effect of sex on bone morphology in gazelles have been conducted (30). In the present study, significant differences between males and females were observed for MFL (median frontal length), FRL (frontal length), UNCL (upper neurocranium length), GLLB (greatest length of the lacrimal bone), OPL (oral palatal length), LUMR (length of the upper molar row) and GNCB (greatest neurocranium breadth).

Conventional radiological methods used for assessing the skull volume employ two-dimensional measurements. Computer tomography based methods present a more precise and noninvasive way for estimating in vivo skull volume (3). Mean skull volumes in females and males were detected as 87.69±1.09 cm^3 and 115.74±2.43 cm^3, respectively. In contrast to the findings in this study, Chanpanitkitchote et al. (4) have reported a skull volume of Grant's gazelles (*Nanger granti*) as 1016±11 cm^3. The differences in the morphometric values between the species have been attributed to inclusion or exclusion of mandible, horn status of the animal, measurement methods used or live weight of the animal.

In conclusion, new technologies like CT presents opportunities for obtaining comprehensive data on skull morphometry in animals. This study was the first reporting the use of CT for morphometric analysis of the skull in goitered gazelle (*Gazella subgutturosa*). The data obtained in this study will be useful for not only the evaluation of CT images from facial, cranial of dental deformities but also for determining the sex based on bone morphometry and for taxonomical studies. However further studies are necessary for comparing the data obtained from 3D modeling and actual measurements on skulls by including larger sample size.

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Conflict of Interest

The authors declared that there is no conflict of interest.

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First molecular data on the validity of *Myxobolus ichkeulensis* (Cnidaria: Myxosporea) from *Mugil cephalus* (Mugilidae) in Turkish waters

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Abstract: To date, there is no report on molecular characterisation of *Myxobolus ichkeulensis* in *Mugil cephalus* (Mugilidae) in Turkish marine waters. The aim of the current research was to provide the first molecular data on *M. ichkeulensis* from *M. cephalus* in Turkish Black Sea coasts. A total of 40 *M. cephalus* specimens were examined for the presence of *M. ichkeulensis* in the Turkish Black Sea coasts during January 2017 and April 2017. In the present study, *M. ichkeulensis* was identified based upon the myxospore morphology, host and tissue specificity, and SSU rRNA sequence. Phylogenetic analysis using neighbor-joining also revealed that our isolate (#GZP-2018-Samsun1) was clustered with other *Myxobolus* species that infect *M. cephalus.* The current study includes the morphological and histopathological descriptions and first molecular data on *M. ichkeulensis* in *M. cephalus* in Turkish marine waters. *M. ichkeulensis* was also reported from the *M. cephalus* in the coast of the Black Sea in Turkey for the first time. Moreover, our current genetic data was recorded as the new valid SSU rRNA sequence (MH374629) for *M. ichkeulensis* in the GenBank database.
Keywords: Histopathology, morphology, *Myxobolus ichkeulensis*, SSU rRNA, Turkey

Türkiye sularında *Mugil cephalus*'tan (Mugilidae) *Myxobolus ichkeulensis*'in (Cnidaria: Myxosporea) geçerliliğine ilişkin ilk moleküler veriler

Özet: Bugüne kadar Türkiye karasularındaki *Mugil cephalus* (Mugilidae)'da *Myxobolus ichkeulensis* türünün moleküler karakterizasyonu ile ilgili kayıt yoktur. Bu araştırmanın amacı Türkiye'nin Karadeniz kıyılarında *M. ichkeulensis* türünün *M. cephalus*'lardaki varlığını hakkında ilk moleküler verileri sağlamaktır. Ocak 2017 ile Nisan 2017 tarihleri arasında Türkiye'nin Karadeniz kıyılarından *M. ichkeulensis* türünün varlığı için toplam 40 adet *M. cephalus* incelendi. Bu araştırmada *M. ichkeulensis* türü spor morfolojisi, konak ve doku spesifitesi ile SSU rRNA sekansına dayanarak teşhis edildi. İzolatımız (#GZP-2018-Samsun1) neighbor-joining metodu kullanılan filogenetik analizde *M. cephalus*'ları enfekte eden diğer *Myxobolus* türleri ile kümelendi. Mevcut araştırma, Türkiye sularında *M. cephalus*'ta *M. ichkeulensis* türünün geçerliliği üzerine morfolojik ve histopatolojik tanımlamalar ile ve ilk moleküler verileri içermektedir. Türkiye'nin Karadeniz kıyılarından ilk kez *M. cephalus*'ta *M. ichkeulensis* türü de bildirildi. Ayrıca mevcut genetik verilerimiz GenBank veri tabanındaki *M. ichkeulensis* türü için yeni geçerli SSU rRNA dizisi (MH374629) olarak kaydedildi.
Anahtar sözcükler: Histopatoloji, morfoloji, *Myxobolus ichkeulensis*, SSU rRNA, Türkiye

Introduction

Thousands of myxosporean parasites are known to cause diseases in various marine and freshwater fish (18, 19). The genus *Myxobolus* Bütschli, 1882 is the largest group of Myxobolidae contains over 850 species described (7, 8). Based on spore morphology, host/ tissue tropism with the molecular marker are especially provided for identification of a new or existing myxozoa species and re-description of incompletely described species (11, 20). *Myxobolus ichkeulensis* was firstly described from the grey mullet *Mugil cephalus* in Ichkeul lagoon in Tunisia by Bahri and Marques (4). Later, there have been few reports on *M. ichkeulensis* in *M. cephalus* from Lake Ichkeul (Tunisia), Ebro River Delta (Spain), Baje de

Gorée (Senegal), Black and Azov Seas (Crimea, Ukraine), Camlık Lagoon (north-eastern Mediterranean, Turkey) and Japan Sea (3, 9, 23, 24, 28, 31). Whereas, this *Myxobolus* species has been reported in different marine sources of the world, there were only two SSU rRNA sequences of *M. ichkeulensis* molecularly characterized and submitted to the GenBank with the accession numbers: AF378337 and AY129315 (3, 14).

Up to now, only four *Myxobolus* species have been morphologically identified and reported in *M. cephalus* from Turkish marine waters: *M. episquamalis, M. exiguus*, *M. ichkeulensis* and *M. muelleri* (2, 6, 23, 30). Whereas those species were morphologically described, there is no detailed molecular and histopathological data of *M. ichkeulensis* in *M. cephalus* in Turkish marine waters. The aim of the current research was to provide first molecular data on the validity of *M. ichkeulensis* from *M. cephalus* in Turkish marine waters.

Material and Methods

Sampling, morphological and histopathological examination: For this study, ethics committee approval was not needed because no handling of live fish specimens were involved. A total of forty freshly caught specimens of *M. cephalus* were periodically purchased from commercial fishermen at Kızılırmak Delta, Samsun coast located by the Black Sea, Turkey (41°44'04.2"N 35°57'23.0"E) in the period between January 2017 and April 2017. After purchase and transportation to the laboratory, *M. cephalus* were examined for *M. ichkeulensis* infections under a dissecting microscope (18). After whitish plasmodia were detected in the gills, plasmodia were isolated with a needle and opened on a slide. Infected gills containing mature plasmodia were fixed in 10% formaldehyde and embedded in paraffin. Paraffin blocks were cut into 5 µ slices using microtome and stained with Hematoxylin-Eosin (H&E). The position of the plasmodia in the gills was classified to Molnár (21). Some fresh spores were prepared in glycerine-jelly into the slide for morphological examination. Subsamples of fresh spores were preserved in absolute ethanol for molecular identifications. Myxospores were morphologically examined as previously reported Lom and Arthur (17) by measuring 20 freshly isolated mature spores in reference slide preparations. The myxospores were photographed and measured by Nomarski DIC optics connected to a digital camera. All measurements are presented in micrometres (µm) with mean and range in parentheses.

DNA extractions and PCR analysis: *Myxobolus* spores were centrifuged at low speed, suspended in digestion solution and incubated at 56°C overnight. DNA was then extracted using a commercial DNA extraction kit (GeneJET Genomic DNA Purification Kit, ThermoFisher Scientific) according to the procedure recommended by the manufacturer. Nested PCR assay was used for identification of *Myxobolus* species. The small subunit ribosomal RNA (SSU rRNA) gene (~1900 bp) was amplified by first PCR using ERIB1 and ERIB10 primer pairs (5). Fifty µl PCR reactions were contained 20-50 ng DNA, 2X Hot start PCR master mix, 0.4 µM of each primer. Amplification of first PCR conditions were: 30 sec at 95°C, 50 sec at 43°C, and 120 sec at 72°C for 30 cycles, and a 10 min extension at 72 °C. Then, second PCR were carried out in a final volume of 50 µl, which contained 1 µl of the first PCR amplicon, 2X hot start PCR master mix, 0.4 µM of each primer. The ~900 bp amplification products using the MyxospecF-MyxospecR primer sets (12) were run with 30 sec at 95°C, 50 sec at 40 °C, 90 sec at 72°C for 35 cycles, and products were subjected to a final extension at 72 °C for 10 min. After amplification, PCR amplicons were electrophoresed on 1% agarose gel in a TBE buffer. Second PCR amplicons were sequenced both directions with MyxospecF-MyxospecR primer pairs by Sanger method (Macrogen, Netherlands).

Phylogenetic analyses: The obtained sequences were controlled by Vector NTI Advance 11.5 (Invitrogen, USA) using phred values. Then, sequences were de novo assembled and edited with using Contig Express (NTI Advance 11.5, Invitrogen, USA) and the created sequences were compared with GenBank accessions using the BLAST research (1). The SSU rRNA sequences were aligned with other known *Myxobolus* species from *M. cephalus* in previous studies (3, 14, 15, 28). Sequence aligments were performed with ClustalW in MEGA 7.0 (29) and adjusted manually. Phylogenetic tree was constructed using neighbor-joining (NJ) analysis in MEGA 7.0 (16). The Kimura two-parameter (K2P) model was used in the analysis. The species *Ceratomyxa shasta* was chosen as the out-group. The mugilid infecting *Myxobolus* sequence sets were built with 1000 bootstrap replications for the NJ reconstruction (10). Bootstrap values ≥ 70 were considered well supported (13).

Results

Whitish cyst-like plasmodia were detected macroscopically along the conjunction line of the gill filaments and arches of *M. cephalus*. We concluded that our isolate (#GZP-2018-Samsun1) is *M. ichkeulensis* based on the spore morphology, biological traits (host/organ specificity and tissue tropism) and molecular data.

Taxonomy of Myxobolus ichkeulensis **(4)**

Host: Grey mullet, *Mugil cephalus* (Mugilidae)

Locality: Kızılırmak Delta, Samsun, Turkey (41°44'04.2"N 35°57'23.0"E)

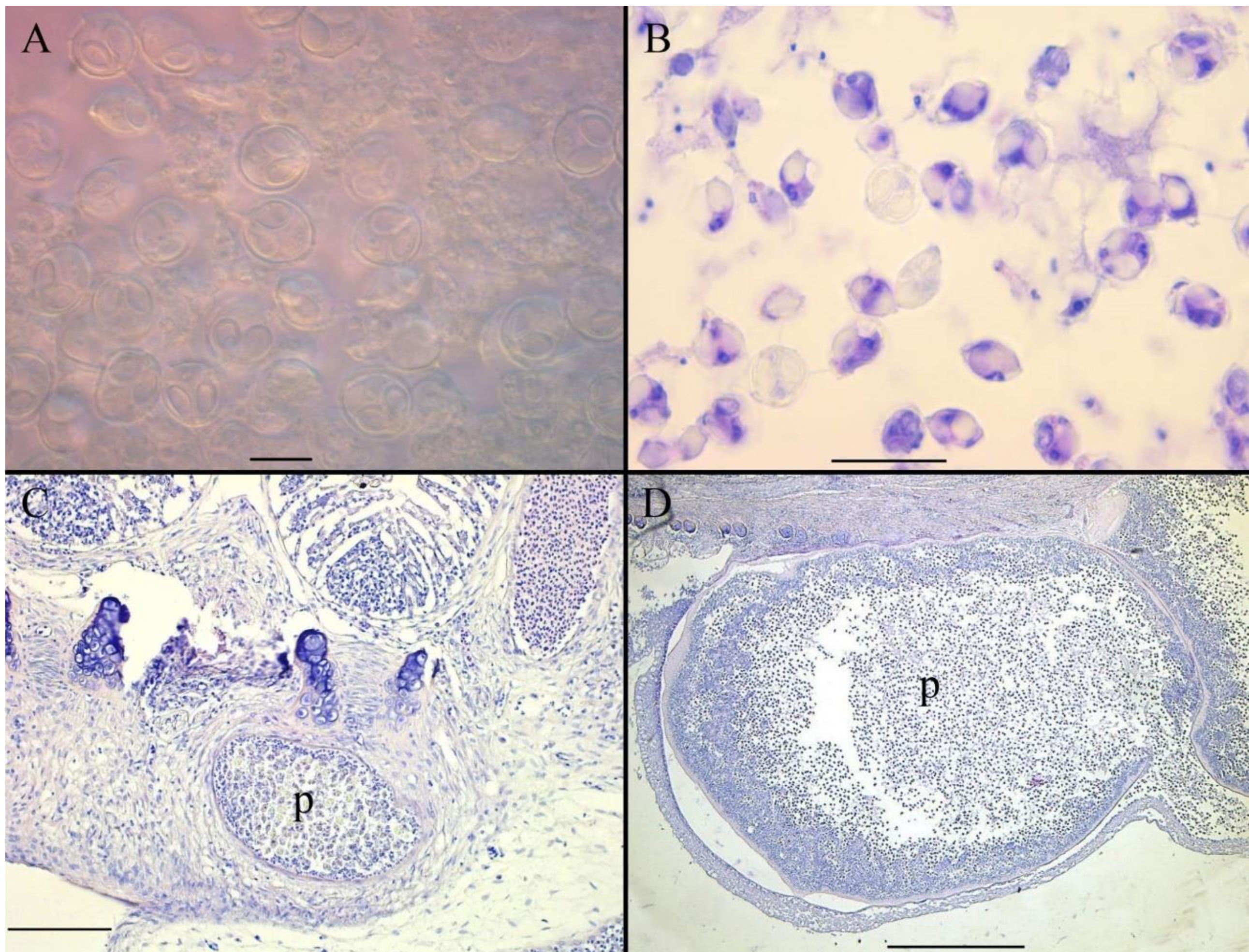

Figure 1. Microscopic photographs of *M. ichkeulensis* infecting the gill arch of *M. cephalus*.
A. Fresh myxospores of *M. ichkeulensis* in glycerine-jelly, scale: 10 µm. **B.** Myxospore of *M. ichkeulensis* inside plasmodium (p), histopathological section, H&E staining, scale: 10 µm. **C.** *M. ichkeulensis* plasmodium (p) located in the connective tissue elements of the gill arch, histopathological section, H&E staining, scale: 100 µm. **D.** A large plasmodium (p) of *M. ichkeulensis* in the gill arch, histopathological section, H&E staining, scale: 500 µm.

Site of infection: Plasmodia were observed macroscopically as whitish cysts along the conjunction line of the gill filaments and arches.

Type material: Reference glycerine-jelly and histopathological sections were deposited in the laboratory. The SSU rRNA sequence of *M. ichkeulensis* was recorded in GenBank as MH374629.

Prevalence of infections: 12.5 % (5 out of 40), 12 to 18-cm-sized fish

Myxospores: The myxospores were round or spherical. The spores were 13.3 (12.06 to 13.72) µm long (n = 20), 11.42 (10.5 to 12.4) µm wide (n = 20), and 8.24 (7.75 to 8.53) µm thick (n = 10). The two polar capsules were oval, equal in size, 5.97 (5.7 to 6.65) µm long (n = 20) and 3.81 (3.4 to 4.18) µm wide (n = 20), and their posterior end reached half the length of the spore. Eight to ten sutural edge markings were easily observed (Figure 1A-B).

Histopathological findings: 250 to 2000 µm in diameter plasmodia were found. The histopathological analysis revealed the development of the cyst-like plasmodia as gill arch type. Moreover, plasmodia were located in the connective tissue elements of the gill arch (Figure 1C-D).

Molecular data: No intraspecific nucleotide variability within three isolate of *M. ichkeulensis* from the Black Sea were observed in the SSU rRNA sequences. A BLAST search indicated that SSU rRNA sequence of our isolate GZP-2018-Samsun1 (MH374629) from *M. cephalus* showed 99.05% similarity to *Myxobolus* sp. voucher Spain6-tp (MF118764), which is also a tail-infecting species and identified as *M. ichkeulensis*, and shared 98.82% similarity with that of *M. ichkeulensis* (AF378337; AY129315). For this reason, our *Myxobolus* isolate (GZP-2018-Samsun1) thus identified molecularly to belong to *M. ichkeulensis*.

Discussion and Conclusion

The flathead grey mullet *M. cephalus* (Mugilidae) is a cosmopolitan coastal fish species distributed worldwide. Several myxosporean parasites have been reported as serious pathogens of mugilid fish species (22, 31). Moreover, a great number of myxosporean species were recorded in *M. cephalus* among other mugiliform fish species. To date, thirty six species of myxosporeans have been reported and amongst them are *M. muelleri*, *M. ichkeulensis*, *M. spinacurvatura*, *M. exiguus*, *M. parvus* and *M. episquamalis* are only six cosmopolite species in *M. cephalus* from worldwide (31).

M. ichkeulensis was firstly described in *M. cephalus* by Bahri and Margues (4) based on traditional criteria, including tissue tropism and detailed light and electron microscopic examination of spore morphology and subsequently Bahri et al. (3) provided a supplemental data on *M. ichkeulensis* from the host type with molecular data of the SSU rDNA sequence (AY129315). Within the current study, the SSU rDNA sequences of our isolate (#GZP-2018-Samsun1) showed 98.82% identity with reference sequence of *M. ichkeulensis* (AY129315). Therefore, we molecularly identified our *Myxobolus* species as *M. ichkeulensis.* Currently, based on spore morphology, host/organ specificity and tissue tropism with the molecular marker are mainly useful for new myxosporean species and re-description of insufficiently described species (3, 25-28, 31). Thus, these approaches combined (morphology, biological traits, and molecular markers) were used for the validation of *M. ichkeulensis* in *M. cephalus* from Turkish waters for the first time. Moreover, this is also first report of *M. ichkeulensis* in *M. cephalus* from the coast of the Black Sea in Turkey. Furthermore, the phylogenetic tree showed that our isolate was clustered with *M. ichkeulensis* species previously known to be sequenced from *M. cephalus* (Figure 2). A comparison of *M. ichkeulensis* Bahri and Marques (4), spore morphometric data isolated from *M. cephalus* at different geographical areas is presented in Table 1.

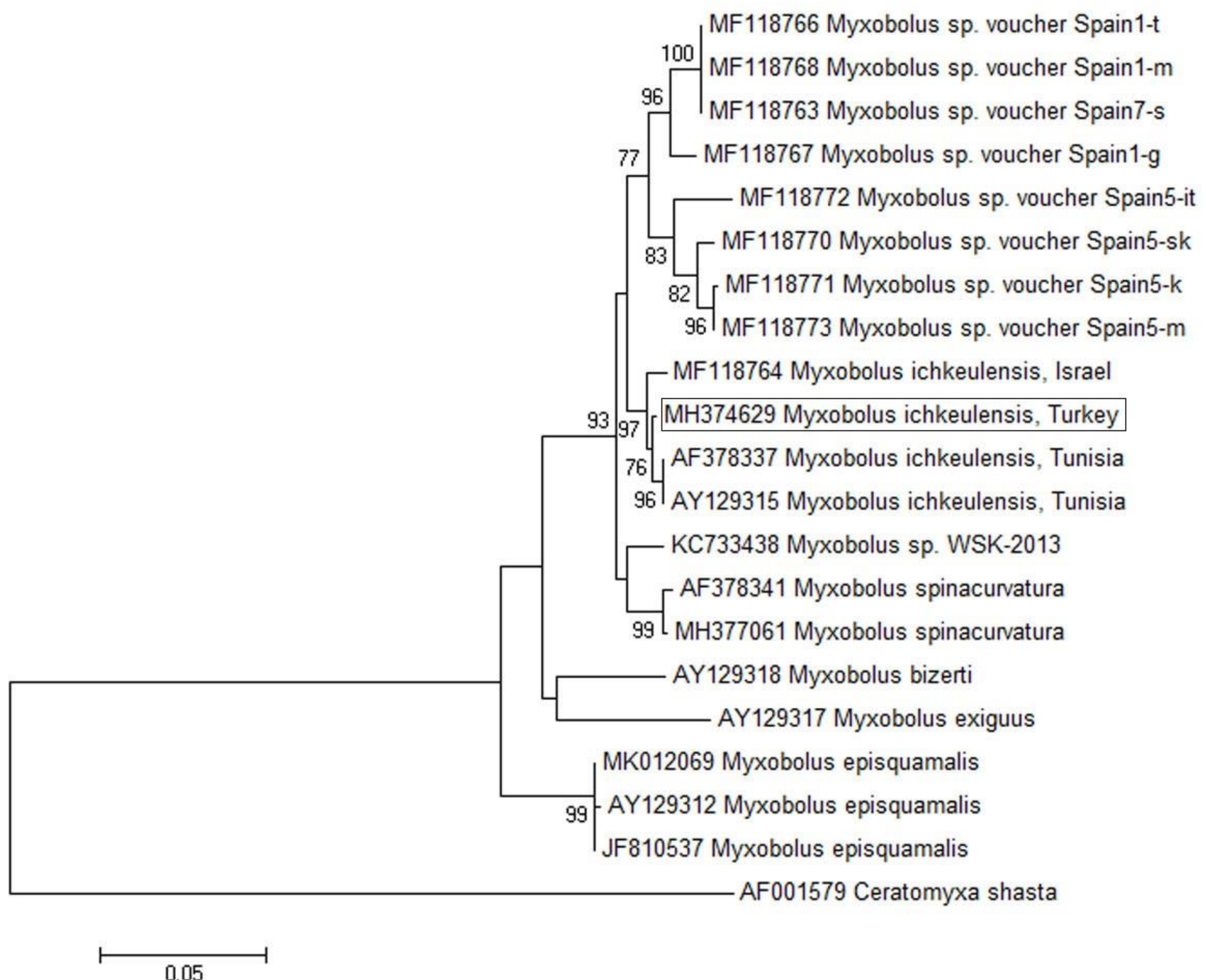

Figure 2. Phylogenetic tree generated by NJ analysis of the SSU rRNA sequences of *M. ichkeulensis* and other *Myxobolus* species infecting mugiliform.
Numbers at nodes indicate the bootstrap values. *Ceratomyxa shasta* was used as the out group.

