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Effects of Waiting Period before Milking on Orotic, Uric and Hippuric Acid Contents of Milks from Shami and Kilis Goats

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ABSTRACT

The organic acids present in milk in varying quantities can reflect the health condition of the animals and the nutritional quality of milk. In rural areas, goats are maintained in pasture during whole day and milking is being started as soon as goats arrive to farm. However, it is not known whether milking during feeding as soon as goats return from pasture or after 1 hour-waiting have any effect on nitrogen-containing organic acids are the body metabolism products. Therefore, in this study we objected to determine the effects of 1 hour-waiting period before milking on orotic, uric and hippuric acids of milks from "Shami (Damascus)" and "Kilis" goat breeds during lactation period. The trial was carried out with 40 goats. The 20 goats from each breed were randomly separated to two groups at equal number. Control and experimental groups of the both breeds were milked during feeding as soon as goats return from pasture and after 1 hour-waiting following feeding, respectively. The milk samples taken with interval 30 days from May to October were used for organic acid analysis at a reverse phase high performance liquid chromatography. During lactation hippuric acid was the most abundant organic acid, followed by orotic and uric acids. Experimental group of Kilis goats had the highest level of hippuric acid. Orotic acid was higher in Shami goats than that in Kilis breed. Uric acid was the highest in control group of Shami breed. The 1 hour-waiting period before milking resulted in a significant decrease in uric acid. In general, orotic and uric acid decreased towards the end of lactation whereas hippuric acid markedly increased in the last 3 months of lactation. It was concluded that the 1 hour-waiting before milking after returning from pasture may be especially suggested to Shami goat raisers due to the low uric acid content of the milk.

Keywords: Lactation; Goat breed; Organic acids containing non-protein nitrogen

Sağım Öncesi Bekleme Süresinin Şam ve Kilis Keçi Sütlerinin Orotik, Ürik ve Hippürik Asit İçeriği Üzerine Etkisi

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ÖZET

Değişen miktarlarda sütte bulunan organik asitler, hayvanın sağlık durumuna ve sütün kalitesine işaret edebilir. Kırsal alanlarda keçiler tüm gün boyunca merada otlatılmakta ve çiftliğe vardıklarında hemen sağıma başlanmaktadır. Ancak, hayvan meradan döner dönmez sağımın ve bir süre bekledikten sonra sağımın, vücut metabolizmasının ürünleri olan azot içeren organik asit içeriği üzerine herhangi bir etkiye sahip olup olmadığı bilinmemektedir. Bundan dolayı çalışmamızda laktasyon boyunca Şam ve Kilis keçi sütlerinde orotik, urik ve hippurik asit içerikleri üzerine sağım öncesi beklemenin etkilerinin araştırılması amaçlanmıştır. Deneme 40 keçi ile gerçekleştirilmiştir. Her bir ırktan 20 keçi rastgele eşit sayıda iki gruba ayrılmıştır. Kontrol ve deneme keçileri sırasıyla meradan döndükten hemen sonra yemleme sırasında ve 1 saat bekletmeden sonra sağılmışlardır. Mayıs ayından Ekim ayına kadar 30 gün aralıklarla alınan örnekler ters faz yüksek performanslı sıvı kromatografisinde organik asitlerin analizi için kullanılmıştır. Laktasyon boyunca hippürik asit en çok bulunan organik asit olmuş, onu orotik ve ürik asitler izlemiştir. Deneme grubundaki Kilis keçileri en yüksek hippürik asit içeriğine sahip olmuşlardır. Şam keçi sütleri Kilis keçi sütlerinden daha fazla orotik asit içermiştir. Sağım öncesi bekletme ürik asit içeriğinde önemli bir azalma sağlamıştır. Genellikle orotik ve ürik asit laktasyon sonuna doğru azalırken hippurik asit laktasyonun son üç ayında önemli bir artış göstermiştir. Sonuçta düşük ürik asit içeriğinden dolayı, keçi yetiştiricilerine özellikle Şam keçileri için meradan döndükten sonra sağım öncesi bekletme uygulaması önerilebilir.

