Turkish Journal of Agriculture - Food Science and Technology, 7(4): 560-566, 2019
DOI: https://doi.org/10.24925/turjaf.v7i4.560-566.1877

Turkish Journal of Agriculture - Food Science and Technology

Available online, ISSN: 2148-127X | www.agrifoodscience.com | Turkish Science and Technology

Effects of Microorganism Count and Physicochemical Properties of Tulum and Kashar Cheeses to Biogenic Amine Formation

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ARTICLE INFO

Research Article

Received : 27/02/2018
Accepted : 18/02/2019

Keywords:
Biogenic amines
Erzincan Tulum cheese
Kars Kashar cheese
Lactic acid bacteria
Hazard limit values

ABSTRACT

In this research, biogenic amine types and quantity of Tulum and Kashar cheeses were determined. In addition, the relationship between biogenic amines and some microbiological-chemical properties of cheese samples were investigated. The contents of tryptamine, phenylethylamine, putrecine, cadaverine, histamine, tyramine of totally 40 samples (20 of each cheese) were examined. While only one sample of Tulum cheeses had no biogenic amines, different levels of biogenic amines were determined in other samples. No significant relation was confirmed between the biogenic amine quantity and total aerobic mesophilic bacteria, lactic acid bacteria, enterococci bacteria count, but there was a correlation between some biogenic amine contents and chemical properties which are tyrosine, lactic acid, pH, protein and ripening coefficient. Biogenic amine levels determined in the cheeses found below hazard limit values according to what legal limit.

Türk Tarım – Gıda Bilim ve Teknoloji Dergisi 7(4): 560-566, 2019

Biyojen Amin Oluşumu Üzerine Tulum ve Kaşar Peynirlerinin Mikroorganizma İçeriği ve Fizikokimyasal Özelliklerinin Etkisi

MAKALE BİLGİSİ

Araştırma Makalesi

Geliş : 27/02/2018
Kabul : 18/02/2019

Anahtar Kelimeler:
Biyojen amin
Erzincan Tulum peyniri
Kars Kaşar peyniri
Laktik asit bakteri
Tehlike sınır değerleri

ÖZ

Bu araştırmada, Tulum ve Kashar peynirlerinde oluşan biyojen aminlerin türü ve miktarları belirlenmiştir. Ayrıca peynir örneklerinin mikroorganizma içerikleri ve bazı fizikokimyasal özellikleri ile biyojen amin oluşumu arasındaki ilişki araştırılmıştır. Toplam 40 peynir örneğinin (her bir peynir çeşidinden 20 adet) triptamin, feniletilamin, putresin, kadaverin, histamin ve tiramin içeriği incelendi. Tulum peynirlerinde sadece bir örnekte biyojen amine rastlanmazken, diğer peynir örneklerinde farklı düzeylerde biyojen amin belirlenmiştir. Biyojen amin miktarı ile toplam aerobik mezofilik bakteri, laktik asit bakteri, enterokok bakteri sayıları arasında önemli bir korelasyon tespit edilemezken, bazı biyojen aminler ile tirozin, laktik asit, pH, protein ve olgunlaşma katsayısı gibi kimyasal özellikler arasında bir korelasyon belirlenmiştir. Her iki çeşit peynirde de belirlenen biyojen amin miktarlarının tehlike sınır değerlerinin altında olduğu bulunmuştur.

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Introduction

Turkey is a rich country concerning traditional cheese varieties. Kasar (like Kashkaval), Tulum (generally it's ripened and packed in small ruminant skin or plastic material) and Lor, Dil, Otlu (with herbs), Cökelek and Mihalic are important cheeses in Turkey (Hayaloglu et al., 2002). Erzincan Tulum and Kars Kashar Cheeses are widespread known and consumed traditional cheese types. Tulum cheese has known with natural mouldy taste and flavour. Tulum cheese is produced from cow, sheep, goat's milk or mix of these milk and are sold after ripening in different packaging materials (eg leather, lacquer can, cloth, plastic drums) in many regions of the country (Keleş 1996; Ünsal, 1997; Tekinşen and Akar, 2017). After the milk is coagulated and whey is separated, curd is salted. The salted curd is ripened at 6-10°C in 85% relative in the goat skin (Tulum) for 3-6 months (Çolak et al., 2007). The sharp flavour and characteristic taste of Tulum cheese comes from the goat skin during the ripening. Most dairy plant use hygienic plastic packaging material to avoid microbial contamination rather than goat skin (Erdogan et al., 2003; Yılmaz et al., 2005).