Table 1. Comparison of spore morphometric data (µm) of *M. ichkeulensis* infection in *M. cephalus.*

Spore length	Spore width	Spore thickness	Polar capsule length	Polar capsule width	Locality	Reference
13.5 (13-14)	12.5 (12-13)	-	5.5 (5-6)	4.2 (4-4.3)	Tunisia: Ichkeul Lagoon	Bahri and Marques (4)
13.5±0.54	12.5±0.54	-	5.5±0.54	4.25±0.27	Tunisia: Ichkeul Lake	Bahri et al. (3)
13.32 (12.49-14.15)	12.24 (11.58-12.9)	7.69 (7.24-8.14)	6.38 (6-6.76)	4.18 (3.95-4.41)	Turkey: Mediterranean coast, Camlık Lagoon	Ozak et al. (23)
13.13 (12.06-13.72)	11.42 (10.5-12.4)	8.24 (7.75-8.53)	5.97 (5.7-6.65)	3.81 (3.4-4.18)	Turkey: Black Sea coast, Kızılırmak Delta	Present study

Myxosporean plasmodia localize in or among gill lamellae, in gill filaments and inside the gill arch cartilage (20). In the present study, histopathological sections showed *M. ichkeulensis* plasmodia in the connective tissue elements of the gill arch (Figure 1C-D). Myxosporean species is strictly connected to a specific tissue of the host (20). Myxosporean plasmodia may develop within the connective tissue layer in skin doublets between the fin rays (20, 21). Supportively, *Myxobolus* plasmodia embedding in the fin tail connective tissue was found in *M. cephalus* and was 99% similar to *M. ichkeulensis* (28). Our study and Sharon et al. (28) indicated that *M. ichkeulensis* in *M. cephalus* has an affinity for connective tissues both in the gill arch and tail fin. Moreover, small and single cysts of *M. ichkeulensis* reveal a basi-filamental type of plasmodial development in the *M. cephalus* (23).

In conclusion, supplementary data of histopathology and SSU rDNA analysis of *M. ichkeulensis* infecting *M. cephalus* as host type were provided in the present study for the first time from Turkish waters. Moreover, the new valid SSU rRNA sequence (MH374629) obtained from *M. cephalus* from Turkish waters has been submitted to the GenBank. This sequence can be also used to construct a phylogenetic tree with other mugiliform-infecting *Myxobolus* species.

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Conflict of Interest

The authors declared that there is no conflict of interest.

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Influence of different water access status of suckling calves on performance, water intake behavior and some blood metabolites

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Abstract: This study was conducted to determine the effects of water accessibility of pre-weaning calves during milk feeding meal on performance, water intake behavior and some blood metabolites. Forty-eight Holstein calves (39.2 ± 5.8 kg of BW) were assigned to 4 treatments, which consisted of different water accessibility: 1) Ad libitum water (AW), 2) Water not available (WNA), 3) Water available after milk feeding (WAM) and 4) Water available before milk feeding (WBM). Resulted showed different water accessibility changed water consumption of calves. The water intake of AW (1.03 kg/d) and WBM (1.07 kg/d) treatments were higher than others. Starter intake of WBM decreased ($P<0.05$) as compared with others. Calves of WBM had higher ($P<0.05$) activity of serum AST. Blood Cl and K did not show the difference ($P<0.05$) among treatments. Serum TAC levels were higher ($P<0.05$) in WNA and WAM calves. Serum protein and HDL in WNA calves were higher ($P<0.05$) than others. These groups had higher ($P<0.05$) serum Na concentration than others. The different water accessibility of calves had no significant effects on health score, respiratory, fecal score and growth parameters. Treatments did not affect behaviors parameters. But the number of visiting water bucket of AW and WBM were higher than other groups. Acetic acid and propionic acid of AW calves significantly increased ($P<0.05$) as versus other treatments. Drinking water around milk feeding meal change water consumption, performance and improve some blood metabolites and rumen parameters of calves but did not influence growth parameters.
Keywords: Blood metabolites, calves, growth factor, performance, water intake.

Introduction

Water is an important nutrient in dairy production (26). According to previous research, 69.5 to 71.6% of the calf body weight is made up of water (6). Too often, it is assumed that if a calf is being fed a liquid diet, its needs for water will be satisfied (27). According to the German enactment of animal welfare and farming of animals (33) calves over 2 weeks of age have to be allowed ad libitum access to water. Feeding calves ad libitum milk during the pre-weaning period is one method to provide more water to the calves at an early stage (17). However, feeding greater amounts of milk have been shown to decrease post-weaning calf growth and decrease starter intake (3). However, milk is the primary source of water during the first few weeks of the calf's life, but represented a decreasing percentage of the total water intake as the calf became older (27). Neonate calves could consume a considerable amount of water via whole milk or milk replacer but it's not clear that amount alone would fulfill the water requirements to support growth and development (35). The drinking water requirement of calves appears to be insignificant when separated from liquid feed intake (35). Milk-fed calves drink little water until weaning, at which time water intake increases markedly (15), and water intake tends to follow the intake of solid food (19). Calves offered supplemental water gained more body weight and consumed more calf starter compared to calves without access to free water (19). Water intake of Holstein bull calves through 56 days of age was measured in 6 different trials over two years (29). In these studies, water intake was very closely correlated with starter intake. As calves consumed more starter diet they drank more water. The study, covering 30 years (27) found a significant correlation of both live-weight gain and calf starter intake with water intake prior to weaning at 5 weeks. In addition, greater water consumption has

been shown to increase early starter intake (5). Studies have shown that consumption of solid feed in starter diets can contribute to optimized development of the rumen in calves, thereby leading to greater potential for early weaning (5). During the pre-weaning period, when calves consume whole milk or milk replacer it bypasses the rumen and goes to the abomasum via the oesophageal groove. This process also prevents a large portion of consumed water from entering the rumen and may influence bacterial growth and the start of rumen fermentation. Therefore, providing clean fresh drinking water to calves is essential for the rumen microbiota development (7). Wickramasinghe (35) reported that offering drinking water to calves from birth itself appeared to offer positive benefits even in systems promoting a large amount of liquid feed (whole milk or milk replacer) intake. Producers also seem to be hesitant to offer water to new-born calves, assuming it would cause diarrhea (4).

Overall, a lack of scientific evidence on drinking water intake of new-born calves and its effect on their performance likely encumbers the effectiveness of efforts promoting the notion of offering drinking water to new-born calves at birth. Depending on the improved starter intake and weight gain in report Kertz et al. (19), it is often hypothesized that drinking water would enhance rumen development, thus increasing nutrient availability for growth and development of calves. However, no systematic attempt has been made to study this link between water intake and nutrient availability to the animal. This also brought attention to the fact that many dairy farmers wait an hour before and after milk feeding meal to offer drinking water to suckler calves even though offering water a full day is the recommendation. Water ideally should be available at all times to young calves, but as minimum warm water should be offered after feeding and midday in cold climates (5). The hypothesis of this study is the question that "Does water drinking around milk meal affects calf health and performance?" Thus, the objectives of our experiment were to determine the effects of different water access status of suckling calves on performance, water intake behavior and some blood metabolites.

Materials and Methods

***Animals, housing and diets*:** The research protocol was approved by the Animal Care and Use Committee of the Iranian Council of Animal Care (1995). Forty-eight Holstein calves ((3 d of age; 39.2 ± 5.8 kg of body weight (BW)) were used in this study. The experiment was carried out at Mohandesiye Dairy cattle farm, Esfahan province, Iran. This study was conducted in late 2017 and early 2018. The month average temperature was decreased monthly. The weather data of Esfahan were for the average temperatures (°C) (19.2, 11.2 and 6.4), Maximum temperature (°C) (31.6, 26.8 and 23.4), Minimum temperature (°C) (6, -2.4 and -4.8) and the average wind speed (m/s) (1.9, 1.1 and 0.9) during October, November and December months (2017), respectively.

The Mohandesiye Dairy cattle farm in Esfahan is one of the biggest farms in Esfahan that had almost 1500 cow. Total parturition days for this study to select and collect the 48 calf were about one week or 7 days. Calves were separated from their mothers within 24 h of birth, weighed, moved into individual pens (1.2 × 1.8 m; width × depth) where they were fed 10% colostrum of their BW for the first 3 d. The pens were cleaned every 2 d by removing all bedding and replacing it with fresh sawdust bedding. Calves were managed according to the standard operating procedures of this research station, in accordance with guidelines set by the Iranian Council of Animal Care (16).

Calf starter was offered ad libitum, and intake was recorded daily, beginning on day 4. The basal diet was formulated to meet Nutrient Requirements of Dairy Cattle (NRC) (27) requirements for calf nutrients. The ingredients, nutrient composition of the basal diet and drinking water quality are shown in Table 1. Chopped dried wheat straw and alfalfa hay were used after 20 days age of calves in the study. The forage was chopped using a total mixed ration mixer (Loewen Horizontal Mixer, Loewen Welding & Manufacturing Ltd., Matsqui, BC, Canada) at 19,000 rpm for 60 min. Three additional buckets were placed in the middle of the area where the new-born calves were housed and also weighted every 24 hours to calculate the evaporation of water. The daily water intake of a calf was calculated by the difference in weight of the buckets of each calf minus average of three bucket evaporation. All calves were fed the same amount of whole milk. Calves were bucket-fed 4.5 L/d of whole milk divided into 3 feedings, at approximately 0800, 1630 and 0030 h from birth until 30 d of age. From d 31 to 50, calves were fed 8 L/d, using the same procedure as described above. Milk volume was reduced by 20 % that means calves were fed 6.4 L/d from d 50 to 60, with milk weaning occurring on d 60. Therefore, the whole experiment period was sixty days, and for fed colostrum in the first 3 days of life, the main experiment period began on the fourth day of calf birth.

On the 4th day, the calves were randomly assigned to 4 treatments: 1) water were ad libitum (AW) 2) Inaccessibility to water an hour before and an hour after milk feeding or water not available (WNA) 3) Inaccessibility before and access to water after milk feeding milk or water available after milk feeding (WAM) and 4) Accessibility before and inaccessibility to water an hour after milk feeding or water available before milk feeding (WBM). A total of 6 water samples were collected and sent directly to a commercial laboratory for analysis (Zagros LAB water quality test, Fars, Shiraz, Z.A.F).

Table 1. Chemical composition of diet and drinking water quality

Diet composition	% of DM	Water quality	Mean±SEM
Corn grain ground	27	Number of samples	6
Barley grain ground	35.5	EC (µs/cm)	2386.00±36.97
Rumen growth factor (RGF)	14	T.D.S[3] (mg/L)	1538.27±22.85
Soybean meal, 45% CP	10	PH	7.27±0.11
Whole soybean (full fat)	0.8	Nitrate(NO_3) (mg/L)	83.25±1.9
Dicalcium phosphate	0.8	Nitrite(NO_2) (mg/L)	0.014±0.004
Sodium bicarbonate	0.6	Phosphate (PO_4^{3-}) (mg/L)	0.03±0.07
Magnesium oxide	0.4	Carbonate(CO_3^{2-}) (meq/L)	0.00±0.00
Salt	0.7	HCO^{3-} (meq/L)	5.00±0.3
Vitamin permix[1]	1.5	Cl^- (meq/L)	11.50±1.05
Mineral permix[1]	1.5	$SO4^{2-}$ (meq/L)	6.00±0.8
Nutrient composition		Sum Anion	22.50±2.4
ME, [2] Mcal/kg	3.03	Ca^{2+} (meq/L)	7.50±1.7
NE_g, [2] Mcal/kg	1.73	Mg^{2+} (meq/L)	2.00±0.06
DM	90	Na^+ (meq/L)	15.58±1.16
CP	22.7	K^+ (meq/L)	0.12±0.09
NDF	16.4	Sum Cation	25.20±1.84
ADF	8.4	*Escherichia coli* (cfu/mL)	ND[4]
Lipid	2.8		
Ash	8.0		
Ca[2]	0.78		
P[2]	0.55		

[1] Contained per kilogram of supplement: 250,000 IU of vitamin A, 50,000 IU of vitamin D, 1,500 IU of vitamin E, 2.25 g of Mn, 120 g of Ca, 7.7 g of Zn, 20 g of P, 20.5 g of Mg, 186 g of Na, 1.25 g of Fe, 3 g of S, 14 mg of Co, 1.25 g of Cu, 56 mg of I, and 10 mg of Se.
[2] Calculated from NRC (27).
[3] Total dissolved solids based on gravimetric analysis.
[4] Not detected.

***The behavioral data recording methods and their description*:** The ingesting water behavior was defined as the calf is ingesting water by drinking from a bucket and the ingesting grain behavior was described as the calf is consuming grain from a bucket. Also, the ruminating activities were explained as the calf is ruminating. The standing status was recorded as the calf is standing with all 4 legs on the ground. The lying in bed position was noted as the calf is resting on the ground; the head may be supported or unsupported by the neck. The object play was monitored as the calf is standing; butting head against milk or water buckets or hutch in a playful manner. The self-grooming was observed as the calf's tongue is out of its mouth and in contact with its own body. The non-nutritive sucking was recorded the calf's tongue is out of its mouth and is in contact with or biting any fixtures of the pen; may include bucket if milk is not available at the time of observation. Other behaviors were described as the calf is urinating, defecating, or performing another behavior not described. All observations were by the same person, who was blind to treatment group's allocation to minimize possible bias in data calves. We observed calf behavior for 6 80-min periods every five minutes one by one at before or after approximately 0800, 1630 and 0030 h from after first 4 days of birth to 60 days of life into 3 calves' meals. Therefore, the visits were 80-min before and 80-min after milk meal in 3 times a day accordingly, means total visits were 8 hour a day that is one replicate. The observations replicated three times.

***Data collection and sampling*:** Starter intake and orts were recorded daily on an individual basis. Feed and refusals were sampled, mixed thoroughly, and ground to pass a 1-mm screen in a Wiley mill (Ogawa Seiki Co., Ltd., Tokyo, Japan). Standard methods were used for determination of DM (dry matter), CP (crude protein), NDF (neutral detergent fiber), ADF (acid detergent fiber), fat and ash as described in AOAC (1). Calves were weighed every 10 d throughout the study. Structural growth measurements of body length, withers height, hip height, heart girth and hip width were recorded on basis (cm) in two periods including d 4 and 50 of the experiment according to Lesmeister and Heinrichs (24). The four body measurements were obtained on all calves as follows:

The body length was the distance from the point of the shoulders to the ischium; withers height was the distance from the floor beneath the calf to the top of the withers directly above the center of the shoulder; hip height of calves be standing straight on a level, hard surface with its head up, and to measure hip height, used a hip stick and place it between the hip bones at a 45-degree angle; heart girth was measured as the minimal circumference around the body immediately behind the front shoulder; hip width was the widest point at the center of the stifle. On the same day the body weights were also obtained. All linear measurements were obtained with specially designed aluminum calipers; or a plastic-coated fiber tape measure available commercially. All measurement devices were in metric graduations (cm). Feed efficiency was calculated according to Khan et al. (20). Fecal scoring was performed every day in the morning (08.00) according to Wisconsin-Madison calf health scoring chart as follows: 0=Normal; 1=Semi-formed, pasty; 2=Loose, but stays on top of the bedding; 3=Water, sifts through the bedding. The health criteria of the calves were observed using the procedure described in Heinrichs et al. (14) and was monitored using the procedure described in Heinrichs et al. (14). Behavior was monitored by direct observation of all the calves on 2 consecutive days (on d 35 of the experiment and before weaning once per week). Calves were observed 8 h around milk feeding during the pre-weaning week. Instantaneous scans were performed for occurrences of lying, standing, water and starter intake, ruminating and non-nutritive oral behaviours (28).

Ruminal fluid samples were obtained using a stomach tube 3 h after the morning feeding on d 35, strained through 4-layer cheesecloth. A sample of the filtered ruminal fluid (5 mL) was added to 1 mL of meta-phosphoric acid (250 g/L) and retained for short chain fatty acids determination. Rumen samples were analyzed for VFA by gas chromatography (Model CP-9002, Chrompack, Middelburg, Netherlands) with a 50-m (0.32 mm internal diameter) fused-silica column (CP-Wax Chrompack Capillary Column, Varian, Palo Alto, CA).

On d 35 and 50 of the study period, jugular blood samples were also collected in evacuated tubes (5 mL) 3 h after the morning feeding without adding any anticoagulant. These samples were centrifuged at 1500 × g for 20 min, and the serum samples obtained were stored at −20 °C until subsequent analyses. After unfreezing, in serum samples biochemical indicators (glucose, total protein, chloride (Cl^-) albumin, cholesterol, HDL, triglycerides and activity of enzymes, ALT, AST) were measured by commercial kits (Pars Azmoon, Tehran, Iran) using an automated analyser (Biotecnica, Targa 3000, Rome, Italy). Glutathione peroxidase (GPx), total antioxidant status (TAS), NEFA, BHBA and MDA was determined by Ransel ® kits (Randox, UK) with UV/VIS Spectrophotometer. The amounts of sodium (Na) and potassium (K) were measured by a flame photometer (Jenway 6105 Clinical, Jenway LTD Felsted England).

***Statistical analysis*:** The corrections of the collected records were on a 35-day base. All the data collected were analyzed using the MIXED procedure (Version 8, SAS Institute Inc., Cary, NC) as a completely randomized design (32). Calf within treatment was included as a random effect that was used to test the main effect of treatment. Time was modeled as a repeated measurement for starter intake, total dry matter intake (DMI), average daily gain (ADG), feed efficiency, and fecal score within d 4–50. The data on blood metabolites, rumen parameters, BW, skeletal growth and feeding behavior were analysed for two periods while those on nutrient digestibility were analysed for one period (weaning). For BW and skeletal growth, initial values were used as the covariate. The statistical model used for the analyses was $Y_{ijklm} = \mu + T_i + P_j + T \times P_{ij} + C_{kij} + l(Cov)_m + e_{ijklm}$, where Y_{ijklm} = observation or the dependent variable, = the overall mean, T_i = the effect of treatment i, P_j = the effect of period j, $T \times P_{ij}$ = the effect of the interaction between treatment i and period j, C_{kij} = calf random effect, l = regression coefficient of observations on birth weight (BW) or skeletal growth as a covariate, and e_{ijklm} = random residual effect. Values were presented as least square means. Treatment differences were declared at $P \leq 0.05$, with trends towards significance considered at $P \leq 0.15$.

Results

It is evident that different water accessibility around calf milk meal changed water consumption of calves (Table 2). AW and WBM significantly increased water intake of calves as compared to other treatments. Also, the water intake of calves increased with age (P<0.05). Results of different calves' water accessibility showed that the calves in treatments of AW and WBM had higher water intake (respectively AW=1.03 kg/d and WBM=1.07 kg/d). The mean daily water intakes of WNA and WAM treatments decreased and were 0.84 kg/d and 0.84 kg/d, respectively. In this study, AW calves in the second decades (11 to 20 d) drank 1.26 kg/d water, that significantly higher than other groups (P<0.05). During decade 3, water intake of both AW (0.95 kg/d) and WBM (0.96 kg/d) treatments increased compared with other groups. In the 4 decades age of calves, WBM groups had more water intake (1.10 kg/d) than other treatments. The water intake of calves in decade 5, did not differ among treatment (P>0.05). In the last decade, water intake of the WAM group significantly decreased compared with other treatments (P<0.05).