Anahtar Kelimeler: Laktasyon; Keçi ırkı; Protein olmayan azot içeren organik asitler

1. Introduction

Due to the small fat globules and the differences in casein fraction and minor components, goat milk and its products have a higher digestive property than cows' milk (Jesse et al 1980; Slacanac et al 2010). This may be essential for babies and elders. Orotic, uric and hippuric acids, are carboxylic acids containing nitrogen, contribute to the non-protein nitrogen (NPN) pool of mammalian milk (Carver & Walker 1995; Gil & Uauy 1995). Their presence in milk is assumed to originate from either lactational cellular secretion, post-secretory metabolism in milk, and/or transfer across the blood-milk barrier (Robinson 1980; Schlimme et al 2000). Additionally, the various factors such as animal breed, species, feeding, milking time and the lactation period affect the organic acid content of milk (Buiarelli et al 2003; Carpio et al 2010). Goat milk had higher levels of uric and hippuric acid than cow and sheep milk whereas had lower orotic acid (Mahdi et al 1990; Indyk & Woollard 2004). The attention on nitrogen-containing organic acids of milk has recently been widely increased due to their potential physiological and nutritional importance to neonates and also technological importance to dairy products.

Orotic acid having neither phosphorus nor ribose is known as uracil-6-carboxylic acid and an intermediate pyrimidine nucleotides synthesis such as uridine nucleotide (Carver & Walker 1995). Thereby it is a component of all cells. Orotic acid is an essential growth factor of *Lactobacillus* subp. *bulgaricus* using for the fermented dairy products production (Tamime & Robinson 2001). With respect to physiological, orotic acid can decrease the level of cholesterol in human due to its interfering with the endogenous synthesis of cholesterol (Anastasi et al 2000). Like orotic acid, uric acid is the acid-soluble nucleotide of milk and a principal degradation product of the purine nucleosides adenosine, inosine, and guanosine (Robinson 1980; Resmini et al 1990). Increases in uric acid content of milk can indicate to increased rumen microbial protein synthesis in ruminant livestock as a microbial marker (Belenguer et al 2002). However, in animal metabolism uric acid is biodegraded to ammonia by urease and allantoicase enzymes. Such enzymes are lack in the human metabolism and uric acid is excreted in urine though the kidneys. If uric acid is elevated in the human body fluid as a result of consuming high uric acid containing-foods, it may cause gout disease (Najafpour 2015). On the other hand, uric acid can increase the oxidative stability of milk and dairy products due to its potential antioxidant property (Ostdal et al 2000), which is an advantage for dairy technology. Both orotic acid and uric acid may be suitable indicators for the determination of the proportion of milk added to foods.

Hippuric acid (benzoyl glycine) is produced in mammals from the metabolism of benzoic acid. Carpio et al (2010) claimed that hippuric acid could be considered as a marker of feeding regime, which was markedly higher in milk from organically fed goats than conventionally fed ones. The concentration of hippuric acid in milk could also be an indicator for the health condition of the animals because gut microflora in animals produces hippuric acid (Buiarelli et al 2003). As mentioned above, the individual organic acid containing nitrogen has the different nutritional and technological properties.

Although several authors have examined orotic, uric and hippuric acids of goat milk taking into consideration various factors such as the effects of different lactation stages, organic and conventional feeding (Johke & Goto 1962; Gil & Medina 1981; Carpio et al 2010; 2013), no data in literature is about the effects of goat breed and waiting period before milking on orotic, uric and hippuric acid contents of milk. Johansson et al (1999) reported that the feeding to cows during milking compared to before and after milking resulted in higher milk production and higher protein and lactose yields, and also an increase in oxytocin and somatostatin secretion which are hormones relating to milk production. Goat raisers believe that the waiting after returning from the pasture result in an increase in milk yield. However, it is not known whether feeding to goats before and during milking after returning from pasture has any effect on orotic, uric and hippuric acids of milk from the different goat breeds. Therefore, the object of this study was to determine the effects of 1 hour-waiting period before milking on concentrations of orotic, uric and hippuric acids in milk from 'Shami' and 'Kilis' goat breeds during lactation period.

2. Material and Methods

2.1. Milk samples

The study was carried out with Shami (Damascus) and Kilis goats raised in Yalaz Village of Hatay Province. Twenty goats (3-4 years old) from each breed were randomly separated to two groups at equal number which were described as control and experimental. The goats were grazed on pasture during whole day and additionally concentrate feed (1 kg per goat day^{-1}) having 2600 kcal ME and 160 g crude protein kg^{-1} was offered to all the goats after returning from pasture. All the goats were milked by hand. Control group goats were immediately milked when reaching to the farm. The experimental group goats were milked after 1 hour-waiting. Milk samples were taken separately from the goats individually with interval 30 days from May to October. After milking, milk samples taken from two goats in each milking day were mixed at the equal volume and a 500 mL milk sample was analyzed for organic acid. In this manner, the five samples from each group at each lactation month were made and then the samples were immediately transferred to laboratory at Food Engineering Department, Mustafa Kemal University using ice boxes. A total of 20 milk samples at each analysis time were used for organic acid analysis.