Another important traditional cheese of Turkey is Kars Kashar cheese. Similar cheeses are produced in Serbia, Romania and Bulgaria countries and known as "Kashkaval" (Öksüz et al., 2001; Sulejmani et al., 2014). There is not much difference from Kashkaval cheese, except the treatment of curd (teleme) in the step following the boiling. The shape of the Kars Kashar cheese resembles the Gruyere of French as a form and its taste flavor of the Emmental cheese of Switzerland (Eralp, 1961).

During the ripening of Tulum or Kashar cheese, decarboxylation of free amino acids which formed enzymatic hydrolysis of protein causes biogenic amine formation. Biogenic amines are low molecular weight organic, nitrogen compounds found in plant, animal and microbial cells (Alvarez and Moreno-Arribas, 2014). It is admitted that the presence of biogenic amine in cheese is an indicator the unsuitable production conditions and exposure to microbial contamination (Edwars and Sandine, 1981, Elsanhoty et al., 2009). It is known that a lot of bacteria like *Escherichia, Enterobacter, Salmonella, Shigella, Achromobacter, Pseudomonas, Alcaligenes, Proteus, Clostridium, Perfringens, Micrococcus, Streptococcus, Lactobacillus* and *Leuconostoc* produce biogenic amine at different levels (Edwards and Sandine, 1981; Joosten and Stadhouders, 1987; Joosten and Northolt, 1987; Joosten, 1988; Sumner et al., 1990; Joosten et al., 1995; Durlu-Özkaya et al., 1999; Marino et al., 2000; Durlu-Özkaya, 2001).

Biogenic amine contents of food are widely investigated due to the potential toxicity of these compounds. Symptoms caused by the biogenic amines are erythema, nausea, fever, vomiting, sweating, higher or lower tension, sore throat, thirst, swollen lips and spots on skin (Taylor et al., 1982; Chang et al., 1985; Joosten and Stadhouders, 1987; Chander et al., 1989; Stratton et al., 1991; Ladero et al., 2010).

Cheese is an ideal media for bacteria to synthesize the biogenic amines. However, the quantity and the type of biogenic amine depend on the cheese type, ripening period and microbial count (Joosten, 1988; Sumner et al 1990; Stratton et al., 1991). Therefore the presence of biogenic amines in various types of cheeses has been widely investigated (Durlu-Özkaya et al., 1999; 2000; Durlu-Özkaya and Tunail, 2000; Durlu-Özkaya, 2001; 2002; Karahan et al., 2001; Öner et al., 2002; Hocalar and Üren, 2002; Yıldız et al., 2010; Şenel et al., 2012; Schirone et al., 2013; Renes et al., 2014)

However, there are a few studies on biogenic amine contents of Kars Kashar and Erzincan Tulum cheeses. The aims of this study were to determine the biogenic amine types and quantities present in these cheeses by HPLC. In addition, some microorganism count and physicochemical properties were also defined and the relationship with formation of biogenic amine was displayed.

Materials and Methods

Material

Twenty Tulum cheeses and twenty Kashar cheeses were purchased from different sale locations in the center of Erzincan and Kars provinces. Approximately 500 g of cheese samples were brought to the laboratory by maintaining the cold chain and analyse were carried out at Ankara University Dairy Technology Department.