Starter intake of calves is presented in Table 2. Starter intake affected by water accessibility treatments.

Calves offered ad libitum water consumed an average of 39% more water than other calves in the second period (P<0.001). At third- and fourth-decade age calve AW and WBM treatments had higher water intake as compared with WNA and WAM treatments. Starter intake did not significantly difference between treatments.

The WBM treatments significantly reduced calves body weight rather than other treatments. Also, the ADG of WBM treatment was the lowest (0.54 kg/d). The result showed that water accessibility of calves before milk feeding had a negative effect on performance. According to Table 2 feed efficiency of calves did not affect by treatments.

Table 2. Water intake and starter intake, performance, feed efficiency and ADG of calves (n = 52) with different water accessibility during the preweaning period (Mean ± SEM)

Item	Treatment				SEM	P		
	AW	WNA	WAM	WBM		Treat	Time	Treat×time
Water intake (kg/d)								
Whole period	1.03±0.06[a]	0.84±0.04[b]	0.84±0.05[b]	1.07±0.07[a]	0.01	0.01	0.01	0.29
1-10 d	0.94± 0.06	0.86± 0.06	0.79± 0.06	0.89± 0.06	0.51	0.24	-	-
11-20 d	1.26± 0.06[a]	0.76± 0.06[c]	0.95± 0.06[bc]	0.98± 0.06[b]	0.06	0.01	-	-
21-30 d	0.95± 0.05[a]	0.69± 0.05[b]	0.78± 0.05[b]	0.96± 0.05[a]	0.05	0.01	-	-
31-41 d	0.92± 0.06[b]	0.73± 0.06[c]	0.76± 0.06[c]	1.10± 0.06[a]	0.05	0.01	-	-
41-50 d	1.14± 0.07	1.08± 0.07	0.94± 0.07	1.07± 0.07	0.03	0.21	-	-
51-60 d	1.28± 0.09[ab]	1.55± 0.09[a]	1.04± 0.09[b]	1.48± 0.09[a]	0.11	0.02		
Starter intake (kg/d)								
Whole period	0.63±0.03[a]	0.62[a]±0.02	0.61[a]±0.03	0.54±0.03[b]	0.02	0.01	0.01	0.01
1-10 d	0.13± 0.01	0.12± 0.01	0.12± 0.01	0.09± 0.01	0.01	0.17	-	-
11-20 d	0.29± 0.02	0.27± 0.02	0.30± 0.02	0.28± 0.02	0.01	0.73	-	-
21-30 d	0.45± 0.03[a]	0.44[ab]± 0.03	0.37[ab]± 0.03	0.36± 0.03[b]	0.01	0.05	-	-
First month	0.29± 0.01	0.27± 0.01	0.26± 0.01	0.24± 0.01	0.01	0.08	0.01	0.16
31-41 d	0.58± 0.03	0.63± 0.03	0.55± 0.03	0.61± 0.03	0.01	0.34	-	-
41-50 d	0.90± 0.03	0.81± 0.03	0.89± 0.03	0.84± 0.03	0.01	0.12	-	-
51-60 d	1.46± 0.08[a]	1.46[a]± 0.08	1.43[a]± 0.08	1.10± 0.08[b]	0.04	0.01	-	-
Second month	0.98± 0.03[a]	0.97[a]± 0.03	0.96[a]± 0.03	0.85± 0.03[b]	0.01	0.01	0.01	0.01
BW, kg								
Initial, 1th day	39.41± 1.74	39.23± 1.74	38.92± 1.74	39.65± 1.74	0.84	0.99	-	-
Final, 56th day	71.69± 2.80[ab]	73.78± 2.80[a]	69.23± 2.80[ab]	65.29± 2.80[b]	2.10	0.03	-	-
ADG (kg/d)	0.70± 0.04[a]	0.72± 0.04[a]	0.62± 0.04[ab]	0.54± 0.04[b]	0.21	0.01	-	-
Feed Efficiency	0.97± 0.08	0.89± 0.08	1.12± 0.08	1.07± 0.08	0.03	0.14		

Treatments: WNA=Water not available, AW=Ad libitum water, WAM= Water available after milk feeding, WBM= Water available before milk feeding.
BW: Body weight, ADG: Average daily gain
[a,b] Values within a row with different superscripts differ significantly at P<0.05.

Table 3. Blood metabolite concentration for different water accessibility of Holstein calves with common starter and milk replacer (Mean ± SEM)

Item	Treatments				SEM	P		
	AW	WNA	WAM	WBM		Treat	Time	Treat×time
Glucose (mg/dL)	128.6± 6.98	139± 6.98	130.5± 6.98	138± 9.87	3.36	0.68	0.42	0.72
Cholesterol (mg/dL)	104.1± 9.8 [a]	103.3± 9.8[a]	72.1± 9.8[b]	71.2± 13.9[b]	5.87	0.03	0.09	0.67
Triglycerides (mg/dL)	30.8±3.05	45.8±3.98	53.6±34.10	44.2±3.45	4.03	0.18	0.08	0.62
AST (u/L)	37.5±4.37 [b]	37.6±4.37[b]	32.1±4.37[b]	53.5± 6.18[a]	2.14	0.01	0.01	0.01
ALT (u/L)	7.6± 0.84	10.1± 0.84	8.1± 0.84	8.0± 1.18	0.45	0.15	0.60	0.37
TAC (mmol/L)	0.19± 0.04[b]	0.37± 0.04[a]	0.44± 0.04[a]	0.20± 0.06[b]	0.04	0.01	0.01	0.01
BHBA (mmol/L)	0.10± 0.02	0.10± 0.02	0.13± 0.02	0.08± 0.02	0.01	0.22	0.47	0.42
Albumin (g/dL)	3.53± 0.09	3.75± 0.09	3.71± 0.09	3.77± 0.12	0.04	0.31	0.67	0.96
NEFA (mmol/L)	0.175± 0.03	0.185± 0.03	0.190± 0.03	0.130± 0.05	0.01	0.58	0.92	0.82
Protein (g/dL)	6.51± 0.14[b]	7.10± 0.14[a]	6.86± 0.14[ab]	6.57± 0.19[b]	0.08	0.01	0.08	0.14
HDL (mg/dL)	30.33± 2.88 [ab]	36.16± 2.88 [a]	23.16± 2.88[b]	23.50± 4.07[b]	1.82	0.01	0.09	0.67
MDA (nmol/mL)	0.91± 0.89	1.05± 0.09	1.13± 0.09	0.85± 0.13	0.05	0.21	0.08	0.72

Treatments: WNA=Water Not Available, AW=Adlibitum Water, WAM= Water available after milk feeding, WBM= Water available before milk feeding
[a,b] Values within a row with different superscripts differ significantly at P<0.05.

Table 3 summarizes the blood parameter data for each group and sampling date. Concentrations of glucose, cholesterol, TG, ALT, BHB, albumin, NEFA and MDA were similar among treatments (Table 3). But the serum concentrations of AST, TAC, protein and HDL significantly changed by different water accessibility. At 24–48 h after birth the level of total protein was in reference range but at other ages was below the reference value. In this study WBM has higher activity of serum AST (53.5 u/L) as compared with other treatments.

TAC levels of serum in this study were significantly higher in group WNA and WAM than others (P<0.05). The treat × time interaction was statistically significant for AST and TAC. Serum concentrations of Protein and HDL in WNA treatment is significantly higher than other groups (P<0.05). But were not influenced by either time or treat × time.

Some blood electrolytes are presented in Table 4. The elements Cl and K did not significantly different among treatments. But Na concentration of blood serum in AW and WAM is 138.08 and 138.01, respectively. These groups had significantly higher serum concentration of Na than others (P<0.05). Water treatments did not affect the fecal score, temperature, and the respiratory score of calves, these parameters are presented in Table 5. The findings of the current study showed that different water accessibility of calves had no significant effects on health, respiratory and fecal score.

Mean body length, withers height, heart girth, body barrel, hip height and hip width during the pre-weaning periods are given in Table 6. There is no difference in frame growth and body condition scores of calves. Rumen fermentation data showed in Table 7. Acetic acid and

Table 4. Blood concentration of some electrolyte for different water accessibility of Holstein calves (Mean ± SEM)

Item	Treatments				SEM	P		
	AW	WNA	WAM	WBM		Treat	Time	Treat× time
Cl (meq/L)	100.5± 9.33	93.83± 9.33	108.33± 9.33	104.75± 13.20	4.55	0.74	0.54	0.49
Na (meq/L)	138.08± 1.16[a]	137.58± 1.16[a]	138.01± 1.16[a]	132.97± 1.64[b]	0.75	0.01	0.03	0.23
K (meq/L)	4.13± 0.11	4.26± 0.11	4.39± 0.11	3.94± 0.15	0.06	0.13	0.12	0.62

Treatments: WNA=Water not available, AW=Adlibitum water, WAM= Water available after milk feeding, WBM= Water available before milk feeding

[a,b] Values within a row with different superscripts differ significantly at P<0.05.

Table 5. Fecal score, temperature, and respiratory score of Holstein calves (Mean ± SEM)

Item	Treatments				SEM	P
	AW	WNA	WAM	WBM		
Health score[1]	1.53± 0.53	2.00± 0.72	2.15± 0.51	2.15± 0.48	0.23	0.78
Nasal discharge	0.30± 0.17	0.53± 0.23	0.61± 0.17	0.61± 0.16	0.07	0.43
Respiratory score	0.61± 0.17	0.76± 0.23	1.23± 0.16	1.15± 0.16	0.13	0.29
Temperature (◦C)	39.07	39.16	39.29	39.31	0.05	0.38
Fecal score	0.76± 0.22	1.15± 0.29	1.53± 0.21	1.15± 0.20	0.13	0.24

Treatments: WNA=Water not available, AW=Ad libitum water, WAM= Water available after milk feeding, WBM= Water available before milk feeding

[1]The day is getting sick until recovery

Table 6. Frame growth measurements and body condition scores for different water accessibility of Holstein calves with common starter and milk replacer (Mean ± SEM)

Item (cm)	Treatments				SEM	P		
	AW	WNA	WAM	WBM		Treat	Time	Treat×time
Body length	60.65± 0.55	60.69± 0.55	60.42± 0.55	60.34± 0.55	0.58	0.96	0.01	0.21
Withers height	82.03±0.82	83±0.85	81.42±0.79	82.38±0.81	0.50	0.44	0.01	0.88
Heart girth	82.92± 0.80	83.88± 0.80	81.65± 0.80	81.46± 0.80	0.69	0.11	0.01	0.76
Body barrel	76.50± 0.94	77.19± 0.94	76.30± 0.94	75.15± 0.94	0.67	0.49	0.01	0.92
Hip height	85.57± 0.70	86.57± 0.70	85.57± 0.70	85.92± 0.70	0.51	0.71	0.01	0.77
Hip width	19.46± 0.23	19.30± 0.23	19.34± 0.23	19.09± 0.23	0.16	0.71	0.01	0.81

Treatments: WNA=Water not available, AW=Ad libitum water, WAM= Water available after milk feeding, WBM= Water available before milk feeding

Table 7. Effects of water treatments on rumen fermentation parameters (Mean ± SEM)

Item	Treatments				SEM	P
	AW	WNA	WAM	WBM		
NH_3 (mmol/L)	23.05±1.25	23.42±1.35	28.77±1.48	31.27±1.65	1.98	0.41
Acetic acid (mM)	58.40± 5.62[a]	57.42± 5.62[a]	36.19± 5.62[b]	23.83± 5.62[b]	5.02	0.01
Propionic acid (mM)	28.39± 4.42[a]	22.00± 4.42[ab]	12.37± 4.42[b]	8.06± 4.42[b]	3.05	0.04
Butyric acid (mM)	7.04± 2.11	6.59± 2.11	8.35± 2.11	2.40± 2.11	1.12	0.29
Isobutyrate (mM)	0.64± 0.53	2.01± 0.53	1.96± 0.53	0.92± 0.53	0.29	0.23
Valerate (mM)	3.17± 0.88	2.93± 0.88	3.36± 0.88	1.17± 0.88	0.45	0.33
Isovalerate (mM)	1.76± 0.46	1.91± 0.46	1.77± 0.46	0.88± 0.46	0.23	0.42

Treatments: WNA=Water not available, AW=Ad libitum Water, WAM= Water available after milk feeding, WBM= Water available before milk feeding, mM=milimolar
[a,b] Values within a row with different superscripts differ significantly at P<0.05.

Table 8. Total times devoted to perform different behaviors during 8 h of observation in preweaning periods (Mean ± SEM)

Item (h)	Treatments				SEM	P		
	AW	WNA	WAM	WBM		Treat	Time	Treat×time
Visit water bucket	0.64± 0.15 [a]	0.01± 0.14	0.40± 0.15 [ab]	0.43± 0.14[a]	0.08	0.01	0.44	0.01
Visit starter bucket	1.07± 0.40	1.61± 0.35	1.93± 0.39	1.25± 0.38	0.19	0.39	0.22	0.07
Rumination	1.07± 0.45	1.11± 0.40	1.73± 0.43	1.00± 0.42	0.21	0.66	0.02	0.78
Standing	1.21± 0.41	1.61± 0.36	1.06± 0.40	1.62± 0.39	0.18	0.66	0.58	0.95
Lying in bed	1.85± 0.97 [b]	3.61± 0.86[ab]	2.80± 0.94 [ab]	5.00± 0.91[a]	0.45	0.02	0.63	0.85
Object play	1.92± 0.49	1.77± 0.43	1.66± 0.47	1.50± 0.46	0.22	0.92	0.54	0.62
Self grooming	1.71± 0.42	1.05± 0.37	2.26± 0.40	1.50± 0.39	0.19	0.17	0.24	0.96
Nonnutritive sucking	3.07± 0.58	2.88± 0.51	3.60± 0.56	3.18± 0.54	0.26	0.83	0.45	0.66
Other	1.00± 0.36	1.11± 0.32	1.60± 0.35	1.00± 0.34	0.16	0.53	0.27	0.75

Treatments: WNA=Water not available, AW=Ad libitum water, WAM= Water available after milk feeding, WBM= Water available before milk feeding.
[a,b] Values within a row with different superscripts differ significantly at P<0.05.

propionic acid of AW group significantly increased as compared with other treatments (P<0.05). As shown in Table 8, treatments did not affect behaviors parameters. But the number of visiting water bucket of AW and WBM were higher than other groups.

Discussion and Conclusion

De Passillé et al. (8) reported that that water intake of calves is low until weaning (0.17 kg/d), whereas other calf studies measured considerable intakes of water (1 kg/d) (19). According to NRC (27), water intake will decrease with increasing cold until 4.4 °C and will remain constant for lower temperatures. In the different decade's age of calves' water intake, between groups had significantly changed, which may have been due to the accessibility of water in these groups during milk feeding calves. Kertz et al. (19) observed that daily water intakes decreased after 3 to 4 d of age compared with initial intakes. Increased DMI, and increased calf body size, which consequentially led to an increase in water requirements. The researchers indicated (3) demonstrated that total body water content of dairy calves decreased from 80 to 70% during the first 40 d. According to Wenge et al. (34) restrictively-milk fed calves drank 1.1 L water per day within the first 3 weeks of life. The NRC (27) reported that water intake increases from 1 kg/d at first week of age to 2.5 kg/d at 4 weeks of age. Lower water drinking of calves in this study may be because of decreasing the air temperature.

The studies were revealed that starter intake affected by water accessibility as shown by Gonzalez (11) that water appears to have a relationship with starter intake as starter intake is a significant variable in predicting the water intake of a calf, especially after day 21 when starter intake and water intake are both increasing. Prior to 21 days, the relationship between water intake and starter intake is not significant but does trend to be related. These results corroborate the positive relationship normally observed between total DM intake and water intake (19). Conversely, when a greater volume of milk is offered (and indirectly, a greater amount of TS), there is a negative relationship between DM intake and voluntary water intake (21), because the liquid feed fills part of the need

for water (2). Unlike water in milk, which is shunted directly to the abomasum, drinking water enters and becomes a part of the developing reticulorumen (hereafter called rumen) of pre-weaned calves (13). Therefore, voluntary water intake of pre-weaned calves (0.75 to 0.82 kg/d) partially represents a water requirement of the developing rumen. In this study higher water intake of AW and WBM groups maybe showed that water in calves after 10 days did not supply the requirements. Thus, calves had the potential to consume more water in this system. These results agree with De Passillé et al. (8) and Hepola et al. (15), who reported higher water intakes in calves fed limited quantities of milk. In contrast, higher starter intakes tend to increase the quantity of water consumed (19).

The contradictory alteration by different water accessibility on blood metabolites was shown by different studies, as Beiranvand et al. (5) reported that addition of water to the starter (up to 50% DM) had no significant effects on blood glucose concentration. Serum albumin concentrations partially reflect hepatic synthesis and its increasing could be related to compensation of decreasing serum osmotic pressure due to globulin levels decline. The level of total protein was below reference value at ages except at 24–48 h after birth. It was approximately similar to Knowles et al. (22) reports. Serum total protein levels were below previously reported reference range from birth up to day 84 of age (9) and from birth up to day 90 of age (36). Knowles et al. (22) revealed that albumin levels in calves were below the reference range of adults until after nine days of age.

It is well known that AST is a nonspecific marker of acute liver damage caused by high-grain diets and associated ruminal lactate production (25). The activities of AST were below the reference range. This is consistent with the Egli and Blum (9) study. Zanker et al. (36) reported a postnatal rise of AST activity had no association with the time of first colostrum intake, indicating that the rise of AST activity was the consequence of endogenous production and was independent of colostrum intake. Serum constituents and hematological profiles evaluated were within normal ranges reported by Kaneko (18).

Reece (30) reported that serum concentration of Na, K and Cl in steers were 141.8, 4.6 and 105 (mEq/L), respectively. Roy et al. (31) indicated that Mean serum-sodium values at birth were 139-140 mEq/L. They revealed that most of the deaths occurred, when the sodium level was low and the potassium level normal. With the increasing incidence of scouring, a greater fall in mean serum-sodium values occurred. Mean serum-potassium levels at birth were 5.8-6.0 mEq/L. For calves that scoured on the 1st day or not at all the mean serum potassium fell gradually to 5.8 mEq/L as the results of Roy et al. (31) study indicated clearly that, with an increased incidence of scouring the serum-sodium levels of calves decline with a concomitant but a slight rise in potassium values. NRC (27) showed that about eighteen times more water and eleven times more sodium and potassium were lost each day in the feces of scouring calves than in those of normal calves. They suggested that this loss was due to the increased osmotic pressure in the lumen of the gut owing to an increase in the number of smaller molecules, such as volatile fatty acids, resulting from bacterial activity. The higher osmotic pressure in the gut thus caused the infiltration of large amounts of water accompanied by electrolytes. It is well known that an increase in potassium concentration in the serum can occur with a cellular deficiency of potassium (30) and NRC (27) have emphasized that potassium should never be given to dehydrated patients because of the danger of an increase in potassium concentration in the serum and cardiac arrest. Wickramasinghe (35) reported that blood sodium concentrations of calves receiving drinking water from birth and calves that did not receive water during the first 17 d after birth were 134.4 and 136.1 mmol / L, respectively. They revealed that this could be due to a greater intake of electrolyte solution given the greater severity of scours among calves did not receive water until days 17. Roy et al. (31) studied changes in serum sodium and potassium concentrations in 150 experimental calves, some of which became affected with diarrhea. They observed that diarrhea caused a decrease in serum sodium and an increase in serum potassium concentrations. Milk is a poor source of the major electrolytes lost in diarrhea (Na^+, Cl^-, $HCO3^-$) (27). Reece (30) reported that in 46 % of the plasma samples collected from diarrhoeic calves, the sodium concentrations were below normal. Since the diarrhoeic calves lost weight, and as part of this loss in body weight was presumably due to a reduction in body water (30), it would appear that these particular samples were from calves which were hypotonically dehydrated. However, as pointed out by Roy et al. (31), the plasma electrolyte concentration only shows the relative proportions of water to electrolytes in the plasma and in itself is not an indication of whether deficits or excesses of water and electrolytes are actually present in the body as a whole. Previous studies have confirmed (30) that in diarrheic calves there is characteristically a loss in body weight, negative sodium and potassium balance.

Overall, the calves in our study were healthy. This result agreed with Gottardo et al. (12) that reported calves receiving water showed a good health status throughout the fattening period, reducing the feed refusal days significantly. Water availability did not lead to the excretion of looser feces; therefore, no consequent detrimental effect on calves' body cleanliness was observed. Wickramasinghe et al. (35) reported that calves

that 17 days delay of the offer first drinking water to new-born calves.