2.2. Organic acid analysis

The organic acids (orotic, uric and hippuric) in milk were analyzed according to the procedure described by Guler (2014) with slight modifications. For this purpose, a 40 mL of 5 mM H_2SO_4 was added to 10 mL of milk. After mixing, the samples were centrifuged at 5697×*g* for 7 min at 5 °C. The upper layer was filtered using a filter paper (Whatman No. 1). The filtrate was filtered again through 0.45 µm syringe filters (Millex PVDF Millipore, Billerica, MA, USA). This filtrate was injected onto the high performance liquid chromatography (HPLC-20 AD Prominence, Shimadzu, Kyoto, Japan) with an ion exchange column (Aminex HPX-87 H, 300×7.8 mm, BIO-RAD, Hercules, CA, USA) since this technique has recently been widely used for the analysis of components of the NPN fraction of milk (Indyk & Woollard 2004). Separation and determination of organic acids were carried out at an isocratic flow rate of 0.6 mL min^{-1} at 60 °C using 5 mM sulfuric acid as a mobile phase and at 210 nm with an UV/VIS detector (SPD-20 AV, Shimadzu, Kyoto, Japan), respectively.

Orotic acid, uric acid and hippuric acid standard (Sigma-Aldrich GmbH, Steinheim, Germany) solutions were prepared in 0.1 N NaOH at the eight different concentrations as the working solutions. As shown in Table 1, linear regression curve-based peak areas were calculated for the individual organic acid. As the linearity of the method is 0.999 for each organic acid it was suitable for quantification.

Table 1- Regression equations for the calibration curves and analysis of the linearity

Acids	*Range (mg L^{-1})*	*Regression equation (y= ax+b)*	R^2	*r*	*RSD* of *f* *(%)*
Orotic	3.2813-420	$y=9.87023.10^{-6}$ x - 1.86361	0.9996	0.9998	7.22
Uric	3.0625-392	$y=1.49714.10^{-5}$ x - 1.58686	0.9998	0.9999	5.92
Hippuric	3.3906-434	$y=1.25897.10^{-5}$ x + 2.40497	0.9993	0.9996	5.57

y, concentration; a, slope; x, response; b, intercept; R^2, coefficient of determination; f, response factor (f was calculated by dividing the area under the peak obtained in the chromatogram and the corresponding concentration); RSD, relative standard deviation

2.3. Statistical analysis

The experiment was a (2x2x6)x5 factorial design of two breeds (Shami and Kilis), waiting period (Control and experimental group) and lactation months (May, June, July, August, September, October). The General Linear Model (GLM) was applied to all data using SPSS statistical program (Version 22.00, SPSS, IBM, NY, USA). The paired comparisons of means were made using the Duncan's Multiple Range Test ($P<0.05$).

3. Results and Discussion

3.1. Validation of method

In order to the validation (accuracy and precision) of analytical method, recovery studies were carried out. Accuracy refers to how close a particular measure is to the true value. Precision is a measure of how reproducible. The firstly, the milk samples without adding standard were analyzed. The secondly, the spiked samples were analyzed. For this purpose, the standard solution (5 levels) of mix of orotic acid, uric acid and hippuric acid with the known amounts were added to the five milk samples (Table 2). For the each sample, concentration was calculated using the calibration curves (Table 1). Recovery values obtaining were close to 100% for all of organic acids (Table 2), which indicated a good accuracy. The precision of the method or its repeatability were expressed as the coefficient of variation (CV) for 10 replications of standard recovery at different concentrations (Table 2) for each organic acid. The coefficient of variation was between 2.75 to 5.02% which indicated a good repeatability. As a rule, a CV below 5% is considered acceptable, although it depends on the type of analysis. A chromatogram sample for the organic acids of goats' milk, standard organic acids used for calibration and spiked samples is shown in Figure 1.