Methods

Microbiological analyses: Total aerobic mesophilic bacteria (TAMB) count, lactic acid bacteria (LAB) and *Enterobacteriaceae* spp. were determined by using cultural counting methods (Harrigan and McCance, 1986; Halkman and Ayhan, 2000). TAMB was inoculated in Plate Count Agar (Merck) and incubated at 32-35°C for 48 h, LAB was inoculated in MRS agar (Merck) and incubated at 40-42°C for 48 h, *Enterobacteriaceae* spp was inoculated in Violed Red Bile-Dextrose Agar (Merck) and incubated at 37°C for 24 h.

Chemical analyses: The methods used for chemical analyses were as follows; total solids by gravimetric method (ISO5534, 2004), fat contents by Gerber method using Van – Gulik butyrometer (ISO3433, 2008), salt contents according to the method described in TSE 591 (Anonymous, 2013), titratable acidity as lactic acid % according to Anonymous (2013). The pH values were measured by digital pH – meter with combined electrode (Mettler Toledo, Switzerland). Total nitrogen (TN), water soluble nitrogen (WSN) and non – protein nitrogen (NPN) contents of cheese samples were determined according to Gripon et al. (1975), tyrosine value according to Hull (1947) spectrophotometrically. Water activity was measured by Novasina thermoconstanter (Axair Ltd., Switzerland). Additionally protease peptone nitrogen (PPN) content was calculated based on NPN content from WSN content and ripening coefficient as the percentage of WSN in TN.

Determination of Biogenic Amines by HPLC

Extraction of the amines: Samples were prepared according to Maijola and Eerola (1993) and Durlu-Özkaya et al. (2000). Five grams of cheese were exposed to Acetonitrile/Percholoric acid (AP) solution (1:1) and 500

µl of 1-7 diamino heptane (Sigma) as an internal standard (IS) were added into this mixture. The mixture was then agitated on a vortex mixer for 10 minutes, and centrifuged at 3000 rpm for 10 min. 200 µl of aqueous layer was transferred into a test tube and 800 µl of acetonitrile, 700 µl of bidistilled water, 200 µl of sodium carbonate solution (0.2 g/ml) and 100 µl of dansyl chloride were added and mixture was agitated on a vortex mixer for 5 second followed by maintained in a water-bath at 37°C for 30 min. 20 µl of Na-glutaminate solution (50 mg/ml) were added, and agitated for 5 sec. Maintaining in water-bath at 37°C for 60 min. again, 1 ml of acetonitrile (ACN) was added and centrifuged at 3000 rpm for 10 min. 1 ml of aqueous layer was placed into eppendorf tube.

Preparation of standard solutions of each biogenic amines mix: 20 ppm of standard solutions of each biogenic amines were prepared separately in 500 ml of AP solution. 5 ml of each of these standard solutions were dissolute in 20 ml of AP solution and 500 µl of IS were added.

Preparation of IS solution: 1 gram of 1-7 diamino heptane (Sigma) was dissolved in 1 liter bidistillated water.

HPLC Equipment: The chromatograms of biogenic amines were obtained according to Bütikofer et al. (1990). High Performance Liquid Chromatographic system (Hewlett Packard, model 1100, CA, US) equipped with reversed-phase Luna C_{18} column (250 × 4.6 mm id., 5 µm), and UV detector (HP-G 1314 A) recording at 254 nm was used to detect biogenic amines. The flow rate was set to 1 mL min^{-1} while the column temperature was set as 35 °C. Injection volume was 20 µL. Mobile phases were prepared in two different ways. First Mobile phase consists of buffer/ethanol/acetonitrile/H_2O:7.5/50/75/117.5 mL and the second mobile phase consists of buffer/ethanol/acetonitrile /H_2O:0.5/112.5/112.5/25 mL. The tris/acetic acid/ H_2O:2/1/2 compose the buffer solution also.

Statistical Analyses

Minitab version 13.2 (Minitab, Inc., State College, PA, USA) was used for data analyses. A two-tailed t test at the 95% confidence interval was conducted to determine differences in microorganism content and biogenic amines, and physicochemical properties and biogenic amines (Rosner 2006).