The excessive intake of drinking water might affect microbial fermentation in the rumen and thus rumen development. No study has examined the effect of water on rumen development of calves. Few studies focusing on the relationship between drinking water and rumen functions in mature cows provide some insight on the potential effects of drinking water in calves. For instance, Fraley et al. (10) found lower molar proportions of butyrate and propionate in the rumen, which were associated with lower liquid weight in the rumen and high fractional liquid passage rates in lactating cows with high water intake. Butyrate and propionate are often considered to be major drivers of morphological and metabolic developments of the rumen in calves (23). Therefore, the increased water intake of WBM calves could be hypothesized to inversely affect rumen development.

The number of drinking bouts recorded by Beiranvand et al. (5) resulted in mean values between 3 and 5 visits per animal per day and this was at a similar level compared to our own results. According to Gottardo et al. (12), the results of the two indicators illustrated that suckler cows showed climate-induced behavioral changes, whereas water intake was not influenced by increasing cold.

The results indicate that suckler calves with different water accessibility had different water intake. AW and WBM treatments drank more water than other groups (P<0.05). Water accessibility was also found to increase AST and Na concentration of serum but serum concentrations of protein and HDL and TAC levels were significantly higher in groups WNA and WAM than others (P<0.05). Different water accessibility of calves had no significant effects on respiratory and fecal score, growth parameters and behaviors. But acetic acid and propionic acid of AW groups significantly increased as compared with other treatments (P<0.05). Overall, in agreement with our hypotheses, giving water to young calves around milk meal had some beneficial effects but resulted in similar growth performance compared with calves that did not access to water. As a conclusion, it can be recommended that water accessibility around milk meal for suckling calves will be resulted in healthier calves.

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Conflict of Interest

The authors declare that they have no competing interests.

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The effects of fumaric and malic acids on the *in vitro* true digestibility of some alternative feedstuffs for ruminants

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Abstract: The aim of this study was to determine the effects of the addition of different amounts of fumaric acid (FA) and malic acid (MA) to the leaves of *Robinia pseudoacacia* (Black locust, acacia), *Prunus laurocerasus* (cherry laurel), *Quercus cerris* (oak), and *Camellia sinensis* (tea factory wastes, TFW), to improve their value as alternative feeds for ruminants. The parameters examined were the *in vitro* true digestibility of feed ($IVTD_{As\ fed}$), dry matter ($IVTD_{DM}$), organic matter ($IVTD_{OM}$), neutral detergent fiber ($IVTD_{NDF}$) and count of protozoans. The digestibility experiments were performed with a DAISY incubator system. Organic acids were not added in the control group and 0.1%, 0.2% or 0.3% FA or MA were added to the experimental groups. Each treatment was replicated 6 times. Samples were incubated for 48 hours. Fumaric acid significantly reduced (P<0.01) all digestibility values of *R. pseudoacacia* leaves. When FA was applied at 0.1% to *C. sinensis* factory wastes, the $IVTD_{OM}$ increased significantly (P <0.05), with the same effect observed for *Q. cerris* (P<0.01). However, for the addition of 0.1% FA, $IVTD_{As\ Fed}$, $IVTD_{DM}$ and $IVTD_{NDF}$ values decreased significantly (P<0.01). Separately, malic acid did not have a significant effect on the *in vitro* true digestibility values determined in this study (P>0.05). Rumen protozoan counts decreased for both organic acid applications compared to counts in the fresh rumen contents. Because 0.1% fumaric acid increased the $IVTD_{OM}$ values of both *C. sinensis* factory wastes and *Q. cerris* leaves they can be considered potential alternative feed sources for ruminants.
Keywords: Fumaric acid, *in vitro*, malic acid, true digestibility

Ruminantlar için fumarik ve malik asitlerin bazı alternatif yem maddelerinin *in vitro* gerçek sindirilebilirliği üzerine etkileri

Özet: Bu araştırmanın amacı farklı düzeylerdeki fumaric (FA) ve malik (FA) asitin ruminant alternatif yem kaynağı olarak *Robinia pseudoacacia* (yalancı akasya), *Prunus laurocerasus* (karayemiş), *Quercus cerris* (meşe), ve *Camellia sinensis* (fabrika atığı, TFW) yapraklarının sindirilebilirlik değerleri üzerine olan etkilerini incelemektir. Araştırmada materyallerin yem bazında ($IVTD_{As\ fed}$), kuru madde bazında ($IVTD_{DM}$), organic maddede ($IVTD_{OM}$), nötral deterjen fiber ($IVTD_{NDF}$) *in vitro* gerçek sindirilebilirlikleri ve protozoa sayıları parametreleri üzerine çalışılmıştır. Sindirilebilirlik parametreleri DAISY inkübatör sistemi ile gerçekleştirilmiştir. Kontrol grubuna FA ve MA eklenmezken deneme grupları ayrı ayrı ve sırasıyla %0,1, %0,2, %0,3 FA ve MA içerecek şekilde düzenlenmiştir. Her örnek 6 kez tekrarlı olacak şekilde araştırmaya dahil edilmiştir. Örnekler 48 saat inkubasyona tabi tutulmuştur. Fumarik asit *R. pseudoacacia* yapraklarında tüm sindirilebilirlik değerlerini önemli (P<0,01) derecede azaltırken değerlerin doza bağlı linear değişimi de önemli (P<0,01) bulunmuştur. Fumarik asit TFW materyaline %0,1 düzeyinde uygulandığında $IVTD_{OM}$ önemli derecede (P<0.05) yükselmiştir. Aynı etki *Q. cerris* için de görülmüş (P<0,01) ancak $IVTD_{As\ Fed}$, $IVTD_{DM}$ ve $IVTD_{NDF}$ değerlerinin önemli seviyede (P<0,01) düştüğü saptanmıştır. Malik asit bu araştırmada *in vitro* gerçek sindirim değerleri üzerine önemli bir etki yaratmamıştır (P>0,05). Rumen protozoa sayıları her iki organik asit uygulamasında da taze rumen içeriğine göre azalmıştır. Sonuç olarak, *C. sinensis* fabrika atıkları ve *Q. cerris* yapraklarına, %0,1 düzeyinde fumarik asit ilavesinin $IVTD_{OM}$ değerlerini artırdığından dolayı, *C. sinensis* fabrika atıkları ve *Q. cerris* yapraklarının ruminantlar için potansiyel alternatif yem kaynağı olarak değerlendirilebileceği kanısına varılmıştır.
Anahtar sözcükler: Fumarik asit, gerçek sindirilebirilik, *in vitro*, malik asit.

Introduction

Organic acids have been used for many years for the purpose of improving the effectiveness of beneficial microorganisms and hence of rumen fermentation in ruminants. Organic acids are described as "Generally Recognized As Safe" (32) for addition to animal feed. The rumen has both a complex biota and ecology. Different species of eukaryotes, prokaryotes, archaeans and bacteriophages play important roles in the functioning of the rumen (20). Dicarboxylic organic acids such as fumaric acid (FA) and malic acid (MA) have been used to manipulate the microbial ecology of the rumen (10, 19). Organic acids can pass through the intestinal mucosal barrier by diffusion and are then involved in the Krebs cycle (32). The use of FA and MA increased the growth rate and number of the bacterium *Selenomonas ruminantium* (25, 32) which uses lactate produced by ruminal bacteria as a source of energy (25). These acids have beneficial effects on fermentation and microbial populations when used in combination with cellobiose and monensin. Organic acids have also been shown to increase the rate of protein hydrolysis (32).

The plant materials used in this research can be found in many geographies and are easy to obtain and naturally contain tannins. The search for alternative feed sources for ruminants has increased the interest in leaves containing tannins as potential resources. Tannins are water-soluble, polyphenolic compounds usually found in plants with high fibre levels. Although there can be adverse effects from tannins, improved feed consumption, feed efficiency and growth have been reported (35). Furthermore, tannins have been included in animal feed for research purposes due to their antioxidant effects against free radicals, metal binding properties and lipid peroxidation inhibition properties (18). However, Tieman et al. (36) reported that plants with high tannin content have low cellulose digestibility. In spite of some anti-nutritional properties, plant sources containing tannins have been used in the feeding of different kinds of animals because they are easy to access and cheap (35).

Against that background, the aim of this study was to use *in vitro* methods to determine the effects of the addition FA and MA on the actual digestibility parameters and count of protozoans associated with four alternative roughage sources.

Materials and Methods

For this study, ethics committee approval was not needed because the rumen samples were collected only from slaughtered animals.

The tree leaves (*Robinia pseudoacacia* (black locust, acacia), *Prunus laurocerasus* (cherry laurel), *Quercus cerris* (oak)), used in the study were collected according to the methodology described in British Columbia Ministry of Forests (5) from Samsun Canik Town forest (41°15' 29" N, 36°21'56" E and 41° 15' 48" N, 36° 22' 05" E), which is located approximately 150 m above sea level. The tea *(Camellia sinensis)* factory waste was obtained from tea factories located in Rize Province at the eastern end of the Black Sea region in Turkey. All tree leaves and tea factory waste samples were collected in May 2019. The FA (≥99% purity) and MA (≥95% purity) were obtained from Sigma Aldrich® (Istanbul, Turkey).

Fresh tree leave samples were weighed as fresh and then dried at 65 ºC for 48h. The dried samples were ground in a mill and then passed through a sieve with a mesh diameter of 1 mm for chemical analysis. Dry matter (DM) content of each sample was determined in air circulation drying oven at 105 °C for 4 hours and the ash content was determined by burning the dried material in an ash oven at 550 °C for 4 hours. The Kjeldahl method was used to determine the crude protein (CP) percentage. Ether extract (EE) was performed according to the methods of AOAC (2). The neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents of the materials that form the cell wall components of the feeds used in this study were determined in accordance with the method reported by Van Soest et al. (37) and were analyzed in a ANKOM 200 Fiber Analyzer (ANKOM Technology Corp. Fairport, NY, USA). The *in vitro* true digestibility (IVTD) analysis was performed with the ANKOM Daisy Incubator (ANKOM Technology Corporation), according to the methodology described in ANKOM (1). The rumen fluid was collected post-mortem from the rumens of four Holstein x Yerlikara hybrid cattle aged three years that were slaughtered in a commercial abattoir in Samsun, Turkey. Animals were fed twice daily with a diet containing grass hay and maize silage (60%) and concentrates (40%). A thermos was used to store and transport the rumen fluid, which was preheated to 39°C and had CO_2 added. The rumen fluid was collected manually by squeezing two handfulls of ruminal contents from each animal's rumen into the same thermos. In the laboratory, the rumen fluid was filtered through 4 layers of gauze. The F57 bags to be used in the analysis were rinsed with acetone (99.5%) for 3 minutes and then the acetone was evaporated at room temperature. All the bags were marked with both acid and alkaline resistant pen. The bags were dried in a drying cabinet at 105 ºC for 2 hours. The tare weights of the bags were recorded. Samples of plants weighing 0.5 g were transferred to separate F57 bags which were then closed. The buffer solution to be used in the analysis was prepared according to the Ankom Daisy *in vitro* fermentation system described in ANKOM (1). Four digestion units, each with a volume of 2 L, were used in this test. The buffer solution was heated to 39 °C and 1.6 L was poured into each digestion unit. Four hundred mL of rumen fluid was added to each unit. A total

of 24 feed samples were used in each digestion unit, and 6 replicates were formed from each feed sample at the same time. The tests with FA and MA were carried out separately but with otherwise identical procedures. While no acid was added to the control group units, 0.1, 0.2 or 0.3% FA or MA was added to the experimental group units. A total of 24 feed samples were added to each of the control and experimental group units, that is, F57 bags, with 6 replicates of each feed sample. The samples were incubated for 48 hours. After the incubation period, all the liquid in the digestion units was removed and the bags were washed under running water. NDF analysis was performed as per the method outlined in ANKOM (1) by placing the bags in the Ankom Fiber Analyzer device. After analysis, the bags were retained in the drying cabinet until they reached a constant weight at 105 °C. The IVTD values of all samples were calculated with the formula reported in ANKOM (1),

$$IVTD\% = \frac{100-(W3-(W1xC1))x100}{W2}$$

where:

W1= Bag tare weight

W2 = Sample weight

W3= Final bag weight after *in vitro* process and sequential ND treatment

C1= Blank bag correction (final oven-dried weight/original blank bag weight)

For the protozoan count, a mixture of 0.6 g methyl green, 8 g sodium chloride (NaCl) and 100 ml 37% formaldehyde was prepared (31). The mixture volume was made up to 1,000 ml with distilled water. One milliliter of the mixture and 1 mL of the liquid containing protozoans that had been taken from the digestion unit were transferred to a Fuchs Rosenthal counting chamber apparatus. For the protozoa count, fresh rumen fluid and samples taken from each digestion unit after the incubation period were studied in parallel. The protozoans were counted on an object slide under a light microscope (Nicone eclipse 80i) with a Fuchs-Rosenthal counting chamber (depth: 0.2 mm, small square area: 0.0625 mm^2) (31).

$$Cell\ number\ per\ CMM = Number\ of\ cells\ counted\ X \frac{1}{Area\ counted\ (mm^2)} X \frac{1}{Depth\ (mm)} X\ Dilution$$

Statistical analysis: The Kolmogorov-Smirnov Test was used to check for normal distribution of the data, and for homogeneity of variance, the data were evaluated with the Levene Test. All traits on digestibility in the study were summarized as the mean of the group and standard error of means (SEM). For the determination of the differences among the groups, the one-way ANOVA model was fitted to the data for chemical composition, $IVTD_{As\ Fed}$, $IVTD_{DM}$, $IVTD_{OM}$ and $IVTD_{NDF}$. ANOVA equation is:

$$Y_{ij} = \mu + a_i + e_{ij} \ (1),$$

Where Y_{ij} is the value for i. group and j. observation; μ is the population mean; and e_{ij} are the individual error terms distributed as N~(0, 1).

To evaluate the differences among the three concentrations of the organic acids, second degree orthogonal polynomial contrasting was used (13). One-way ANOVA and the other statistical tests and calculations were executed with SPSS Software (34).

Results

Chemical composition of the four alternative feeds are stated in Table 1. The $IVTD_{As\ Fed}$, $IVTD_{DM}$, $IVTD_{OM}$, and $IVTD_{NDF}$ values for all doses of FA applied to *R. pseudoacacia* leaves were significantly different from the control (P<0.01) (Table 2). Fumaric acid negatively affected *in vitro* digestion across all parameters in *R. pseudoacacia* leaves. In contrast, MA did not significantly affect *in vitro* digestion in any of the examined digestion parameters (P>0.05) in *R. pseudoacacia* leaves. However, when MA was administered at 0.1%, the values of all parameters for *in vitro* digestion were numerically higher in *R. pseudoacacia* leaves.

Fumaric acid was found to be significantly (P<0.05) effective only on $IVTD_{OM}$ and the highest increase was seen in 0.1% dosing in TFW (Table 3). On the other hand, *in vitro* digestion was found to be numerically higher to all parameters when administered at doses of 0.1% and 0.2% in TFW. Malic acid did not significantly (P>0.05) affect *in vitro* digestion of TFW. However, it was observed that *in vitro* digestion values increased numerically to all parameters although it was not statistically (P>0.05) significant depending on the doses in TFW.

Table 1. Nutrient composition (g/100g DM) of alternative feed sources for ruminants.

Feed sources	DM	Ash	CP	EE	ADF	NDF
C. sinensis	93.5	4.8	18.2	1.16	34.6	40.5
Q. cerris	95.7	4.3	10.1	3.2	32.2	40.1
R. pseudoacacia	92.3	5.5	27.5	2.4	15.8	18.2
P. laurocerasus	90.7	10.8	8.5	1.1	11.3	23.1

DM: Dry matter, CP: Crude protein, EE: Ether extract, ADF: Acid detergent fiber, NDF: Neutral detergent fiber.

Table 2. Effects of the addition of different concentrations (%) of organic acids to *R. pseudoacacia* leaves (n=6) on *in vitro* true digestibility values (Mean±SEM).

Fumaric acid	**$IVTD_{As\ Fed}$**	**$IVTD_{DM}$**	**$IVTD_{OM}$**	**$IVTD_{NDF}$**
Control 0%	60.98±0.01	60.66±0.01	60.81±0.03	17.46±0.02
0.1%	60.24±0.31	59.85±0.34	60.02±0.34	16.22±0.53
0.2%	58.13±0.27	57.58±0.29	57.73±0.20	12.72±0.45
0.3%	57.94±0.02	57.37±0.02	57.55±0.01	12.41±0.03
P				
Combined	0.001	0.001	0.001	0.001
Linear	0.002	0.002	0.001	0.002
Malic acid	**$IVTD_{As\ Fed}$**	**$IVTD_{DM}$**	**$IVTD_{OM}$**	**$IVTD_{NDF}$**
Control 0%	59.75±0.62	59.32±0.68	59.43±0.69	15.40±1.05
0.1%	60.80±0.16	60.46±0.17	60.52±0.15	17.16±0.26
0.2%	60.86±0.39	60.53±0.42	60.69±0.44	17.27±0.66
0.3%	57.93±0.96	57.35±1.04	57.39±0.91	12.38±1.60
P				
Combined	0.077	0.077	0.058	0.077
Linear	0.943	0.943	0.861	0.943

IVTDAs Fed: *In vitro* true digestibility as fed, $IVTD_{DM}$: *In vitro* true digestibility of dry matter, $IVTD_{OM}$: *In vitro* true digestibility of organic matter, $IVTD_{NDF}$: *In vitro* true digestibility of neutral detergent fiber.

Table 3. Effects of addition of different concentrations (%) of organic acids (fumaric acid, malic acid) to *C. sinensis* (n=6) factory waste product (TFW) on *in vitro* true digestibility values (Mean±SEM).

Fumaric acid	**$IVTD_{As\ Fed}$**	**$IVTD_{DM}$**	**$IVTD_{OM}$**	**$IVTD_{NDF}$**
Control 0%	50.96±0.90	49.21±0.96	49.73±0.96	12.19±1.27
0.1%	53.21±0.10	51.63±0.11	52.13±0.10	15.36±0.14
0.2%	51.26±0.35	49.54±0.38	50.12±0.37	12.67±0.51
0.3%	50.06±0.36	48.25±0.38	48.73±0.36	10.94±0.53
P				
Combined	0.051	0.051	0.048	0.054
Linear	0.057	0.057	0.061	0.062
Malic acid	**$IVTD_{As\ Fed}$**	**$IVTD_{DM}$**	**$IVTD_{OM}$**	**$IVTD_{NDF}$**
Control 0%	50.20±0.23	48.41±0.25	48.89±0.24	11.17±0.35
0.1%	51.38±0.87	49.67±0.93	50.26±0.94	12.79±1.24
0.2%	51.97±0.75	50.30±0.81	50.91±0.80	13.65±1.03
0.3%	52.98±0.37	51.38±0.40	51.97±0.42	15.06±0.56
P				
Combined	0.128	0.128	0.117	0.131
Linear	0.536	0.536	0.525	0.527

IVTDAs Fed: *In vitro* true digestibility as fed, $IVTD_{DM}$: *In vitro* true digestibility of dry matter, $IVTD_{OM}$: *In vitro* true digestibility of organic matter, $IVTD_{NDF}$: *In vitro* true digestibility of neutral detergent fiber.

Fumaric acid produced only a numerical increase (P> 0.05) in the *in vitro* digestion parameters of the leaves of *P. laurocerasus* (Table 4) when applied at a dose of 0.3%, however MA had no significant (P>0.05) effect on any of the parameters.

It was found that *in vitro* digestion levels were significantly (P<0.005) reduced for $IVTD_{As\ Fed}$, $IVTD_{DM}$ and $IVTD_{NDF}$ parameters by adding FA to *Q.cerris* (Table 5) leaves however, $IVTD_{OM}$ digestion was found to be significantly (P<0.005) higher when 0.1% dose was applied. At the same time dose-dependent changes of the differences were also significant (P<0.05). It was found that MA had an enhancing effect on the *in vitro* digestion of *Q. cerris* leaves at a dose of 0.1% for all parameters, but the increase was not statistically significant (P>0.05).

The effects of different concentrations of organic acids on the total count of rumen protozoans are stated in Table 6. Compared to the fresh rumen content group, a

decrease was observed in the experimental groups. Fumaric acid at all level of 0.1% and 0.3% increased the count of protozoans numerically in comparison to the control group and in contrast MA numerically reduced the count of protozoa inversely proportional to increasing dose.

Table 4. Effects of addition of different concentration (%) of organic acids (fumaric acid, malic acid) to *P. laurocerasus* (n=6) leaves on *in vitro* true digestibility values (Mean±SEM).