3.2. Organic acids

To our best knowledge, this study is the first report on the effects of 1 hour-waiting period before milking on orotic, uric and hippuric acids in goat milk at the different lactation stages of the Shami and Kilis goat breeds. Therefore comparative data are scarce. As shown in Table 3, orotic acid in milk samples analyzed ranged from 18.09±1.00 mg L^{-1} to 130.57±3.80 mg L^{-1}, which was significantly

Table 2- Percentage recovery of organic acids added to milk and repeatability (as coefficient of variation) of the method

Organic acid	*Replicate*	*Added organic acid (mg L⁻¹)*	*Original organic acid in milk (mg L⁻¹)*	*Organic acid recovered (mg L⁻¹)*	*Recovery of added organic acid*
	1	5.50	5.12	11.05	107.92±0.51
	2	12.39	3.64	15.59	96.44±0.02
Orotic	3	26.09	2.19	28.87	102.27±0.02
	4	51.79	1.56	54.89	102.98±1.06
	5	105.73	4.69	113.65	103.05±0.75
Mean					102.53±4.08
Repeatability*					3.97%
	1	5.24	2.97	8.67	108.84±0.03
	2	11.62	2.85	14.50	100.19±1.93
Uric	3	24.30	4.36	27.79	96.44±0.02
	4	48.23	2.89	50.26	98.22±0.95
	5	100.41	6.91	104.59	97.28±0.44
Mean					100.19±5.03
Repeatability*					5.02%
	1	8.88	10.35	18.59	92.83±0.03
	2	14.51	7.83	21.45	93.85±0.12
Hippuric	3	27.48	7.40	33.58	95.26±0.08
	4	50.29	7.32	56.36	97.51±1.78
	5	101.29	19.89	120.44	99.27±1.36
Mean					95.74±2.64
Repeatability*					2.75%

*, as coefficient of variation

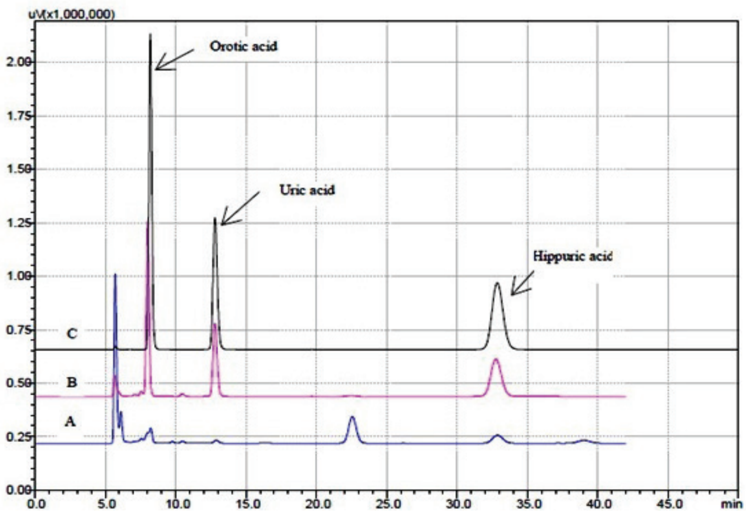

Figure 1- Chromatogram sample for milk (A), organic acid standard (B) and milk added organic acid (C)

($P<0.001$) decreased towards the end of lactation. This trend was consistent with the findings of Jesse et al (1980) and Indyk & Woollard (2004). Decreasing in orotic acid may be due to the increases in fat content (data not shown) since there was an inverse relationship between fat and orotic acid contents of cows' milk (Anastasi et al 2000). Although the minimum value of orotic acid was within ranges (10-22 mg L^{-1}) reported by other researchers (Gil & Medina 1981; Indyk & Woollard 2004; Wehrmüller et al 2008), the maximum values obtained from lactation months between May and August were by far high. The changes in goats' metabolism and feed regime depending on lactation period may have favored high orotic acid production from May to August. The 1 hour-waiting period before milking and interaction between 1 hour-waiting before milking and lactation period had no effect on the mean concentration of orotic acid from both breeds whereas the interaction between breed and 1 hour-waiting period had a significant ($P<0.001$) effect on its. Independently of lactation period and 1 hour-waiting period before milking, breed had a significant ($P<0.001$) effect on orotic acid. Shami goats had higher orotic acid content (76.69±6.67 mg L^{-1}) than that (66.56±4.94 mg L^{-1}) of Kilis breed. This could be attributed to the differences in goats' amino acid metabolism depending on genotype since amino acids glutamine and aspartate are precursors for orotic acid synthesis (Brosnan & Brosnan 2007). The effect of interaction among lactation period, 1 hour-waiting period before milking and breed on orotic acid content was found to be statistically significant ($P<0.001$). While milk from experimental Kilis breed group at September month had the lowest level of orotic acid, milk from control Shami breed group at May month had the highest. In light of the present observations, it is probably that orotic acid content of goats' milk is markedly influenced by lactation stage and breed rather than the 1 hour-waiting period before milking. With respect to high orotic acid content, goat milks obtained from first- and mid-lactation terms may be more valuable for nutritional purposes.