Results and Discussion

The mean, minimum and maximum counts TAMB, LAB and *Enterobacteriaceae* spp. of samples were illustrated in Table 1.

The high TAMB counts of Erzincan Tulum Cheese can be the result of raw milk use in the manufacture, long production time (minimum 10 days and microbial growth at this time) and not ripening in brine contrary to other cheese types. However, the TAMB counts that are determined were lower than the values determined by Kurt et al (1991), Dığrak et al. (1995) and Güven et al. (1995) (2.13×10^9 cfu/g, 1.8×10^9 cfu/g, $2.4\times10^6 - 7.5\times10^7$ cfu/g). High lactic acid bacteria count is desired in Erzincan Tulum Cheese. Otherwise growth and activity of coliform group bacteria and especially proteolytic microorganisms can not be prevented (Kurt et al 1991). While the determined LAB counts in samples were in agreement with results found by Kurt et al. (1991) (8.5×10^6 cfu/g), it was found lower than that of values reported by Dığrak et al. (1995) (1.1×10^7 cfu/g). The fecal enterobacteria (FE bacteria) number was below 100 cfu/g in 18 samples of all the Tulum Cheese samples. The mean values of TAMB and LAB counts in Kars Kashar Cheese were higher but LAB counts were lower than the TAMB and LAB values reported in Kıvanç (1989) respectively. FE counts were lower than 100 cfu/g in 20 Kashar Cheese samples.

This cheese can be characterized by its high dry matter and fat content, hard to crumble structure, semi hard, homogenous texture and certain acidic taste (Kurt et al., 1991). According to Table 2, dry matter and fat contents of Tulum cheese samples were found to be high. Also water activity of samples was found to be low while the titratable acidity was high. It can be seen from the relevant table that Kashar Cheese was a ripened cheese type with high dry matter and fat content.

The mean value of total biogenic amine contents of Tulum cheese was determined as 1.9± 0.37 mg/100g (Table 3). Only one sample had no biogenic amines, yet the presence of tryptamine, phenylethylamine, putrecine, cadaverine, histamine and tyramine values were found as 55, 60, 75, 85, 65 and 70%, respectively. It was determined that the highest biogenic amine level was found for tyramine (0.56±0.11 mg/100g) and it was followed by putrecine, cadaverine, phenylethylamine, tyriptamine and histamine, respectively. Tulum cheese sample and standard chromatograms are seen in Figure 1.

The amount of biogenic amines in Tulum Cheese was not found high and it was recognized that the detected levels were very much lower than the toxic doses for biogenic amines (for histamine 10 mg/100g, for tyramine 80 mg/100mg and for phenylethylamine 3 mg/100g (Halasz et al., 1994).

It wasn't determined an important correlation between microorganism count and biogenic amine content. Because of the ability of producing biogenic amine of every bacteria was different, Valsamaki et al (2000) reported that it was not determined correlation between formed biogenic amine content and microorganism count.

Table 1 Microbiological Analyses Results of Cheese Samples (cfu/g)

Microorganisms	Erzincan Tulum Cheese			Kars Kashar Cheese		
	Min.	Max.	Mean	Min.	Max.	Mean
TAMB	1.0×10^5	1.9×10^7	$4.3\times10^6\pm1.1\times10^6$	2.0×10^4	2.3×10^7	$2.6\times10^6\pm1.2\times10^6$
LAB	5×10^3	3.1×10^6	$1.1\times10^6\pm2.4\times10^5$	1.0×10^4	1.4×10^7	$9.4\times10^5\pm6.9\times10^5$
FE	<100	4.0×10^2	$1.1\times10^2\pm1.5\times10$	<100	<100	<100

TAMB: Total Aerobic Mesophilic Bacteria; LAB: Lactic Acid Bacteria; FE: Fecal Enterobacteria