Fumaric acid	$\mathbf{IVTD_{As\ Fed}}$	$\mathbf{IVTD_{DM}}$	$\mathbf{IVTD_{OM}}$	$\mathbf{IVTD_{NDF}}$
Control 0%	59.76±0.58	58.90±0.64	58.99±0.63	14.33±0.94
0.1%	59.49±0.71	58.60±0.79	58.98±0.86	13.89±1.16
0.2%	59.04±1.49	58.11±1.64	58.45±1.61	13.16±2.41
0.3%	60.51±0.11	59.73±0.12	59.96±0.11	15.55±0.18
P				
Combined	0.706	0.706	0.747	0.706
Linear	0.736	0.736	0.719	0.736
Malic acid	$\mathbf{IVTD_{As\ Fed}}$	$\mathbf{IVTD_{DM}}$	$\mathbf{IVTD_{OM}}$	$\mathbf{IVTD_{NDF}}$
Control 0%	61.17±0.56	60.46±0.61	60.63±0.61	16.62±0.90
0.1%	61.73±0.14	61.07±0.15	61.23±0.17	17.51±0.22
0.2%	61.58±0.01	60.91±0.02	61.04±0.02	17.28±0.03
0.3%	61.17±0.42	59.73±0.12	60.63±0.47	16.61±0.69
P				
Combined	0.634	0.634	0.659	0.634
Linear	0.787	0.787	0.750	0.787

IVTDAs Fed: *In vitro* true digestibility as fed, $IVTD_{DM}$: *In vitro* true digestibility of dry matter, $IVTD_{OM}$: *In vitro* true digestibility of organic matter, $IVTD_{NDF}$: *In vitro* true digestibility of neutral detergent fiber.

Table 5. Effects of addition of different levels (%) of organic acids (fumaric acid, malic acid) to *Q. cerris* (n=6) leaves on *in vitro* true digestibility values (Mean±SEM).

Fumaric acid	$\mathbf{IVTD_{As\ Fed}}$	$\mathbf{IVTD_{DM}}$	$\mathbf{IVTD_{OM}}$	$\mathbf{IVTD_{NDF}}$
Control 0%	46.75±0.14	45.47±0.15	45.40±0.89	12.86±0.14
0.1%	46.64±0.11	45.35±0.12	46.01±0.12	11.88±0.55
0.2%	45.82±0.25	44.49±0.26	44.94±0.37	9.64±0.62
0.3%	45.69±0.29	44.36±0.30	44.81±0.15	9.17±0.07
P				
Combined	0.004	0.004	0.004	0.005
Linear	0.020	0.020	0.017	0.020
Malic acid	$\mathbf{IVTD_{As\ Fed}}$	$\mathbf{IVTD_{DM}}$	$\mathbf{IVTD_{OM}}$	$\mathbf{IVTD_{NDF}}$
Control 0%	46.95±0.09	45.68±0.10	46.36±0.09	11.48±0.12
0.1%	47.52±0.43	46.28±0.46	46.94±0.46	12.33±0.58
0.2%	46.76±0.62	45.47±0.66	46.08±0.56	11.21±0.98
0.3%	46.73±0.75	45.44±0.79	46.13±0.80	11.16±1.15
P				
Combined	0.499	0.499	0.464	0.520
Linear	0.227	0.227	0.190	0.240

IVTDAs Fed: *In vitro* true digestibility as fed, $IVTD_{DM}$: *In vitro* true digestibility of dry matter, $IVTD_{OM}$: *In vitro* true digestibility of organic matter, $IVTD_{NDF}$: *In vitro* true digestibility of neutral detergent fiber.

Table 6. Effects of addition of different concentrations (%) of fumaric acid (FA) and malic acid (MA) on the total ruminal protozoa count per mL.

Additives	**Protozoa count in fresh rumen liquid**	**Protozoa count after 48 hours incubation**			
		Control 0%	**0.1%**	**0.2%**	**0.3%**
FA	917262	161938	186731	143438	190625
MA	950000	198929	182929	165954	161928

Discussion and Conclusion

Chemical composition (DM, Ash, CP, EE, ADF and NDF) of feedstuffs is known to be important in terms of animal nutrition. For *P. laurocerasus,* the parameters mentioned above have not been investigated previously. Özyılmaz (28) reported that TFW had 93.42% DM, 14.07% CP, 4.69% ash, 1.07% EE, 47.76% NDF and 40.93% ADF levels and that these values varied according to the organic or conventional cultivation of the tea plants and their harvest periods. Parissi et al. (29) and Luginbuhl and Mueller (24) reported that *R. pseudoacacia* leaves had 27.3% CP (g/100g DM) and 28.0% (g/100g DM) respectively, which is consistent with the results of the present study.

For *Q. cerris*, Kaya and Kamalak (15) reported 91.6% DM, 4.3% CP, 23.6% NDF and 18% ADF. Also for *Q. cerris* leaves, Canbolat et al. (8) reported 94.6% DM, 8.4% CP, 5.5% ash, 43.5% NDF and 36% ADF levels. While some of the values obtained in our study are in relative agreement with the results presented for other studies, other results differed markedly. These differences may have been due to the growing of the plants in different environments, different variety of plants and harvesting of leaf samples at different times.

In the current study, the effects of FA and MA on the *in vitro* true digestibility and protozoa count of four alternative feedstuffs for cattle were varied considerably. In a trial (4) in which these acids were used in conjunction with paddy straw, no concentrations had a statistically significant effect on IVTD ($P > 0.05$). In another study reported that rumen digestion and sodium retention were not affected by adding a salt of MA to corn silage (21). Furthermore, Ebrahimi et al. (12) reported that both FA and MA had no effect on digestibility as measured through DM, OM, NDF and ADF. Similarly, in our study, both FA and MA did not have a significant effect on IVTD values when they were used in combination with *P. laurocerasus* leaves ($P>0.05$).

In our study, the fact that FA dramatically reduces the *in vitro* digestion values of acacia leaves may be related to its high level of crude protein. As a matter of fact, Chen (11) attributed the low *in vitro* digestion values of *R. pseudoacacia* leaves to higher levels of crude protein compared to carbohydrate levels, despite low levels of NDF and ADF. They also pointed out that the *R. pseudoacacia* leaves had high level of condensed tannin and lectin that could prevent fermentation. A similar description of the *in vitro* digestive properties of *R. pseudoacacia* leaves are described by Burner et al. (6).

Sirohi et al. (33) reported that FA (0, 5, 10 and 15 mM) added to rations that included berseem, sorghum and wheat straw containing different proportions of cellulose had a positive effect on $IVTD_{DM}$. In this study (33), the highest digestion percentage was seen in the group with the lowest cellulose level and 10 mM concentration of fumaric acid. There was a significant increase ($P<0.05$) in microbial biomass for all cellulose levels in the berseem group. Furthermore, for the sorghum group, FA at 15 mM concentration significantly ($P<0.05$) decreased the $IVTD_{DM}$, proportional to the amount of cellulose. In addition, the count of protozoa was significantly reduced in all sorghum and berseem groups ($P <0.05$) and this effect was not correlated with the amount of cellulose (33). The fact that the data obtained in our research on $IVTD_{DM}$ is not compatible with the study of Sirohi et al. (33) may be attributed to the different nutrient and cell wall structure of plant materials. Sirohi et al. (33) reported that FA increased the $IVTD_{DM}$, but the true digestibility values for the groups that received the highest concentrations of FA and MA were low, which is in agreement with the results of the current research. The same researchers reported that the different types of feed may have had different effects on $IVTD_{DM}$ values; this argument is supported by the results of our studies.

In the researches, the findings of the effects of MA administered on different doses and durations on rumen fermentation vary. Carro and Ranilla (9) reported that a 10 mM/L of malate and a 17-hour incubation period was insufficient for the complete fermentation of treatments that included corn, barley, wheat and sorghum separately. Among the feeds used, the most fermentation occurred in the corn group. However, Callaway and Martin (7) reported that the application of malate at a concentration of 7.5 mM/L resulted in complete fermentation in ruminal fluid within 10 to 24 hours. It therefore appear that no any significant ($P<0.05$) enhancing effects of MA on *in vitro* digestion were observed in the present study for any plants because of the low content of fermentable material they have.

According to Castillo et al. (10), organic acid salts may be more useful in facilitating rumen fermentation due to their buffering properties. Montano et al. (26) reported that MA added to a highly concentrated feed had no effect on ruminal digestion of OM, ADF, starch level, microbial numbers, microbial digestion and protein level. Similar findings were reported for a high roughage ration supplemented with MA (21). The high solubility of this acid contributes to the effects it has on chemical reactions (25). In the study of Kara (14) in which MA was added to corn silage at 0.5%, 1%, and 1.5%, $IVTD_{OM}$ was not affected ($P>0.05$) by the applications. This finding is supported by the results of our research. Kara (14) suggested that the effects of MA on NDF were due to the increased solubility of the cellulose in silage. Khampa et al. (16) demonstrated that Dimethyl (DL) -malate had no effect on digestion of DM, OM, CP and NDF but increased ADF digestion for cassava ($P>0.05$). The use of MA at high doses did not have a statistically significant effect on

in vitro digestion in other studies (4, 7, 9, 12). Consistent with these data, in our study, when MA was used at 0.1% dose, it was observed that *in vitro* digestion levels increased only numerically in *R. pseudoacacia*, TFW and *P. laurocerasus* leaves. Different findings obtained from *in vitro* digestion studies with different feed materials can be explained by the efficacy of antinutritional factors such as tannin and other polyphenols that affect rumen microbial activity.

In the present study, only 0.1% and 0.3% dose of FA increased protozoa counts numerically and MA showed a reducing effect on protozoa counts with increasing dose. This finding is consistent with the reports of other studies (17, 27, 32) investigating the effects of organic acids on rumen microorganisms. Ok et al. (27) reported that FA and MA have different effects on bacteria, protozoa and other microbial community. They reported that these acids had an increasing effect on rumen bacteria, but decreased the number of methanogenic archaea species that could form complexes with protozoa. Sahoo and Jena (32) reported that MA increases the number of lactate utilizing *S. ruminantium* in rumen, leading to a decrease in lactic acid levels. Therefore the count of ruminal protozoa may also be adversely affected due to the change in pH. This situation may be explained as the anionic effects of organic acids may adversely affect microbial life (32).

Li et al. (22) stated that the numbers of fumarate-utilising bacteria (*Fibrobacter succinogenes, S. ruminantium*) did not change significantly in the presence of FA and also that DM digestibility ratios were not affected by the application. Lopez et al. (23) reported that the application of sodium fumarate to rumen fluid did not change the total number of bacteria during a 48-hour incubation period but increased the number of cellulolytic bacteria three fold ($P<0.01$).

Partanen (30) stated that the effects of organic acids on rumen bacteria vary according to the chemical properties of the acids. Gram (+) bacteria are sensitive to long chain acids whereas Gram (-) bacteria are sensitive to acids with less than 8 carbon atoms. The authors of the study suggest that these effects should also be investigated with respect to protozoans. In addition, Asanuma and Hino (3) stated that the increasing effects of higher amounts of organic acids on DM digestibility are associated with an increase in the cellulolytic bacteria population, along with an increase in H_2 transfer. Therefore, the author(s) of the present study recommend that the determination of true digestibility should also take into consideration the counts of protozoans, ruminal bacteria and methanogen archaea together.

In this study, it was observed that the digestion of organic matter of *C. sinensis* factory wastes and *Q. cerris* leaves could be increased by treating with 0.1% fumaric acid. It can be concluded that this application may be beneficial in using *C. sinensis* factory wastes which are considered as undesirable material as an economical alternative feed source in ruminant nutrition. Malic acid has no negative effect on *in vitro* true digestibility values for *R. pseudoacacia, C. sinensis* factory waste, *P. laurocerasus* and *Q. cerris* in this study. On the other hand, *in vivo* studies with similar organic acids and alternative feed raw materials are needed.

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Conflict of Interest

The authors declared that there is no conflict of interest.

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Insulin-like growth factor-1 gene expression in the endometrium of repeat breeder cows after the administration of presynch-10 and ovsynch protocol

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Abstract: The aim of this study was to investigate the effects of using presynch-10 and ovsynch synchronization protocols on endometrial expression and serum levels of insulin-like growth factor-1 (*IGF-1*) that thought to play roles during endometrial healing process in cows diagnosed with repeat breeder syndrome (RBS) and subclinical endometritis (SE). A total of 40 Holstein-Friesian cows identified as having RBS and SE were used. The animals in Group 1 (G1, n=20) were applied presynch-10 and ovsynch furthermore, biopsy and blood samples were collected both before and after administration. Biopsy and blood samples were taken from the animals in Group 2 (G2, n=20) without performing any synchronization. The *IGF-1* gene expression profiles were determined in all samples using RT-qPCR. Even though the level of *IGF-1* expression increased in samples taken after synchronization in G1, it was not statistically significant (P>0.05). There was no statistical difference in serum *IGF-1* levels between G1 and G2 (P>0.05). In conclusion, the expression of the *IGF-1* gene in the endometrial tissue of RBS cows with SE was identified, but the evidence suggests that the synchronization protocols in question had no significant effect on the gene expression profile being investigated.
Keywords: Gene expression, *IGF-1*, ovsynch, presynch-10, repeat breeder

Presynch-10 ve ovsynch protokolleri uygulamasından sonra repeat breeder ineklerde endometriyal insülin benzeri büyüme faktörü-1 gen ekspresyonu

Özet: Bu çalışmada; repeat breeder sendromu (RBS) olan ve subklinik endometritis (SE) olduğu belirlenen ineklerde uygulanan presynch-10 ve ovsynch senkronizasyon protokollerinin, endometriyal iyileşme sürecinde rol oynadığı düşünülen insülin benzeri büyüme faktörü-1'in (*IGF-1*) endometriyal ekspresyonu ve serum düzeyleri üzerine etkilerinin araştırılması amaçlanmıştır. Çalışmada RBS olan ve SE tespit edilmiş toplam 40 baş Holstein-Friesian inek kullanılmıştır. Grup 1'de bulunan hayvanlara (G1, n=20), presynch-10 ve ovsynch uygulanmış, ayrıca uygulama öncesi ve sonrasında biyopsi ve kan örnekleri alınmıştır. Grup 2'de bulunan hayvanlara (G2, n=20) herhangi bir senkronizasyon uygulaması yapılmadan sadece biyopsi ve kan örnekleri alınmıştır. Tüm örneklerde RT-qPCR ile *IGF-1* geninin ekspresyon profilleri belirlenmiştir. G1'de senkronizasyon sonrası alınan örneklerde *IGF-1* ekspresyon düzeyinde bir yükselme şekillenmiş olsa da istatistiksel olarak bir önem bulunamamıştır (P>0,05). G1 ve G2 arasında serum *IGF-1* seviyesi açısından da istatistiksel bir fark oluşmamıştır (P>0,05). Sonuç olarak; SE tespit edilen RBS'li ineklerin endometriyal dokularında *IGF-1* geninin eksprese olduğu belirlenmiş ancak kullanılan senkronizasyon protokollerinin, incelenen genin ekspresyon profili üzerine anlamlı bir etkisinin olmadığı sonucuna varılmıştır.
Anahtar sözcükler: Gen ekspresyonu, *IGF-1*, ovsynch, presynch-10, repeat breeder.

Introduction

Repeat breeder syndrome (RBS) is a substantial problem that causes infertility in the dairy herds (36) and occurs at the frequency of 7-36% (19, 38, 39). RBS causes significant economic losses since especially it prolongs calving-conception interval and increases the culling rates of cows in herds (14, 34). Even though there are a number of factors that play a role in the etiology of RBS, the major causes can be listed as subclinical endometritis (SE), luteal deficiencies, delayed ovulation and malnutrition, which cause embryonic death or fertilization errors (1, 19, 23, 38). One of these problems is luteal insufficiency that

leads to low serum progesterone levels and embryonic deaths (20). Low progesterone levels may lead to lower interferon-tau secretion of the embryo, which causes early lysis of the corpus luteum (21). Similarly, endocrine asynchronization, which means that hormones are not released when they should be, causes delayed or absence preovulatory luteinizing hormone (LH) surge. Depending on this situation; progesterone level remains low and, embryonic deaths and RBS may occur (3, 4, 11). Another condition that causes RBS is SE. It is a superficial inflammation characterized by degradation and congestion in the epithelial cells of the cows' endometrium and infiltration of inflammatory cells without any purulent discharge and uninterrupted continuation of the sexual cycle (15). Cows with SE do not show any clinical signs of endometritis and they have very low chances to become pregnant after artificial insemination (AI). Studies have shown that the prevalence of SE in cows with RBS varies between 15% and 50% (12, 24, 28). A number of factors play a role in the development of these disorders that cause RBS. In this context, reproductive activities in cows, as in all other mammals, are regulated by the harmonious interaction of the hypothalamic-hypophyseal-ovarian axis. It has been revealed that during this interaction, growth hormone and insulin-like growth factor play crucial roles, like as hypothalamic-hypophyseal-ovarian axis derived hormones (18, 31). *IGF-1* is synthesized and secreted in the liver due to the effect of growth hormone (26). *IGF-1* does not only affects ovarian functions, oocyte quality and viability of the embryo, but also the endometrium (33). For instance; it was determined the members of the *IGF* system that have a stimulating effect on the regeneration of damaged epithelial cells in the process of tissue repair and in the production of new cells (5). Endometrial cells are also specific epithelial cells of the uterus and, *IGFs* are expressed in varying amounts in endometrium in various species such as rodents (8), pigs (29), cattle (7) and, sheep (32). Moreover, it has been demonstrated that *IGFs* have both progressive or regressive effects on uterine tissue depending on the stage of the sexual cycle. In this context, it was determined that post-partum cows with low *IGF-1* concentrations are more sensitivity to endometritis (35). There are many causative factors in the etiology of bovine RBS, therefore, treatment methods are also preferred according to the etiology. In the treatment for RBS that develops due to hormonal dysfunction or SE, it should be focused on the treatment of endometritis or regulation of ovarian function. From this perspective, one of the preferred treatments of RBS with endometritis is the application of the "ovsynch" protocol which controls ovarian functions (25). The ovsynch method is preferred the treatment of RBS with SE because it ensures regular follicular development and ovulation, stimulates the regeneration of endometrial epithelium. Furthermore, it stimulates the immune system due to the resulting estrus and, the prostaglandins used in this method are thought to stimulate the local immune response of the uterus. The aim of this study was to determine how endometrial *IGF-1* gene expression levels and serum *IGF-1* levels in RBS cows cytologically diagnosed with SE, are affected by presynch-10 + ovsynch applications.

Material and Methods

Animals: The animal material for the study has consisted of 40 Holstein-Friesian cows bred on dairy farms and identified as having RBS. All of the procedures conducted on the cows were approved by the Local Ethics Committee on Animal Experiments (Approval no: 2019-519). Repeat breeder cows were identified by examining farm records and conducting gynecological examinations. Cows that had given birth at least once and were inseminated in three consecutive estrus cycles yet failed to get pregnant were identified based on farm records. Rectal palpations and USG examinations were conducted on these cows to determine whether there were any pathological issues in the uterus or ovaries. Clinical pathological conditions were excluded from the study. The cows included in the study had corpus luteum on the ovaries and body condition scores between 3.0-3.5 under the scoring system with 1 to 5 scales. An endometrial cytology sample was taken from all of the cows included in the study to determine the endometrial polymorph-nuclear lymphocyte (PMNL) ratio, an endometrial biopsy to determine gene expressions, and a blood sample from the tail vein to measure serum *IGF-1* levels. The days in milk (DIM) information and the calving-first service interval of cows were determined based on farm records. In the cytological evaluation, cows with PMNL ratios ≥ 3% were considered to be SE (28) and included in the study. The cows were randomly divided into two groups (G1 and G2).

Experiment 1: The presynch-10 + ovsynch protocol was administered to the cows in Group 1 (G1, n=20) and the same samples were repeated 10 days after the last injection (Figure 1).

Experiment 2: No synchronization protocol was administered to the cows in Group 2 (G2, n=20). After waited for the length of time required by the synchronization protocols administered to G1 (34 days), estruses were monitored. Ten days after estruses were determined, rectal examinations were performed and all samples were collected again from cows with corpus luteum (Figure 2).