Regarding on uric acid, it was significantly ($P<0.001$) affected by the interaction among lactation period, breed and 1 hour-waiting before milking. As shown in Table 3, uric acid was found in the range of 31-95 mg L^{-1}. This was markedly higher than that (10-26 mg L^{-1}) reported by Larson & Hegarty (1979) for goat milk stored at 4 °C or -18 °C. Becker (1993) reported that the cold storage could lead to a decrease in uric acid content of milk since it is converted to allantoin and glyoxylate at the cold conditions. In this study, the conversion may not be carried out as being used fresh raw goat milk. At July and August months the highest concentrations of uric acid in milk samples analyzed were obtained. This may be due to the conversion of xanthine to uric acid by xanthine oxidase which has a relatively high activation rate at high environmental temperatures (Fox & Kelly 2006). On the other hand, the high level of uric acid may be related to an increase in microbial nitrogen yield in duodenum of goats at high seasonal temperatures as reported by Tas & Susenbeth (2007). Regardless of the lactation period and 1 hour-waiting period before milking, the mean concentration of uric acid with a value of 61 mg L^{-1} was significantly ($P<0.05$) higher in milk from Shami breed than that (54 mg L^{-1}) from Kilis one. This may be due to the physiological differences as previously reported by Indyk & Woollard (2004). Independently lactation period and breeds, there was observed a significant ($P<0.001$) decrease in uric acid of experimental group compared to control group. This may be due to decrease in intake energy with the rest after returning from pasture since a significant increase in uric acid excretion in cows' milk with increase in energy intake was observed by Giesecke et al (1994). With respect to technological, high uric acid level may play an important role for milk products as an antioxidant but it may be indicator for poor hygienic quality of milk and also may carry a risk for health due to gout, urolithiasis and possible cardiovascular diseases.

Hippuric acid contents of all milk samples from both breeds showed a significant ($P<0.001$) variation during lactation whereas they were fluctuated as increase or decrease. A similar

Table 3- The effect of waiting before milking in Shami and Kilis goats on concentrations of nitrogen-containing organic acids (mg L^{-1}) in milk during lactation (n= 5)

Organic acids	*Lactation month*	*Shami*			*Kilis*				
		Control†	*Experimental*‡	^{1}P	*Control*	*Experimental*	^{1}P	^{2}P	^{3}P
	May	130.57±3.80^{a}	97.28±6.96^{ab}	**	72.72±5.97^{a}	97.23±8.16^{a}	*	***	ns
	June	111.68±5.27^{b}	82.73±13.12^{b}	ns	76.33±3.15^{a}	100.30±5.53^{a}	**	ns	ns
	July	94.30±7.45^{c}	109.90±7.06^{a}	ns	71.03±3.68^{a}	91.25±3.34^{a}	**	***	*
Orotic	August	84.66±6.16^{c}	77.66±7.21^{b}	ns	67.19±5.14^{ab}	72.73±4.83^{b}	ns	ns	ns
	September	24.54±1.70^{d}	32.96±3.45^{c}	ns	21.65±1.84^{c}	18.09±1.00^{d}	ns	***	ns
	October	38.57±4.26^{d}	35.37±2.65^{c}	ns	55.12±4.49^{b}	49.46±6.63^{c}	ns	**	ns
	Mean	80.72±7.27	72.65±6.09	ns	60.86±3.92	72.27±5.95	ns	***	ns
P value for lactation		***	***		***	***			
	May	84.86±2.58^{b}	47.63±6.36 ^{ab}	***	44.52±3.80^{b}	44.11±4.58^{c}	*	ns	ns
	June	74.79±3.09^{b}	47.33±3.71 ^{ab}	***	59.01±4.16^{a}	58.55±5.63^{ab}	ns	ns	*
	July	95.71±3.60^{a}	62.56±8.87^{a}	**	71.74±4.47^{a}	67.48±3.84^{a}	**	ns	***
Uric	August	82.31±2.51^{b}	46.19±3.26 ^{ab}	**	71.35±4.70^{a}	55.82±4.00^{abc}	**	ns	***
	September	58.84±2.43^{c}	38.78±3.40 ^{b}	**	32.34±2.84^{b}	31.20±1.21^{d}	***	*	ns
	October	42.22±6.37^{d}	54.80±4.87 ^{ab}	*	63.07±6.47^{a}	53.54±1.49^{bc}	*	ns	ns
	Mean	73.12±3.57	49.55±2.45 ^{ab}	***	56.79±3.19	51.72±2.65	**	*	***
P value for lactation		***	*		**	**			
	May	112.61±2.63^{d}	73.96±5.68^{d}	***	70.72±14.87^{de}	80.31±5.20^{e}	ns	*	ns
	June	79.90±5.94^{de}	54.87±3.86^{d}	**	96.19±15.07^{d}	120.99±2.11^{d}	**	***	ns
	July	66.33±5.28^{e}	66.26±6.55^{d}	ns	62.15±12.03^{e}	83.63±2.02^{e}	**	ns	ns
Hippuric	August	208.73±9.31^{b}	192.90±7.17^{c}	ns	181.29±16.57^{c}	215.49±11.8^{c}	*	ns	ns
	September	356.65±26.20^{a}	361.88±8.60^{a}	ns	406.08±45.05^{a}	339.37±16.07^{b}	ns	ns	ns
	October	155.48±16.76^{c}	235.04±13.21^{b}	**	216.36±12.32^{b}	395.36±11.26^{a}	***	**	**
	Mean	163.28±19.02	164.15±20.92	ns	170.61±124.59	199.32±23.06	*	*	*
P value for lactation		***	***		***	***			