Table 2 The chemical analyses results of cheese samples, n=20

Chemical properties	Erzincan Tulum Cheese	Kars Kashar Cheese
	Mean	Mean
Water activity, aw	0.93±0.01	0.94±0.00
Lactic acid, %	1.95±0.05	1.43±0.07
pH value	4.75±0.10	5.20±0.02
Dry matter, %	55.95±0.71	58.31±0.45
Protein, %	19.84±0.60	26.06±0.28
Tyrosyne, mg/5g	4.25±0.28	3.34±0.26
TN, %	3.11±0.09	4.09±0.04
WSN, %	0.86±0.03	1.06±0.06
Ripening coefficient, %	28.13±1.28	26.04±1.56
NPN, %	0.54±0.02	0.74±0.04
PPN, %	0.32±0.02	0.33±0.03
Fat, %	31.48±0.82	25.74±0.48
Fat in dry matter, %	56.15±0.99	44.17±0.86
Salt, %	2.32±0.18	2.14±0.16
Salt in dry matter, %	4.13±0.30	3.98±0.28

TN: Total Nitrogen; WSN: Watersoluble Nitrogen; NPN: Non-protein Nitrogen; PPN: Protease Peptone Nitrogen

Table 3 The biogenic amine levels of Tulum Cheese samples (mg/100g).

Biogenic Amines	Determined sample number	Minimum	Maximum	Mean
Tryptamine	11	0.12	0.39	0.13±0.03
Phenylethylamine	12	0.07	0.95	0.16±0.05
Putrecine	15	0.06	2.21	0.51±0.13
Cadaverine	17	0.02	2.13	0.45±0.15
Histamine	13	0.08	0.32	0.12±0.02
Tyramine	14	0.20	1.54	0.56±0.11
Total	19	0.02	6.58	1.93±0.37

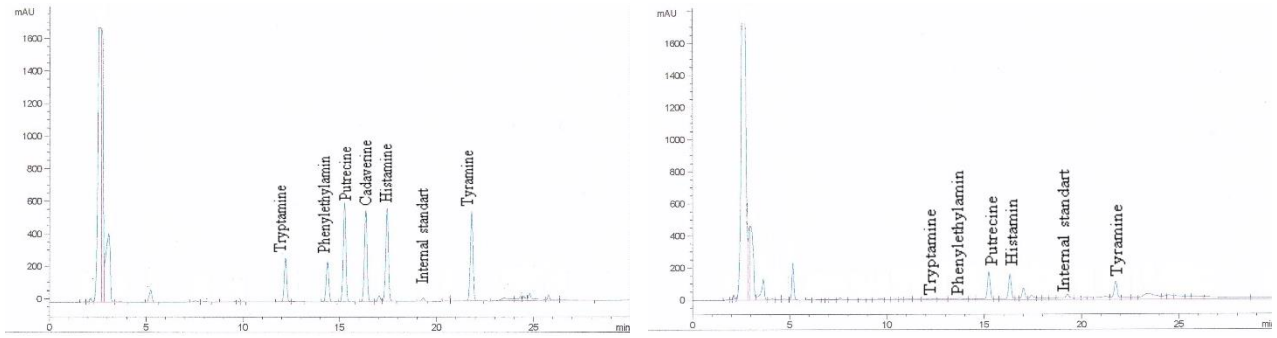

Figure 1 The standard chromatogram and Tulum cheese sample

There was a positive correlation between tryptamine and tyrosine which was a measuring of proteolysis (r=0.482; P<0.05) (Table 4). It was admitted that the important factor for biogenic amine formation in cheese was presence of free amino acid as a substrate and amine formation at toxic levels depended on adequate free amino acid counts (Chang et al., 1985; Stratton et al., 1991; Antila et al., 1984; Joosten and Van-Boekel, 1988).The correlation between histamine and protein count was determined as P<0.05 (r=0.454). When protein amount increased, also biogenic amine formation increased, due to increasing of substrate count which was necessary for forming amine by microorganism (Chang et al., 1985; Stratton et al., 1991; Joosten and Van-Boekel, 1988). There was a negative correlation between phenylethylamine and ripening coefficient (r=- 0.443; P<0.05). In parallel with ripening progressive, it might be expected that biogenic amin counts increased under normal condition. However it can be seen different situation for biogenic amine formation. Similarly, it was determined that during the ripening while qualification of tyramine, cadaverine and putresine increased , the levels of other biogenic amines decreased in another research (Ordonez et al 1997).