Collection of endometrial cytology samples: A cytobrush (Cytobrush®, Ref.17214/2960, Minitube, Germany) was used to determine the neutrophil concentration. In order to prevent the cytobrush from being contaminated during this procedure, it was inserted

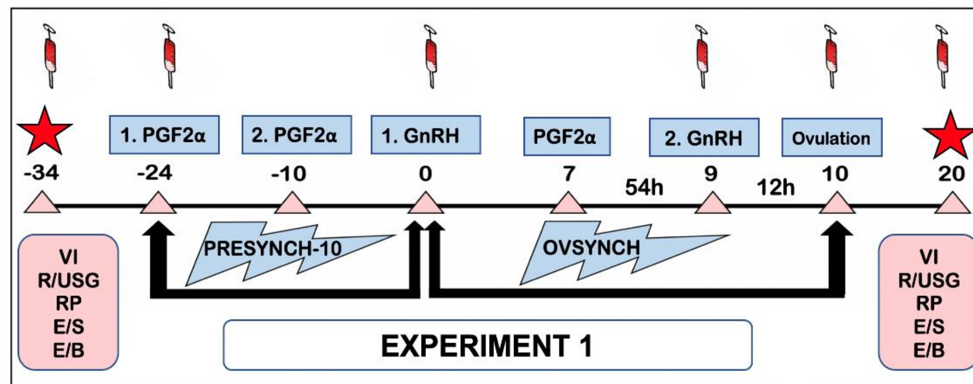

Figure 1. Presynch-10 + ovsynch protocol
PGF2α: Cloprostenol, 500 µg, im, Estrumate®, Intervet; GnRH: Buserelin acetate, 10 µg, im, Receptal®, Intervet; VI: Vaginal Inspection; R/USG: Rectal ultrasonography; RP: Rectal palpation; E/S: Endometrial swab; E/B: Endometrial Biopsy; Syringes: collecting blood samples; Stars: collection tissue samples.

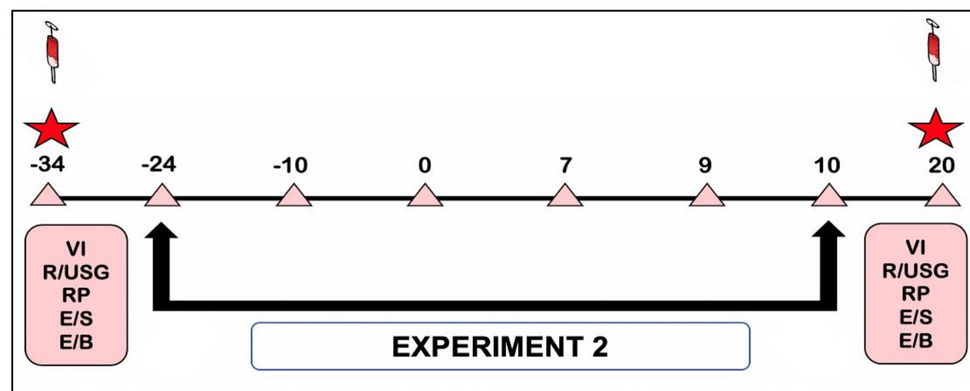

Figure 2. Control group
VI: Vaginal Inspection; R/USG: Rectal ultrasonograpy; RP: Rectal palpation; E/S: Endometrial swab; E/B: Endometrial Biopsy; Syringes: collecting blood samples; Stars: collection tissue samples.

into the uterus lumen inside a stainless-steel metal catheter approximately 50 cm long. After the sample was taken, it was smeared on a slide with the brush. The collected samples were stained using the May Grunwald Giemsa staining method and 200 cells were counted in each preparation microscopically.

RNA isolation and cDNA synthesis: The endometrial biopsy samples were collected from the dorsal wall and intercaruncular site of the uterus with biopsy forceps (Kruuse Biopsy instrument, Denmark). The collected tissues were placed in 1.5 ml microcentrifuge tubes and frozen in liquid nitrogen. The samples were taken to the laboratory in a nitrogen tank and stored in a deep freeze at -86°C until the analyses were performed. Homogenization of frozen endometrial tissue samples was performed using the TRIzol® (Invitrogen, USA) method. DNA digested with DNase-I (Fermentas). And, the commercial kit (Thermo Scientific RevertAid™ First Strand cDNA Synthesis Kit) was used to cDNA synthesis.

RT-qPCR: YHWAZ was used as the reference gene as it is most stable and suitable for the study (2). And, a pair of specific primers [Forward: 5'-CTGAGCAAGGAGCTGAATTATC-3' and Reverse: 5'-CTCTGTATTCTCGAGCCATCT-3 for YHWAZ (NM_174814.2)] was designed using the idt/PrimerQuest and ncbi/tools/primer-blast software to amplify fragment of the YHWAZ gene consisting of 162 bp. (2). For the IGF-1 gene, the primers designed by Yoshida et al. (37) [Forward: 5'-TCAGTTCGTGTGCGGAGACA-3' and Reverse: 5'-ACTTCCTTCTGAGCCTTGGG-3' for IGF-1(NM001077828)] were used to amplify a region consisting of 222 bp. Primer efficiencies were determined using two-fold serial dilutions of cDNA. The expression profiles for YHWAZ and *IGF-1* genes were identified with RT-qPCR, using a ROCHE Nano Lightcycler®. The reaction was prepared using SYBR Green Master Mix (2X). This procedure was performed twice. Melting curve analyzes were performed.

Hormone measurements: Hormone analyses were performed in a private laboratory with international accreditation (TÜRKAK, TS EN ISO/IEC 17025:2005 Experimental Laboratory) using ECLIA® (Electrochemiluminescence Immunoassay) for estradiol and progesterone, and *IGF-1* specific ELISA kit (CusaBio®, Bovine insulin-like growth factor 1, ELISA Kit).

Statistical analysis: MINITAB 16.0© was used for the statistical analysis of the data. It was later normalized with the data reference gene obtained from the qPCR analyses based on the delta delta Ct mathematical model reported by Livak and Schmittengen (16). The paired t test was used for data collected from the same group while the T test and One-way ANOVA were used to identify differences between the groups. Box plot and Interval Plot graphics were created for a visual representation of the data.

Results

The calving-first service interval and the DIM numbers were 85.95±19.79 and 205.6±55.60 for G1 and 90.05±21.77 and 236.25±54.51 for G2 respectively. The mean DIM numbers for the cows in G1 and G2 were 205.60±55.60 (min. 150, max. 349), so cows with PMNL >%3 during the cytological evaluation were considered to have SE (31).

Expression of IGF1 gene: The *IGF-1* gene was expressed in all samples. The resulting RT-qPCR products using YHWAZ as the reference gene and the *IGF-1* gene in bovine endometrium were separated with electrophoresis on 2% agarose gel and stained with ethidium bromide (Figure 3).

When the *IGF-1* expression profile was examined depending on days of applied synchronization protocols, there was more expression in the endometrial biopsy samples taken after synchronization. However, it was not statistically significant (P>0.05). Expression levels between the biopsy samples taken in G2 remained almost the same. The Box Plot graphics that compare the data obtained after normalization of the *IGF-1* gene RT-qPCR results with each sampling group are provided in Figure 4.

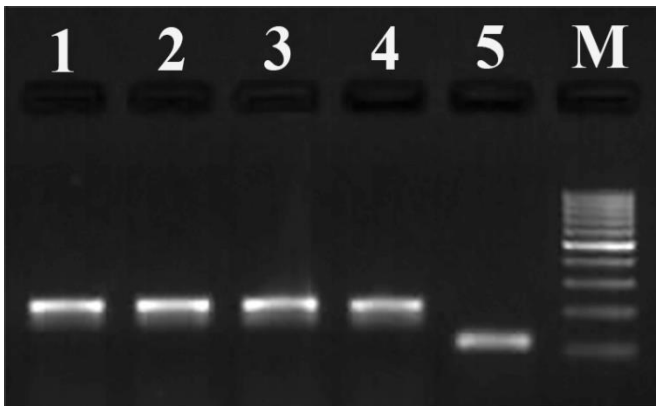

Figure 3. Expression of YHWAZ and IGF-1 genes at mRNA levels in bovine endometrium on 2% agarose gel.
Lanes 1-4: PCR products of IGF-1 (222 bp), lane 5: YHWAZ (162 bp), M: 100 bp DNA ladder

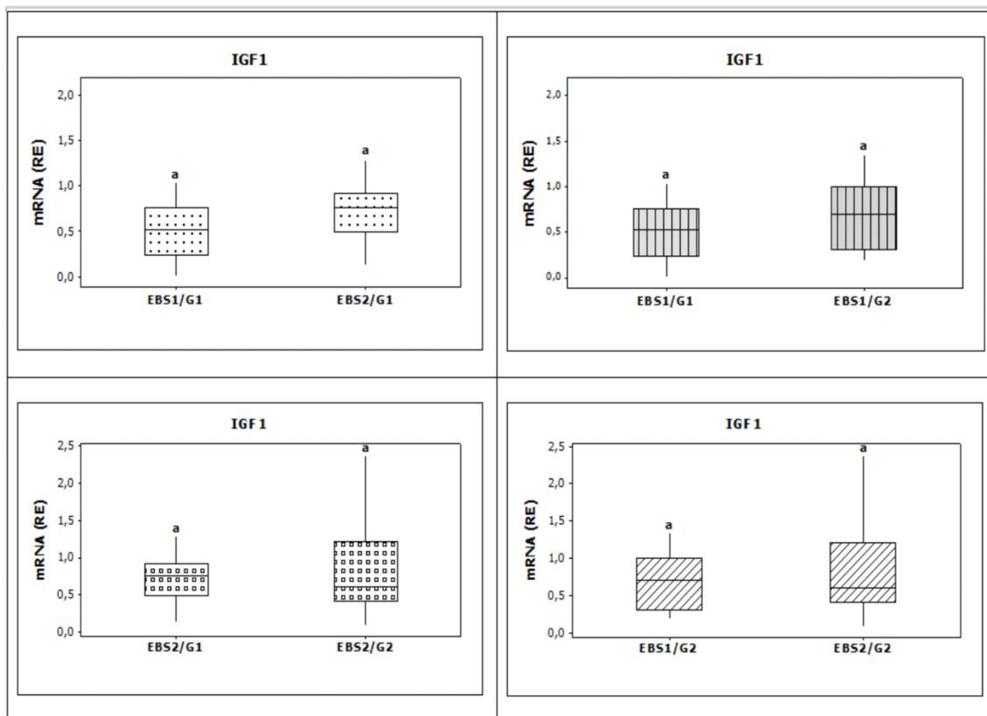

Figure 4. Box plot graphics showing the differences between sampling groups.
EBS: endometrial biopsy sample.

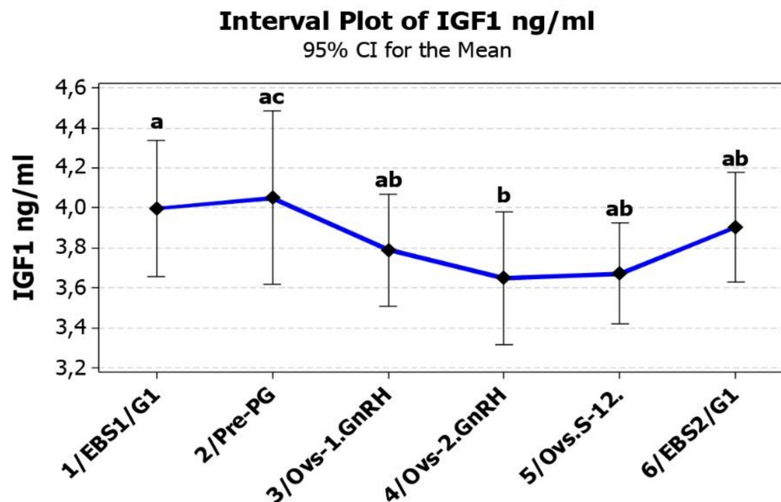

Figure 5. Serum IGF-1 levels according to administration days in G1.
a:b, P<0.05; c:b, P<0.05, 1/EBS1/G1: Group 1 Endometrial biopsy sample 1; 2/Pre-PG: presynch-10 1st Prostaglandin injection; 3/Ovs-1st GnRH: ovsynch 1st GnRH injection; 4/Ovs-2nd GnRH: ovsynch 2nd GnRH injection; 5/Ovs.S-12: After ovsynch 12 hours; 6/EBS2/G1: Group 1 Endometrial biopsy sample 2.

When serum *IGF-1* levels were examined, it was showed that the *IGF-1* level of 3.99 ng/ml on the day the first sample was taken in G1 fell to 3.64 ng/ml on the day that the second GnRH administration of ovsynch was performed and that this decline was statistically significant ($P<0.05$). No statistically significant difference was found between the *IGF-1* levels (4.04±0.92 pg/ml) measured on the day when the 1st PGF2α injection of presynch-10 was performed and on the day when 2nd GnRH injection of ovsynch (3.64±0.54 pg/ml) ($P<0.05$). There was also no statistically significant difference in terms of serum *IGF-1* levels between the day on which the first sample was taken and the days on which the second sample was taken ($P>0.05$) (Figure 5).

No statistically significant difference was found between *IGF-1* levels in the blood samples collected on the days when the first and second samples were taken in G2 (1st *IGF-1* value: 3.99±0.73, 2nd *IGF-1* value: 4.09±0.75).

Discussion and Conclusion

The healing of damaged epithelial tissue consists of proliferation and remodeling processes (10). *IGF-1* is an important factor that functions in the tissue repair process by stimulating epithelization and regeneration (5). Physiological regeneration of endometrial tissue takes place during the post-partum period in cows. Increased endometrial *IGF-1* gene expression has been determined during the post-partum period in cows, and it has been demonstrated that the *IGF-1* may play an important role in the endometrial repair process (17). Because a certain amount of endometrial injury occurs in cases of bovine SE, there might be a connection between *IGF-1* and the injury and subsequent healing that occurs. In a study of the issue, endometrial *IGF-1* expression was reported to be higher in cows diagnosed with metritis or clinical endometritis than in healthy cows or cows with SE. The same study found that when RBS cows with SE were compared with cows that did not have SE, endometrial *IGF-1* expression was higher in the cows with SE. It was concluded that both of these findings could be related to the tissue damage in the endometrium and the healing process (13). Ovarian steroids are reported to have an effect on endometrial *IGF-1* expression. In cows, it has been shown that endometrial *IGF-1* expression peaks during the estrus stage when the estradiol level is highest in the estrus cycle (27). In this study, presynch-10 and ovsynch administrations given for treatment purposes to RBS cows induced ovulation, meanwhile, increased serum estradiol levels due to the follicular development that occurs. When the *IGF-1* expression profile based on days of administration examined that showed an increasing in *IGF-1* expression but the difference between the biopsy samples taken before and after synchronization in G1 was not statistically significant. It is thought that the increase in the expression of *IGF-1* may be associated with the fact that the endometrium is exposed to estradiol as a result of the synchronization protocols applied.

In a study that examined bovine serum *IGF-1* levels during ovsynch administration that the serum *IGF-1* levels decrease after the 1st GnRH injection of ovsynch but later increase until day 9 after the PGF2α in cows and this occurred due to follicular development (6). It has also been demonstrated that *IGF-1* is synthesized not only in the follicles of in bovine ovaries but in luteal tissue as well. However, after lysis of the luteal structure due to PGF2α

stimulation, there is no significant decrease in *IGF-1* expression has been demonstrated (22). Moreover, it has been shown that ovulation occurred before the peak of plasma estradiol level at the time of second GnRH injection during ovsynch administration in cows (30). This finding indicates that the *IGF-1* concentration in circulation is controlled by several different mechanisms.

It is thought that the reason why there is no difference in serum *IGF-1* levels between on the day of the first PGF2α of presynch-10 and on the day of the second GnRH of ovsynch may be due to this complex biological mechanism. We think that this situation may be the reason that a correlation could not be established between serum *IGF-1* levels and endometrial *IGF-1* expression levels.

In conclusion, in addition to this study, which examined the *IGF-1* expression profile in conjunction with administration of the presynch-10 + ovsynch protocol for the purpose of treating SE, new studies that investigate all of the elements of the *IGF* system and not only endometrial expression levels but also expression in follicles, corpus luteum and liver tissue could help develop new approaches when it comes to solving the repeat breeder problem, which is important to livestock breeders.

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Conflict of Interest

The authors declared that there is no conflict of interest.

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Short Communication / *Kısa Bilimsel Çalışma*

PCR detection of *Mycobacterium genavense* DNA in fecal samples of caged birds

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Abstract: In this study, pathogenic mycobacteria were investigated in fecal samples of caged birds by PCR. A total of 47 feces samples collected from 4 different aviaries in Ankara. DNA extraction from fecal samples was performed with a commercial kit using spin column technology. PCR was performed with designed primers respectively amplifying 274 base pairs (bp), 128 bp, 102 bp and 219 bp nucleotide sequences of specific genes (16SrRNA, ISI245, IS901 and hypothetical 21kDa protein gene) of *Mycobacterium* spp., *Mycobacterium avium* complex (MAC), *Mycobacterium avium* subsp. *Avium* and *Mycobacterium genavense*, respectively. Five samples were positive and harbored the sequence for the *Mycobacterium* spp., of 4 of these 5 samples was identified as *M. genavense* by PCR. As a conclusion of this study, which is the first announcement of the detection of *M. genavense* DNA in fecal samples of caged birds in Turkey, PCR was seen to be a rapid, sensitive, and a reliable method in detection of avian mycobacteriosis.
Keywords: Bird, feces, mycobacteriosis, *Mycobacterium genavense*

Kafes kuşu dışkı örneklerinde *Mycobacterium genavense* DNA’sının PZR ile saptanması

Özet: Bu çalışmada, kafes kuşlarının dışkı örneklerinde PCR ile patojenik mikobakterilerin varlığı incelendi. Çalışmanın materyalini Ankara'da 4 farklı kuşhaneden toplanan 47 dışkı örneği oluşturdu. Dışkı örneklerinden DNA ekstraksiyonu, spin kolon teknolojisi kullanılarak ticari DNA ekstraksiyon kiti ile yapıldı. PCR, *Mycobacterium* spp., *Mycobacterium avium* complex (MAC), *Mycobacterium avium* subsp. *avium* ve *Mycobacterium genavense*’nin spesifik genlerinin (16SrRNA, ISI245, IS901 ve hipotetik 21kDa protein geni) sırasıyla 274 baz çiftini (bp), 128 bp, 102 bp ve 219 bp nükleotit sekanslarını çoğaltan tasarlanmış primerlerle gerçekleştirildi. PZR sonucunda, 5 dışkı örneği *Mycobacterium* spp. için pozitif bulundu. Örneklerin 4'ü PZR’da *M. genavense* olarak tanımlandı. Türkiye'deki kafes kuşlarının dışkı örneklerinde *M. genavense* DNA'sının saptanmasına yönelik ilk duyuru olan bu çalışmanın sonucu olarak, PZR'nin kanatlı mikobakteriyozisi tespitinde hızlı, duyarlı ve güvenilir bir yöntem olduğu görülmüştür.
Anahtar sözcükler: Dışkı, kuş, mikobakteriyozis, *Mycobacterium genavense*

Avian tuberculosis is a chronic wasting disease in cages, exotic, zoo and wild birds in the world. In recent years, for exotic birds, *Mycobacterium genavense* has been reported as agent of tuberculosis (2,12,16,17,18,20,22). *M. genavense*, in bird species, especially avian Passeriformes and Psittaciformes, described as the most frequent etiologic agent of avian tuberculosis (4,14). *M. genavense* was first described on AIDS patients in 1990 (17). In people with weakened immune systems, children and other animal species including the dog could cause the infection (1,3,7,8,11,14,20). The disease occurs sporadically and can also be seen in intensive breeding companies (6).

Mycobacteria can exist for months in the environment. The main sources of infection are infected animals, contaminated soil and water (12,20). *M. genavense* is transmitted through the digestive system by fecal-oral route (10). Feces of infected birds are one of the important source for infection. In intensive breeding, stool contains a large number of bacteria for bird to bird contact (9,16,20). People may inhale the bacteria as a result of direct contact with infected birds (9,10).

Mycobacteriosis generally occurs in adult birds more than young birds. Avian tuberculosis causes direct and indirect economic losses (19).

M. genavense shows similar clinical and histopathological lesions of *Mycobacterium avium* complex (14,15,17). The deaths occur after several months because it is a chronic infection. Infected birds usually are greater than one year of age (12). Also, they are weakened with a weight loss (5,12,15,20). Although some infected birds, which may exhibit a normal appearance and behavior, could be found dead. The most common route of transmission is by fecal-oral route. Because of that, lesions can be seen in the digestive tract (20).

M. genavense is a fastidious and slow growing bacterium (5). For this reason, it is very difficult to culture this bacteria (11,17,20). Because of this, the diagnosis based on Ziehl-Neelsen staining of asidoresistance bacteria from infected samples (2,17). Since *M. genavense* is a fastidious bacterium, PCR is a rapid and accurate method for detecting the small amount of DNA from clinical samples (2,9,15,19,20). Results are obtained within hours (19). Fresh tissues and feces can be used for the diagnosis of infection in PCR method (20). Because of the risk of zoonotic infection between individuals with a weakened immune system and their animals, rapid diagnosis is very important to prevent serious infection (11).

In this study, presence of *M. genavense* DNA was investigated by PCR from cage bird feces samples.