†, control indicates the goats milked immediately after returning from pasture; ‡, experimental indicates the goats milked following 1 hour-waiting after returning from pasture; means in the same column followed by different letters are significantly different (at *, P<0.05; **, P<0.01; ***, P<0.001); ^{1}P, significance between control and experimental groups of each breed at each month; ^{2}P, significance between breeds irrespectively the control and the experimental groups; ^{3}P, significance between control and experimental groups at each month irrespectively goat breed

tendency was observed for Czech White Shorthaired breed (Hornickova et al 2014). Hippuric acid was significantly (P<0.001) influenced by interaction among 1 hour-waiting period before milking, breed and lactation period. Milk from experimental Shami breed group at June month had the lowest level of hippuric acid with a value of 54.87±3.86 mg L^{-1} but milk from control group of Kilis breed at September month had the highest hippuric acid value with 406.08±45.05 mg L^{-1}. Regardless of 1 hour-waiting period before milking and breeds, August, September and October months of lactation favored high hippuric acid production compared with May, June and July months. This could be attributed to the variations in feeding regime and goats' physiological as progressing lactation. Carpio et al (2010) reported that hippuric acid is a marker of feeding regime and is significantly high in milk from organically fed goat. The 1 hour-waiting period before milking for Kilis goat breed resulted in a significant increase in hippuric acid but it had no significant effect on Shami goat breed. Regardless of 1 hour-waiting period before milking and lactation period, hippuric acid with a mean of 185 mg L^{-1} was significantly ($P<0.05$) higher in milk from Kilis breed than that (164 mg L^{-1}) in milk from Shami breed. This could be attributed to the mainly differences in genotype since the other conditions were almost identical. The mean result for hippuric acid were within ranges (72.59-188.96 mg L^{-1}) reported by Carpio et al (2010) for milk samples from organically fed goats but it was higher than values (5.78-25.11 mg L^{-1}) reported by Hornickova et al (2014) for milk from Czech White Shorthaired goat breed. This could be attributed to the differences in genotype and in feed regime between our studies and theirs.

Briefly, orotic, uric and hippuric acids were significantly influenced by the interaction among period of lactation, breed and 1 hour-waiting period before milking. The waiting period before milking had not effect on orotic acid only.

4. Conclusions

Determination of orotic, uric and hippuric acids in milk by HPLC method had a good repeatable for both breeds. These organic acids were significantly affected by the interaction among lactation period, 1 hour-waiting period before milking and breed. As conclusion; 1) lactation stages of the breeds affected the orotic, uric and hippuric content of milk produced in all groups, 2) irrespectively 1 hour-waiting before milking and lactation period, breed differences affected the nitrogen-containing organic acids of milk, 3) milk from Kilis goat breed had higher hippuric acid than that from Kilis goat breed whose milk contained more orotic acid and uric acid and 4) one hour-waiting before milking of goats is resulted in low uric acid level in milk that may have an advantage for human health. However, more studies are needed to elucidate the effects of waiting before milking on the other milk components.

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