Tyramine was the highest biogenic amine type in Kashar Cheese (0.68 ± 0.14 mg/100g) and it was followed by histamine, phenylethylamine, cadaverine, putrecine and tryptamine, respectively (Table 5). The presence of tryptamine, phenylethylamine, putrecine, cadaverine, histamine and tyramine were determined as 30, 45, 65, 80, 75 and 75%, respectively. The total biogenic amine content of one sample was found as 1.62±0.26 mg/100g in average. Kashar cheese sample and standard chromatograms are seen in Figure 2.

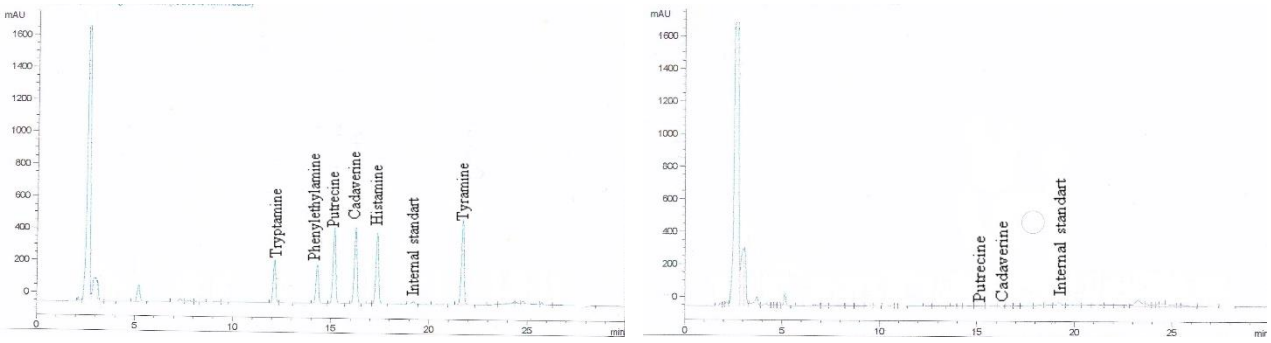

Figure 2 The standard chromatogram and Kashar cheese sample

Table 4 Correlation coefficient between biogenic amines and microbiological and physicochemical properties in Tulum cheese (r)

Chemical Properties	TRY	PHEN	PUT	CAD	HIS	TY	TA
TMAB	-0.281	-0.380	-0.290	-0.343	-0.365	-0.366	-0.448
LA	0.193	-0.369	-0.180	-0.408	0.289	0.091	-0.214
FE	-0.211	-0.173	-0.210	-0.154	-0.269	-0.273	-0.275
Water activity, aw	0.285	0.115	0.153	0.202	0.365	0.335	0.298
Tyrosyne, mg/5g	0.482*	0.068	0.163	-0.033	0.152	0.123	0.142
Lactic acid, %	0.472*	-0.074	0.159	-0.087	0.300	0.332	0.170
pH value	0.220	-0.168	0.081	-0.122	0.210	0.198	0.048
Protein, %	0.381	0.238	0.126	-0.007	0.454*	0.249	0.211
TN, %	0.380	0.238	0.125	-0.007	0.453*	0.249	0.210
WSN, %	0.147	-0.341	0.081	-0.045	0.179	0.193	0.046
Ripening coefficient, %	-0.163	-0.443*	-0.072	-0.073	-0.240	-0.090	-0.172
NPN, %	0.180	-0.326	0.000	-0.138	-0.101	0.028	-0.082
Salt, %	-0.122	0.022	0.016	0.007	-0.080	-0.044	-0.018
Dry matter, %	0.092	0.358	0.204	0.029	0.201	0.220	0.217
Fat, %	0.131	0.382	0.204	0.099	0.289	0.114	0.227