This study was carried out in Ankara University Faculty of Veterinary Medicine Department of Microbiology. A total of 47 feces samples were collected from four different aviaries in Ankara region, Turkey. There were canaries, parrots, budgerigars, pigeon, phesant and dove birds in aviaries. The number of birds in cages ranged from 1-23. From the first aviary 10 feces samples were taken. 12, 13 and 12 feces samples were taken from the second, third and fourth aviaries, respectively. Feces samples were collected from cages floor and put into the sterile containers. The amount of each feces samples were approxiametly 25 grams. Symptoms were evaluated following clinical examinations and anamnesis was obtained from the owners. Respiratory system symptoms of the birds were changing from slight to heavy. They showed conjuctivitis and diarrhoea, some birds without any symptoms.

PCR was used for the investigation DNA of *Mycobacterium* spp., *Mycobacterium avium* complex (MAC), *M. avium* subsp. *avium* and *M. genavense* from birds feces. DNA extractions were performed with the QIAamp DNA Stool Mini Kit (Qiagen), which is works by spin column technology, from birds feces.

The primers used in this study, were designed by Barış Sareyyüpoğlu with Primer3 (21). PCR was performed with designed primers respectively amplifying 274 base pairs (bp), 128 bp, 102 bp and 219 bp nucleotide sequences of specific genes (16SrRNA, ISI245, IS901 and hypothetical 21kDa protein genes) of *Mycobacterium* spp., MAC, *M. avium* subsp. *avium* and *Mycobacterium genavense*, respectively. (Table 1). The PCR were performed for all target genes, in a total reaction volume of 25 µl, containing 2.5 µl 10x PCR buffer, 3 µl 25 mM magnesium chloride, 250 µM of each deoxynucleotide triphosphate, 1.25 U Taq DNA Polymerase, 20 pmol of each primer and 25 ng of template DNA. The reaction conditions for the *M. genavense* specific PCR are 35 cycles of denaturation at 94°C for one minute, annealing at 54°C for one minute and extension at 72°C for one minute, followed by a final extension step at 72°C for 7 minutes. The amplified products were detected by staining with 10 mg/ml ethidium bromide after electrophoresis at 80 V for two hours in 2% agarose gels. Results were screened from agarose gel by molecular imaging system (Gene Genius, Syngene, England).

M. genavense DNA used as positive control in PCR tests, was supplied by Enrico Tortoli from Regional Reference Centre for Mycobacteria, Florence, Italy. Also *M. avium* subsp. *avium* strain (German Collection of Micrroorganisms and Cell Cultures-DSMZ; DSM NO:44156) used as a positive control in PCR tests.

Table 1. Primers, target genes, sequences and product sizes

Primers	Name of primer	Target gene	Product size (bp)	Primer sequence
***Mycobacterium* spp.**	Myco1F	16SrRNA	274	TGGGTACTAGGTGTGGGTTTCC
	Myco1R			TTAACCCAACATCTCACGACAC
MAC	MAC1F	ISI245	128	TGGCCGGCTCGGTACTCGTT
	MAC1R			GGCTGTGGGGGCAATGGTTT
M. avium* subsp. *avium	Masa1F	IS901	102	CTCGATGCTCACCGCCATCTT
	Masa1R			ATTTCGCCCGGAGTGCACATAG
M. genavense	Mgen1F	hypothetical 21kDa protein genes	219	TGACTGGTCGTTTGAGATGAAT
	Mgen1R			GATCGGAGGCAGTTCAATGTAG

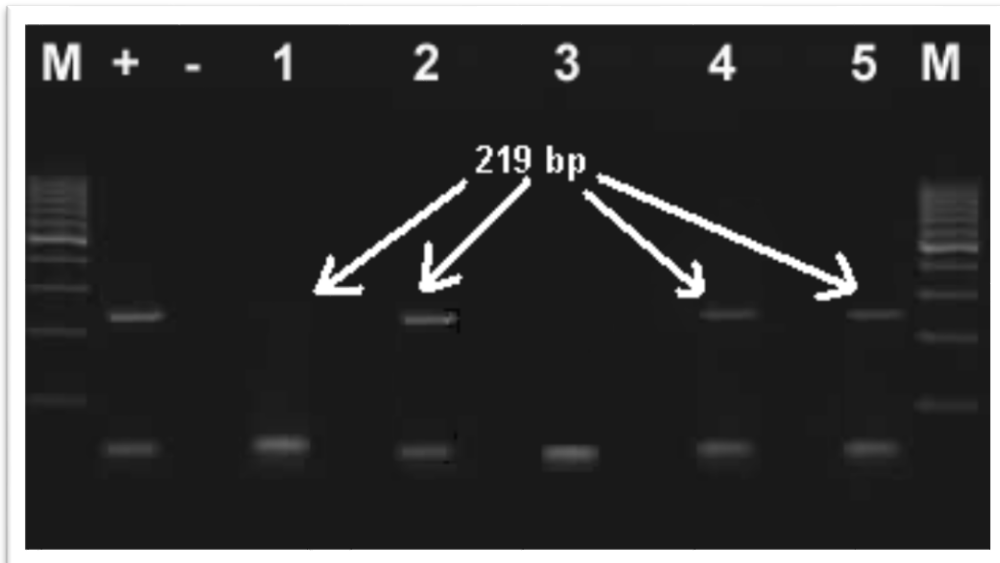

Figure 1. PCR results for *Mycobacterium genavense*.
M: marker (100bp) +: positive control -: negative control 1-5: feces samples.

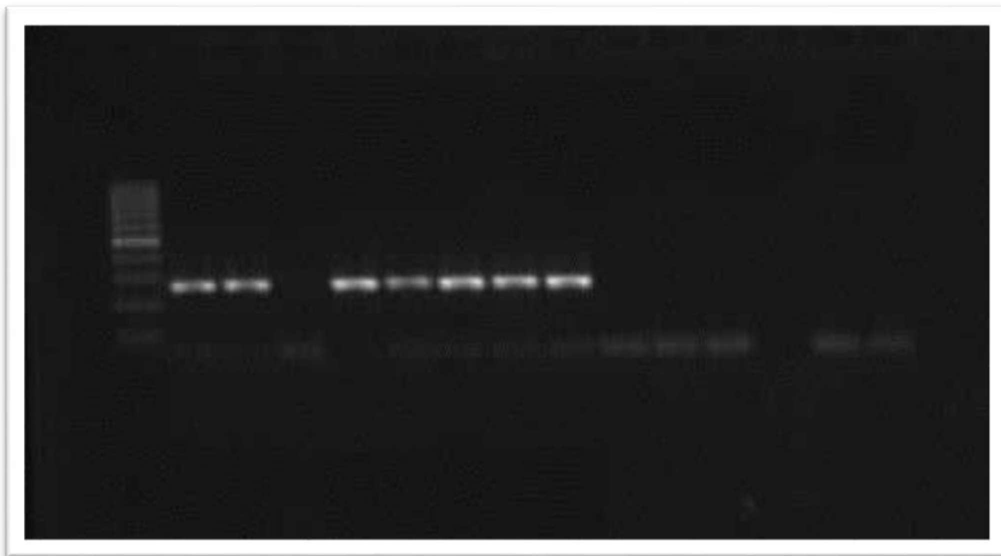

Figure 2. PCR results for *Mycobacterium* spp. (274bp)
M: marker (100bp) Line 1-2: positive controls, Line 3: negative control, Line 4-8: positive feces samples for *Mycobacterium* spp.

As a result of the molecular analysis of all samples, a total of 5 samples were found positive in terms of the sequence for the *Mycobacterium* spp. (Figure 2). Four of these 5 samples, positive for the *Mycobacterium* genus, were identified as *M. genavense* DNA by PCR (Figure 1).

In Turkey, *M. genavense* DNA was found for the first time in cage bird's feces with PCR. In birds, the primary source of infection is contaminated environment for mycobacterium infections. Feces of infected birds play an important role for the spread of infection among birds (20). The results of this study showed that feces are not only important for spreading of the infection but also detection of the mycobacteriosis. The important role of fecal contamination in avian tuberculosis has been seen once more with this study.

Importantly, all *M. genavense* positive specimens were detected in cages of the same aviary. This result can indicate that the contamination by horizontal route from bird to bird.

In several studies, PCR has been used to diagnose mycobacterial infections from poultry organ samples. *M. genavense* is difficult to cultivate unlike other Mycobacteria species. PCR is a rapid and reliable method for the diagnosis of *M. genavense* infections (19,20). Tell et al. (19) reported that they found *M. genavense* in 67% of poultry organ samples. The *M. genavense* primers,

which were used in this study, were designed from the hypothetical 21kDa protein gene. Ledwon et al. (14) designed the primers from the same gene for the diagnosis of *M. genavense* from the organ samples of budgerigars and identified positive samples. Patino et al. (13) reported 3 (8 %) *M. genavense* DNA in free living birds' feces using *16s rRNA* gene specific primers of *Mycobacterium genavense*. Addition, Schimitz et al. (16) were used probe targeting the hypothetical 21kDa protein gene in their real-time PCR protocol. According to of all these studies, it was seen that hypothetical 21kDa protein gene could be used for the diagnosis of *M. genavense* in PCR.

Mycobacterium genavense-specific primers, tested for the first time in this study in Turkey, can be used in PCR tests performed with clinical materials other than feces (liver, spleen, bone marrow, tracheal swab, air sacs, lungs, intestines) especially in patients with suspected tuberculosis in cage birds. The results of this study showed that fecal screening can be performed especially for cage birds in terms of *M. genavense* for zoonotic risks.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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Case Report / *Olgu Sunumu*

The first case of *Bovicola (Werneckiella) ocellatus* (Piaget, 1880) (Phthiraptera: Ischnocera: Trichodectidae) on a donkey (*Equus asinus* Linnaeus, 1758) in Turkey

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Abstract: In the clinical examination of a donkey, which brought to the Ownerless Animal Care and Rehabilitation Center of the Hatay Metropolitan Municipality due to neglecting and senility, local hair losses, ruffling and opacity were observed. A few louse specimens were detected on the hair shafts on the back of the donkey during the macroscopic examination. They were collected with a forceps, and preserved in a tube with alcohol 70%. Then they examined in detail in microscope, and identified as *Bovicola ocellatus* (Piaget, 1880). The presence of *B. ocellatus* on donkey has been reported for the first time in Turkey in this paper.
Keywords: *Bovicola ocellatus,* donkey, louse, Turkey

Türkiye'de bir eşekte (*Equus asinus* Linnaeus, 1758) ilk *Bovicola (Werneckiella) ocellatus* (Piaget, 1880) (Phthiraptera: Ischnocera: Trichodectidae) olgusu

Özet: Hatay Büyükşehir Belediyesi Sahipsiz Hayvan Bakım ve Rehabilitasyon Merkezi'ne yaşlılık ve bakımsızlık nedeniyle getirilen bir eşeğin klinik muayenesinde tüylerinin çok karışık ve matlaşmış olduğu, yer yer döküldüğü gözlenmiştir. Yapılan makroskobik incelemede eşeğin sırt bölgesindeki tüylerin dip kısımlarında bir kaç bit saptanmıştır. Bitler bir pens yardımıyla alınmış ve içinde %70'lik alkol bulunan bir tüpte saklanmıştır. Daha sonra mikroskopta ayrıntılı olarak incelenen örnekler *Bovicola ocellatus* olarak teşhis edilmiştir. Bu makaleyle *B. ocellatus*'un Türkiye'deki varlığı ilk kez bildirilmektedir.
Anahtar sözcükler: Bit, *Bovicola ocellatus*, eşek, Türkiye

The lice are hemimetabolous and obligatory insects. They complete all their life on the hosts. The lice belonging to the Ischnocera and Amblycera suborders are parasitized on mammals and poultry, and they feed on the feathers or hairs of their hosts. Anopluran species has only seen in mammals, they suck blood of their hosts and cause anemia in heavy infestations. The chewing lice belonging to the genus *Bovicola,* in family Trichodectidae, suborder Ischnocera are found on cattle, sheep, horses, donkeys and deers. These species are similar to each other morphologically and they are specific to their host (1, 2, 11). The lice complete their development in onemonth average and their lives vary between 1-2 months (1, 2). Although they can be observed on the hosts during the year, however, they are seen more common in winter months. Lice infestations cause clinical symptoms such as clutter hairs, dandruff, hair losses, pruritus and anemia. Lice are usually found on the head, neck and sides of the body. In severe infestations, they may spread on the whole body on Equidae (11).

Bovicola (Werneckiella) ocellatus (Piaget, 1880) belong to the family Trichodectidae, in the suborder Ischnocera, order Phthiraptera, class Insecta (4, 12). According to Werneck (13), Piaget described this species as *Trichodectes parumpilosus var. ocellata* in 1880. Later, this species was described as *Trichodectes parumpilosus ocellata* by Kellogg in 1908; as *Trichodectes ocellatus* by Harrison in 1916; as *Bovicola ocellata* by Bedford in 1932; as *Werneckiella ocellata* by Eichler in 1939; *as Damalinia (Werneckiella) ocellata* by Hopkins in 1949.

According to Moreby (9), this species was described as *Werneckiella equi asini* by Eichler in 1953.

There is no data that *B. ocellatus* were seen in donkeys, in Turkey. Morphological information will be given about the female of *B. ocellatus* detected on a donkey in Hatay province of Turkey in this paper.

The material of this article consisted of *B. ocellatus* samples collected from a donkey, which brought to the Ownerless Animal Care and Rehabilitation Center of the Hatay Metropolitan Municipality due to neglecting and senility. Hair losses, ruffling and opacity were observed on the donkey. A few louse specimens were detected on the hair shafts on the back of the donkey during the clinical examination. The louse samples were collected with a forceps and they preserved in a tube with alcohol 70%. After that they sent to Department of Parasitology, Faculty of Veterinary Medicine, Selçuk University. The samples (2 ♀ 3 nymphs) were cleared in 10% KOH for 24 hours, rinsed in distilled water and passed through series of ethyl alcohol, mounted on the slides in Canada Balsam. After drying, they were examined and identified as *B. ocellatus.* No male of *B. ocellatus* was detected.

The head of female *Bovicola ocellatus* slightly rounded in front and it is flat or slightly concave in median. Marginal carina separated from each other by a narrow notch in the anteromedian. The mandibula well developed and notched in the apical. The antenna with three segments; the segment III is slightly longer than the others. The pre-antennal region is slightly shorter and narrower than the post-antennal region. The thorax is narrower and shorter than the head (Figure 1c). It is trapezoidal-shaped, narrowed to posterior. All the legs are weak and with a single claw. The first pair of legs shorter than the others pair and bent in the front. The abdomen is quite larger and longer than the head and thorax. The tergal and sternal plates are well sclerotized in the median, and with a short setae row posteriorly. Tergal plate of the second abdominal segment is thinner than the other tergal plates. The other tergal plates thickened from anterior to posterior. The paratergal plates well developed and became smaller towards to the posterior. The head length of female *Bovicola ocellatus* (Figure 1b) is 0.5 mm, head width 0.52 mm, thorax length 0.28 mm, thorax width 0.35 mm, abdominal length 1.32 mm, abdominal width 0.87 mm and total length was 2.07 mm.

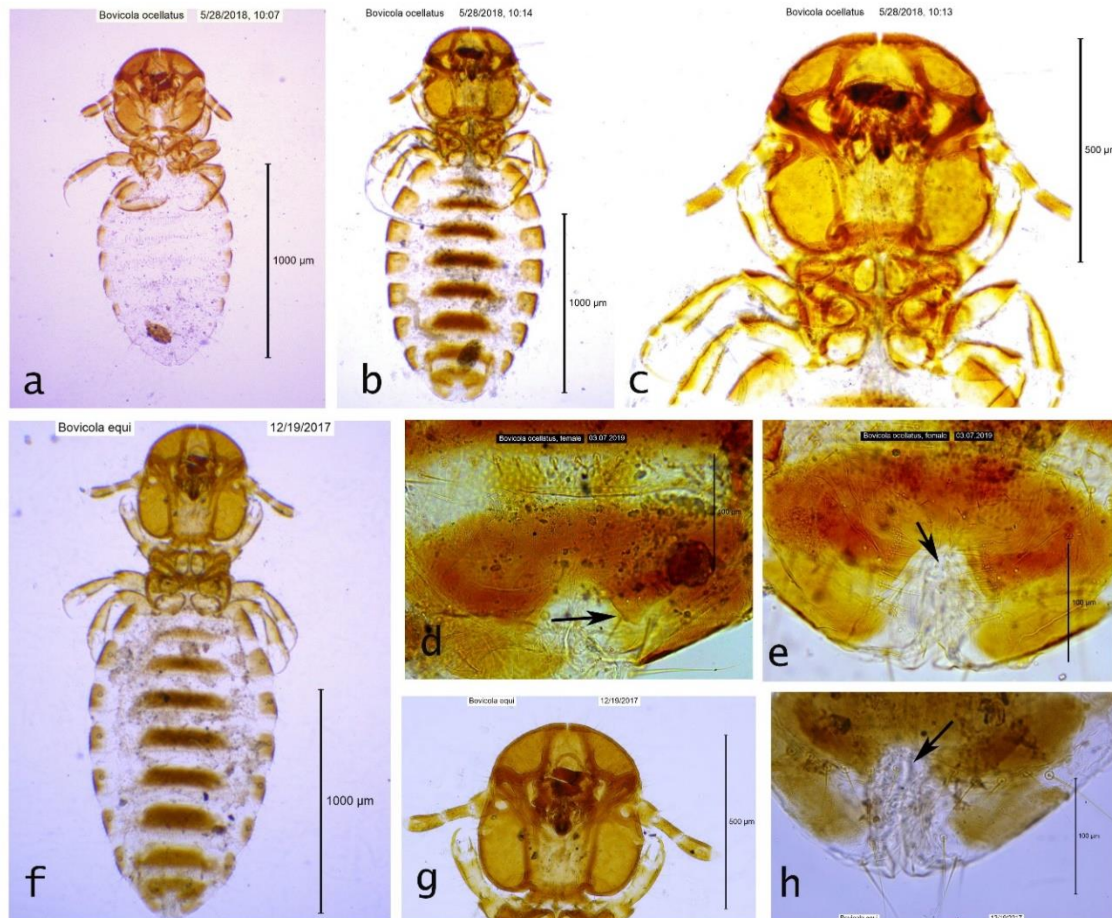

Figure 1. *Bovicola ocellatus* and *Bovicola equi*; **a.** *Bovicola ocellatus*, nymph III. **b**. *Bovicola ocellatus*, female. **c.** *Bovicola ocellatus* head, thorax, original. **d.** *Bovicola ocellatus*, female, lower part of tergite IX is pointed (arrow). **e.** genitalia apertura (arrow). **f.** *Bovicola equi*, female. **g.** head of *B. equi* **h.** post-vulval sclerite of *B.equi*

There were three nymphs; two nymphs III (Figure 1a) and one nymph II. The nymph II is 1.14 mm; the nymph III is 1.5-1.6 mm. The nymphs are very similar to the adult, but tergal, sternal and paratergal plates of nymph II are not developed. The head of nymph III is concave in anteromedian, paratergal plates are weak developed whereas sternal and tergal plates not developed yet.

Moreby (9), stated that five specimens of *B. ocellatus* from zebra (*E. burchellii burchellii*), three specimens from horses (*E. caballus*) and 41 specimens from donkeys (*E. asinus*) have been collected until that day. Only females of *B. ocellatus* detected, but male could not find. Firstly, Werneck (13) described male of *B. ocellatus* and gave its morphological characteristics. Later, he reported that he misidentified this species and corrected this species as *B. aspilopygus* (*B. aspilopyga*) (14). Although three males of *B. ocellatus* among the hundreds louse specimens taken from donkeys were reported, but there was no photo or explanation about the male of this species in that paper (3). It is known that *B. equi* (Denny, 1842), *B. bovis* (Linnaeus, 1758) and some other lice species reproduce parthenogenetically and produce male only during the rapid growth of the population (15). Probably for this reason, male of *B. ocellatus* could not found in this case.

Bovicola ocellatus can be separable easily from *B. equi* by its distinctive features. In female of *Bovicola ocellatus*; the head is widen, and flattened in anterior, the antennal cavity highly sclerotized, pleural plates are large, distinctive and well sclerotized, the post-vulval sclerites widen. In *B. equi*; the head is narrowed and rounded in anterior (Figure 1g), pleural plates are narrowed, moderately distinctive and weakly sclerotized (Figure 1f), post-vulval sclerites are different in shape (Figure 1h). Although tergite IX. is similar in both species, however in *B.equi,* it is oval whereas in *B. ocellatus* it is pointed posteriorly (Figure 1d). In *B. equi*, the genital opening relatively resembles to hourglass while bell-shaped in *B. ocellatus* (Figure 1e). *B. ocellatus* can be distinguished from *B. aspilopygus* and *B. zuluensis* by having vulval-post scleritis, and from *B. neglectus* due to the narrower anterior marginal carina of the head, from *B. zebrae* due to with the absence of transparent circular areas in the tergite IX. and the wider paratergal plates (9).

Moreby (9) reported that; the mean of the head length of the females of *B. ocellatus* samples collected from zebras was 0.51 mm, the abdominal width was 0.88 mm, while the mean head length of the *B. ocellatus* samples taken from the donkey was 0.46 mm, the width of the abdomen was 0.79 mm, and the average total length of samples collected from both host species was measured as 2.12 mm. In this study, the mean head length of two females of *B. ocellatus* collected from the donkey was 0.5 mm, abdomen width 0.87 mm, total length 2.07 mm. These measurements are consistent with those reported by Moreby.

According to Seguy (10), Harrison considered that *B. ocellatus* was a different species from *B. equi* whereas Keler stated that this species was the synonym of *B. equi.* In Turkey, we did not find any paper about the presence, prevalence and the other features of *B. ocellatus* as well as *B.equi.* Although, some authors (7,8) reported that the *Bovicola* specimens found in horses and donkeys were considered as *B. equi*. Unfortunately, they (7,8) did not publish any paper or figures of these specimens. In the light of the above information, some other authors (1,6) have also stated that the species seen on horses and donkeys was *B. equi*. Therefore, it is unclear that the *Bovicola* samples collected from donkeys are *B. equi* or *B. ocellatus*. These findings and considerations can be mistaken, at least partly, and it is not possible to verify all this information today. On the other hand, only one article was found about *B. equi* was seen in horses in Turkey (5). However, there are no morphological characteristics and figures of *B. equi* in that paper.

In conclusion, this is the first report of *B. ocellatus* was seen on a donkey in Turkey. In addition, some morphological characteristics and figures of *B. ocellatus* and *B. equi* were explained here. Further studies should be needed to detect the male of *B. ocellatus*, as well as prevalence and biological characters of this species.

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Conflict of Interest

The authors declared that there is no conflict of interest.

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Review / *Derleme*

The role of micronutrients in high-yielding dairy ruminants: Choline and vitamin E

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Abstract: This review addresses the potential role of antioxidants and methyl-group sources in optimising the metabolic health of dairy ruminants. The productivity of high-yielding dairy cows has increased over the past 40 years and the milk yield has doubled. Such increases in milk production have been observed not only in dairy cows but also to some extent in other dairy ruminants such as ewes, goats and buffaloes (*Bubalus bubalis*). As a consequence, in all specialized dairy ruminants it is essential to optimize the macro and micro-nutrient supply, especially during the most critical period in the animals' production cycle i.e. from parturition until the peak of lactation. In this critical phase, an array of factors can enhance the balance between the intake and demand for nutrients, although the availability and supply of the selected micronutrients is also important. The supplementation of dietary antioxidants or boosting the endogenous methyl group status, via vitamin E, selenium and choline are proposed as possible strategies in maintaining stable metabolic health and optimising milk production.

Keywords: Choline, dairy cows, health, milk production, vitamin E.

Introduction

In high-yielding dairy ruminants the antioxidant and methyl group status are important in defining the metabolic health of the animals. However, the supplementation of both methyl group sources and antioxidants are usually addressed separately. This review highlights the key knowledge on vitamin E and choline supplementation in dairy ruminants and their role in optimizing milk production and metabolic health.

Vitamin E

Historically, deficiencies in vitamin E or selenium have been associated with high somatic cell counts (SCCs) in bulk and individual cow milk, and also with increased incidence and severity of intramammary infection (IMI) and mastitis. The positive effect of vitamin E on the SCC depends on adequate dietary Se levels, as reported since the 1980s. When dietary selenium was adequate, vitamin E supplementation significantly reduced the incidence of IMI and clinical mastitis (38). The supplemental vitamin E also improves the killing ability of neutrophils and enhances macrophage function in cows (6, 8, 16, 17, 33).

The current requirement for supplemental vitamin E is approximately 500 and 1000 IU/day for lactating and dry cows, respectively. Although NRC requirement published in the 2001 was substantially higher than that of the 1989, other data suggest that higher supplementation rates may be warranted in some situations. Cows supplemented with 4000 IU of vitamin E per day (in combination with 0.1 ppm of supplemental selenium) during the last two weeks before calving and 2000 IU/day during the first week of lactation were found to have significantly fewer mammary gland infections and clinical mastitis compared with 1000 and 500 IU/day during the dry and early lactation periods (42). Subsequent studies (3, 7, 23) confirmed these findings.

A meta-analysis (23) encompassing around 34 published papers between 1984 and 2003 on the relationship between vitamin E and mammary gland health, established that different supplementation levels, up to a maximum of 4000 IU during the dry and early lactation periods, were associated with lower somatic cell counts (SCCs), IMI, and clinical mastitis. Overall, vitamin E supplementation reduced milk SCC by a factor of 0.70,

the risk of IMI by 14%, and the occurrence of clinical mastitis by 30%.

A further disorder in which antioxidant status plays an important role is the retention of fetal membranes (RFM). RFM was one of the first conditions to be attributed to depleted antioxidant defenses and may also be related to impaired neutrophil function. RFM in dairy cows is a cause of endometritis, subsequent ovarian cycle delay and hence delayed pregnancy, resulting in serious economic losses (19). In fact, there is considerable evidence that oxidative stress is enhanced in RFM compared to animals with non-retained placenta. Thus, cows with low fast-acting antioxidants (including alpha-tocopherol) in plasma, and low glutathione peroxidase in red blood cells have been shown to have a higher incidence of retained placenta than cows with higher antioxidant levels (10, 11). LeBlanc et al. (21) showed that vitamin E (in association with selenium) was involved in RFM, but also highlighted that there are other important contributors, one of which may be the energy supply for immune function.

Supplementing dairy cattle with adequate amounts of vitamin E and Se is thus now a widely-accepted practice. However, adequate levels of these micronutrients do not completely control oxidative stress around calving (3, 6, 27). Numerous studies in dairy cows (34) have shown that the plasma vitamin E concentration decreases gradually around parturition, reaching the lowest values around calving and then increasing gradually afterwards. The inadequacy of micronutrient supplementation in this situation is probably due to increased milk yields, and a greatly reduced feed intake, resulting in a substantial increase in the recommended intake levels for this animal (24, 43).

In dairy cows the intake of vitamin E is generally considered adequate when alpha-tocopherol plasma levels are over 3-3.5 mg/ml or above 2 mg/mg relative to plasma cholesterol. Beyond these levels, no further benefits are observed (3). Reaching an adequate vitamin E status however, is difficult in dairy cows. Plasma vitamin E levels, expressed as alpha-tocopherol, fall significantly around parturition in all dairy ruminants (cows, ewes, goats, buffaloes - Figure 1), and it is difficult to maintain levels that are thought to be adequate for health. It has been suggested that this is one of the reasons for the decline in peripartum innate and acquired immune defense in dairy ruminants. Supplementation levels should therefore be re-considered, choosing high bioavailability sources. In this respect, the bio-potency and bioactivity of naturally occurring RRR-a-tocopherol are known to be higher than its synthetic all-rac counterpart (34, 41).

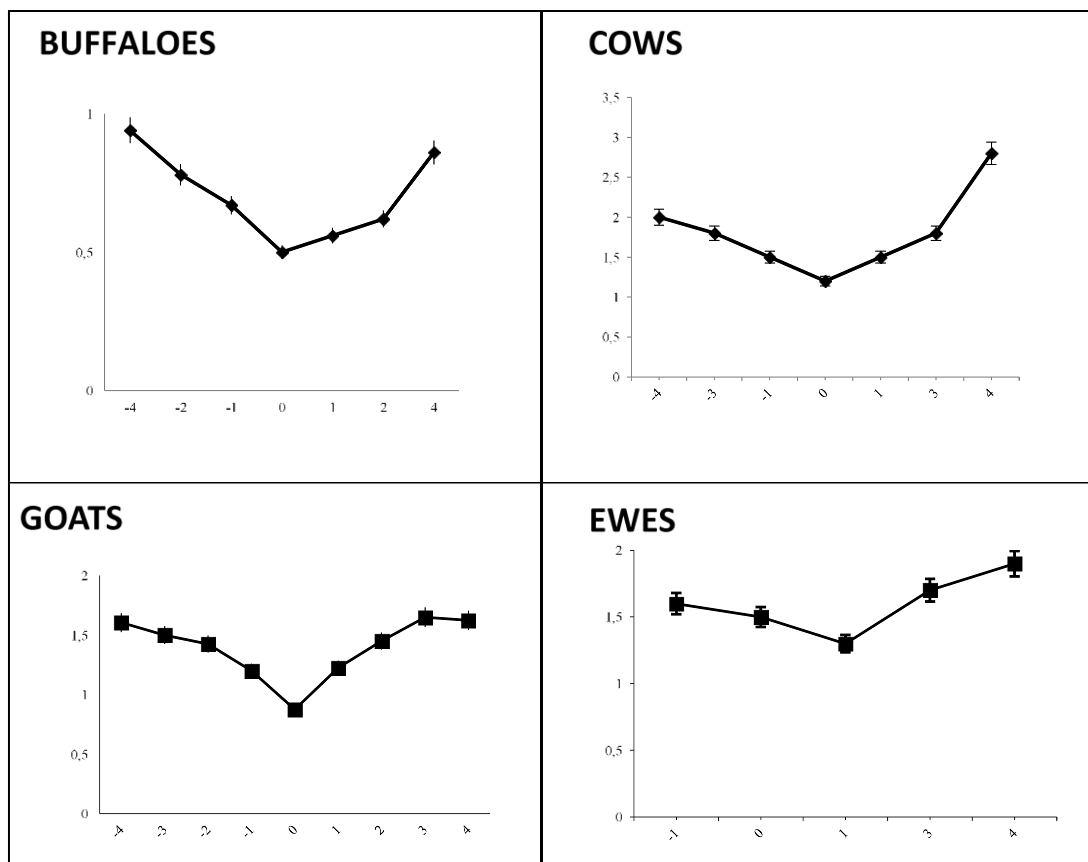

Figure 1. Changes in plasma alpha-tocopherol around parturition (day 0 in *x* axis) in various dairy ruminant species.
Data from Hogan et al. (17), Panda et al. (25), Pinotti et al. (32), Toker (40). x-axes = days relative to parturition; y- axes = alpha-tocopherol, mg/l.

Advances in feed manufacturing technology (including microencapsulation, and polymer coat protection) now make it possible to design products that deliver specific nutrients to the absorption sites of the small intestine. It is also clear that the formulation (natural vs. synthetic; inorganic vs. organic form; protected vs. unprotected) may not only influence intestinal bioavailability, but may also affect the uptake from target organs, tissue utilization, transfer to the new-born animals (1), and food quality (e.g. milk).

Choline

Choline supplementation increases productivity as well as metabolic health in dairy ruminants (2, 29, 32). Choline is often considered as a vitamin however, unlike classical vitamins, its endogenous synthesis is possible, and a choline deficiency syndrome usually goes undetected in healthy mammals. It has therefore been suggested that choline may be an essential nutrient for mammals, irrespectively of whether or not it is classified as a vitamin-like compound. Choline has an important role in energy and protein metabolism especially for those pathways that take place in the liver. It is involved in lipid metabolism, and more precisely in lipid transport as a lipotropic agent.

Choline, an important labile methyl group donor, takes part in the biosynthesis of other methylated compounds in the body. This latter function is "code shared" with methionine, thus choline and methionine are interchangeable being the two primary methyl donors in animal metabolism.

However, the metabolic pathways of choline and methyl groups are different in ruminants. In adult ruminants, choline is extensively degraded in the rumen; and as a consequence, unable to contribute significantly to the choline body pool. The methyl group metabolism is generally conservative with a relatively low rate of methyl catabolism, and a high rate of de novo synthesis of methyl groups via the tetrahydrofolate (THF) system.

In ruminants there is a conservative methyl group metabolism, which works perfectly in positive energy balance animals (e.g. beef cattle). However, this can become exacerbated in lactating dairy ruminants, for which the availability of dietary choline is still limited, while the output of methylated compounds in milk is high, and precursors from the tetrahydrofolate pathway (such as glucose and other gluconeogenetic precursors) are limiting, especially at the beginning of lactation.

On the basis of these assumptions, the hypothesis that choline may be a limiting nutrient for milk production has been proposed (29, 32). A great number of studies conducted to assess the possible effects of choline in milk production, established that from a technical/feeding point of view, choline must be rumen-protected in order to be effective in ruminant nutrition.

Knowledge about transition and early lactation periods of dairy cows proposes that greater choline availability improves not only milk production (5, 9), but also lipid (12, 13, 26, 30, 32) and methyl group metabolism (5). These results have also been investigated in a few meta-analyses. In 2010, Sales et al. (37) quantified the dietary effects of RPC (rumen-protected choline) on the production traits of dairy cows. In Sales' review, by increasing RPC supplementation from 6 to 50 g/d, which can also be effective in increasing milk yield, no dose response was observed. Milk yield in fact, decreased from 131.5 to 0.037 g of milk for each g of dietary RPC supplemented in the diets from 6 up to 50 g/d. In the same supplementation range, milk fat content decreased linearly at a rate of 0.00339% for a 1g/d increase in dietary RPC, confirming that choline has no effect on the milk fat content. Although the reasons for this are not clear, an interaction between choline and methionine has been proposed (37).

More recently two other meta-analyses on choline in dairy cow nutrition have been published. In 2019 using data from 27 studies, Humer et al. (18) reported that choline supplementation increased postpartum dry matter intake by 4% and milk yield by 3.4% in dairy cows. A higher milk yield also induced a higher milk fat yield and milk protein yield, without affecting their concentration. However also in this meta-analysis, no dose/response effects were observed. Providing RPC had no effect not only on the main plasma metabolites (such as non-esterified fatty acids, beta-hydroxybutyrate, glucose, and cholesterol) but nor on theincidence of ketosis, and mastitis across all studies in this meta-analysis.

These results seem to be in contrast with several studies in which choline was shown to improve the metabolic health and liver function (5, 12, 13, 26, 30). In fact, Zenobi et al. (44) stated that feeding pregnant, non-lactating Holstein cows with increasing amounts of RPC (from 0 to 25.8 g/d) decreased the concentration of hepatic triglycerides in a linear manner. Dietary choline thus seems to improve fat metabolism in the liver and increase the apparent absorption/traffic of triglycerides in the bloodstream, further confirming the beneficial effects of supplemental RPC for adult ruminants.

The most recent meta-analysis conducted by Arshad et al. (2) was based on 21 experiments including a total of 1313 cows, with a range of prepartum choline supplementation from 5.0 to 25.0 g/d. The results confirm that milk yields and energy-corrected milk (ECM) in multiparous cows increase linearly with the amount of dietary choline supplemented during the transition period. In addition, across the studies included in this meta-analysis, feeding RPC tended to reduce retained placenta and mastitis, although the optimum dose of choline was not established. Arshad et al. (2) recommended more than

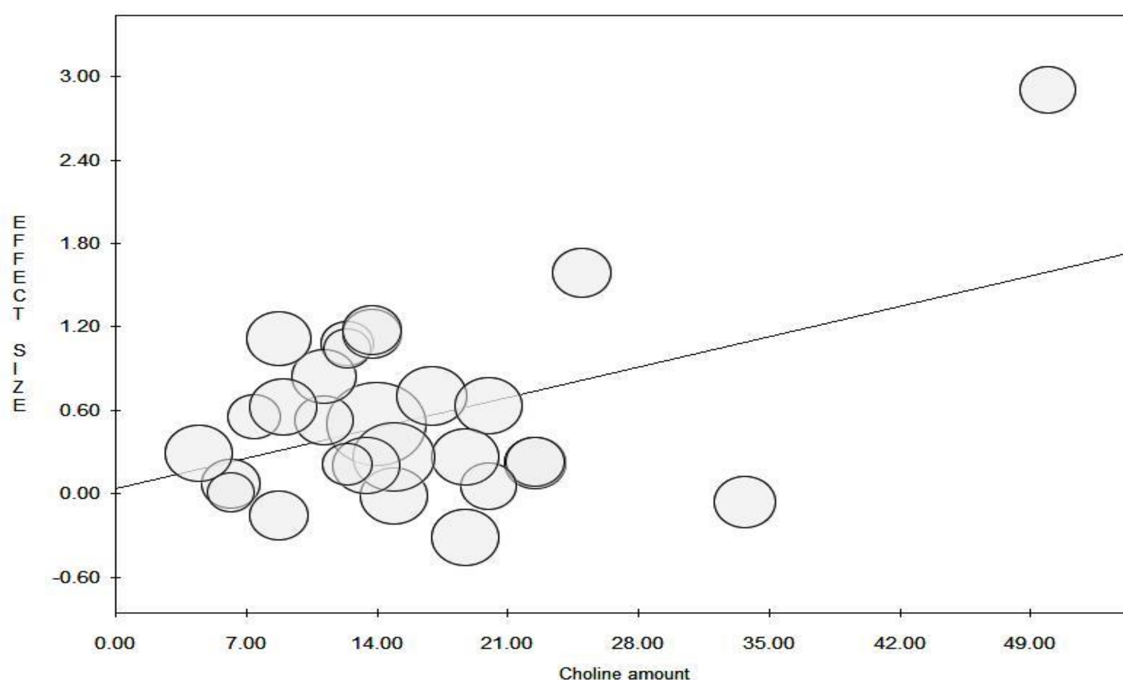

Figure 2. Meta-regression of choline amount (g/d of choline chloride) on effect size (MY) based on 17 studies.

13 g/d of choline, provided in a rumen-protected form. This quantity, however should be considered with caution since the dose–response relationship between dietary choline and milk yield needs to be addressed more thoroughly (Figure 2). Thus, several physiological and dietary factors are probably related to the obtained responses with dietary RPC supplementation necessitating further research to investigate the precise mechanism of choline action in lactating dairy cows.

In the case of other dairy ruminants, the effects of RPC have been less investigated. In dairy buffaloes 15 g/d of choline in a rumen-protected form were shown to increase milk yield by 15% (15). Using the same RPC dose, Kumar et al. (20) obtained a 20% increase in milk yield in choline-supplemented animals compared to the controls. The effect of RPC on milk yield recorded in these studies on dairy buffaloes are among the highest registered in dairy ruminants. Although the exact mechanism for such a large response is still unknown, the co-presence of supplemental fat in the diets cannot be ruled out. In small ruminants, Lobley et al. (22) studied the effects of choline infusion in sheep on transmethylation reactions, while Emmanuel and Kennelly (14) investigated methionine and choline incorporation into the plasma and milk of lactating goats. In both cases however, the main goal was to assess the fate and metabolism of methyl groups and the interchangeable nature between choline and methionine. Other researchers (4, 32) also assessed the efficacy of RPC supplementation in dairy goats. Pinotti et al. (32) supplemented periparturient dairy goats with 4 g/d of RPC starting four weeks prior to expect kidding and continuing for five weeks after parturition. Supplemental choline dose was derived from the experiments with dairy cows (32) corrected for the metabolic body weight ($BW^{0.75}$) of the goats at the beginning of the experiment. In RPC supplemented goats during early lactation, milk yield and fat-corrected milk (4% FCM) yield increased by 7 and 12%, respectively, compared to non-supplemented goats. RPC supplement also increased the milk fat concentration, and fat and protein yield while plasma metabolites did not differ between treatments. By contrast, in dairy ewes (39), RPC supplementation in combination with methionine and betaine had no effect on milk yield, although the dose of choline used was extremely low. It is worth noting that the number of studies on ovine species is still limited.

Overall these results suggest that greater choline availability is essential for optimizing milk production in dairy ruminants, although other factors such as dietary composition, dietary crude protein content, and post-ruminal methionine supply merit further investigation.

This review has highlighted that the dietary supply of antioxidants and methyl group sources may not always be sufficient to maximize milk production in dairy ruminants. Although the requirement of these nutrients may in theory be satisfied by specific supplementation (vitamin E and selenium) or by other nutrients (in the case of choline), it is unlikely that this happens in practice especially at the onset of lactation. The magnitude of the production response is likely to be affected by the basal diet composition, the dose of the nutrient, its form of

presentation/supply, and the stage of lactation (5, 24, 28, 41). However, it is also evident that our knowledge is incomplete regarding the potential role of antioxidants and methyl group sources in optimising metabolic health in dairy ruminants. For example, there are indications that both methyl group precursors (including choline) and other co-factors (folate and vitamin B12) are important for the optimal metabolic support of milk production, although methionine may not always be involved in this scenario (35). At the same time there are limited indications (*in vitro*, 36) that antioxidant and methyl sources interact positively in bovine mammary epithelial cells. In conclusion, a nutritional approach based on vitamin E and choline supplementation in dairy ruminants could be beneficial in terms of optimizing their milk production and metabolic health. However, the supply and use of individual nutrients should be reconsidered in order to meet the novel findings about the synergic effects of these compounds in ruminant diet.

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Conflict of Interest

The authors declared that there is no conflict of interest.

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