TRY: Tryptamine, PHEN: Phenyl-ethylamine, PUT: Putrecine, CAD: Cadaverine, HIS: Histamine, TY: Tyramine, TA: Total amine, TAMB: Total Aerobic Mesophilic Bacteria; LAB: Lactic Acid Bacteria; FE: Fecal Enterobacteria TN: Total Nitrogen; WSN: Watersoluble Nitrogen; NPN: Non-protein Nitrogen; PPN: Protease Peptone Nitrogen, *: P<0.05

Table 5 The biogenic amine levels of Kashar Cheese samples (mg/100g)

Biogenic Amines	Determined sample number	Minimum	Maximum	Mean
Tryptamine	6	0.08	0.36	0.07±0.03
Phenylethylamine	9	0.07	1.35	0.22±0.09
Putrecine	13	0.05	0.25	0.09±0.02
Cadaverine	16	0.03	0.47	0.18±0.03
Histamine	15	0.13	3.78	0.39±0.18
Tyramine	15	0.31	2.41	0.68±0.14
Total	20	0.20	5.36	1.62±0.26

Table 6 Correlation coefficient between biogenic amines and microbiological and physicochemical properties in Kashar cheese (r)

Chemical Properties	TRY	PHEN	PUT	CAD	HIS	TY	TA
TMAB	0.184	-0.177	0.339	-0.085	-0.120	-0.077	-0.152
LA	0.265	-0.088	0.345	-0.105	-0.076	-0.005	-0.046
FE	-	-	-	-	-	-	-
Water activity, aw	0.158	0.061	0.420	-0.010	-0.257	-0.167	-0.205
Tyrosyne, mg/5g	0.587**	0.373	0.469*	0.408	-0.031	-0.167	0.152
Lactic acid, %	0.316	0.368	0.319	0.132	-0.195	-0.272	-0.091
pH value	-0.357	0.076	-0.576**	-0.136	0.267	0.376	0.322
Protein, %	-0.085	-0.360	0.008	0.120	0.089	0.157	0.034
TN, %	-0.054	-0.369	0.032	0.146	0.084	0.145	0.028
WSN, %	0.278	0.094	0.350	0.211	0.035	-0.185	0.034
Ripening coefficient, %	0.286	0.140	0.337	0.187	0.012	-0.209	0.017
NPN, %	0.343	0.099	0.437	0.209	-0.020	-0.187	0.008
Salt, %	0.226	0.264	-0.005	0.260	-0.003	0.010	0.143
Dry matter, %	-0.075	-0.079	-0.296	-0.185	-0.034	0.120	-0.036
Fat, %	-0.163	0.351	-0.116	-0.032	-0.247	-0.028	-0.101

TRY: Tryptamine, PHEN: Phenyl-ethylamine, PUT: Putrecine, CAD: Cadaverine, HIS: Histamine, TY: Tyramine, TA: Total amine, TAMB: Total Aerobic Mesophilic Bacteria; LAB: Lactic Acid Bacteria; FE: Fecal Enterobacteria TN: Total Nitrogen; WSN: Watersoluble Nitrogen; NPN: Non-protein Nitrogen; PPN: Protease Peptone Nitrogen, *: P<0.05; **:P<0.01

According to the obtained results, the biogenic amine contents of Kars Kashar Cheese was not high and found below toxic doses (Halasz et al 1994).

The correlation coefficient between tryptamine and putresine with tyrosine was r= 0.587 (P<0.01), r= 0.469 (P<0.05), respectively (Table 6). As expected, when the tyrosine count increased, tryptamine and putresine counts increased. It was determined a remarkable negative correlation between putresine and pH value (r= -0.576) and this was important statistical (P<0.01). This situation was same as reported by Joosten (1988) and Chander et al (1989) that when pH value increased, formation of biogenic amine increased, on the contrary decreased.

Acknowledgments

This study was supported by BAP (Ankara Universty, Scientific Researches Projects) with the Project number of "20030711071".

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