Effect of some external factors on the content of biogenic amines and polyamines in a smear-ripened cheese

Komprda, Rejchrtová, Sládková, Zemánek, Vymlátilová

Dairy Sci. & Technol. (2012) 92:367–382
DOI 10.1007/s13594-012-0075-4

ORIGINAL PAPER

Effect of some external factors on the content of biogenic amines and polyamines in a smear-ripened cheese

T. Komprda · E. Rejchrtová · P. Sládková · L. Zemánek · L. Vymlátilová

Received: 25 February 2012 / Revised: 14 June 2012 / Accepted: 14 June 2012 /
Published online: 18 July 2012

Abstract Ripening cheeses belong to the risky product category due to the formation/presence of biogenic amines (BA) and polyamines (PA). Various ambient and technological factors can affect the content of these toxicologically important substances in ripening cheeses. The aim of the study was to assess the safety of a smear-ripened cheese with regard to BA and PA. The content of amines was determined in cheeses produced in two shapes (discs and bars) during the winter, spring, and summer seasons, and stored until the age of 66 days at 5 and 20 °C. Storage temperature, shape, cheese age, and season accounted on average for 46, 26, 15, and 13 % of the explained variability in biogenic amines content. Tyramine, cadaverine, and putrescine content in cheeses increased ($P<0.001$) during storage by 13.5, 14.6, and 9.7 mg.kg^{-1}.day^{-1}. Tyramine content was twice as high ($P<0.05$) in the disc-shaped cheeses (834 mg.kg^{-1}) as compared to the bars (419 mg.kg^{-1}). Average tyramine, cadaverine, and putrescine content in cheeses stored at 20 and 5 °C was 1,332 and 353, 1,416 and 127, and 784 and 255 mg.kg^{-1}, respectively. Higher counts of both lactic acid bacteria (8.22 vs. 7.63 log cfu.g^{-1}; $P<0.05$) and enterococci (6.33 vs. 6.02 log cfu.g^{-1}; $P<0.05$) were found in the cheeses stored at 5 °C in comparison to 20 °C. It was concluded that high-risky consumers should store this type of cheese at refrigerated temperature for no longer than 2 weeks and to favor the bar-shaped products.

环境因素对涂抹成熟干酪中生物胺和多胺含量的影响

摘要: 由于干酪成熟中能够产生生物胺(BA)和多胺(PA), 成熟干酪被列为高风险食品。环境因素和技术因素影响成熟干酪中这些有毒物质的形成。本文基于BA和 PA数据对涂抹成熟干酪的安全性进行风险评估。两种形状(饼形, 块状)的干酪分别在冬季、春季和夏季, 5 °C-20°C条件下成熟66 天, 测定了其中酪胺、组胺、色胺、2-苯乙胺, 尸胺、腐胺、亚精胺和精胺的含量。贮存温度、干酪形状、成熟时间和季节对应的干酪中平均生物胺的含量分别为46、

T. Komprda (✉) · E. Rejchrtová · P. Sládková · L. Zemánek · L. Vymlátilová
Department of Food Technology, Mendel University in Brno, Zemědělská 1, 613 00 Brno,
Czech Republic
e-mail: komprda@mendelu.cz

26、 15 和13%。干酪贮存过程中酪胺、尸胺和腐胺日增长量分别为13.5、14.6 和9.7 mg kg^{-1} (P<0.001)。饼形干酪中酪胺的含量 (834 mg kg^{-1}) 是块状干酪 (419 mg kg^{-1}) 的两倍 (P<0.05)。在20 °C和5 °C两种贮存温度下,干酪中酪胺、尸胺和腐胺平均含量分别为1332和353,1416和127,及784和255 mg kg^{-1}。在5 °C下存储的干酪中乳酸菌 (8.22 vs. 7.63 log cfu g^{-1}; P<0.05) 和乳球菌(6.33 vs. 6.02 log cfu g^{-1}; P<0.05)高于20 °C下存储的干酪。因此,块状干酪在冷藏温度下贮存不应超过2周。

Keywords Tyramine · Putrescine · Enterococci · Smear-ripened cheese · Food safety

关键词 酪胺 · 腐胺 · 肠球菌 · 涂抹成熟干酪 · 食品安全

1 Introduction

The production of biogenic amines (BA) in foods and their toxicological importance has previously been summarized in the reviews of Stratton et al. (1991) and more recently by McCabe-Sellers et al. (2006).

Currently, the only BA controlled by legal regulations in the European Union is histamine (an upper limit of 100 mg.kg^{-1} in fish; EC 2005). The toxicological limits for tyramine are difficult to establish due to the wide interindividual differences in the robustness of the detoxifying system. Eerola et al. (1997) reported a wide range of tyramine toxicity threshold values from 100 to 800 mg.kg^{-1}. According to Latorre-Moratalla et al. (2008), patients treated with a new generation of monoamine oxidase inhibitor drugs (MAOI) can tolerate 50–100 mg tyramine.

The polyamines (PA) spermidine and spermine, including their precursor putrescine (chemically a diamine), are currently classified as a distinct group within BA. PA can be formed by an alternative metabolic pathway, are required for cell growth and proliferation, and are taken up by tumour cells (Walter et al. 2004).

During the ripening of cheeses, the conditions required for the formation of BA/PA are present: presence of free amino acids, microorganisms with amino acid-decarboxylase activities, and conditions enabling the growth of microorganisms (Innocente and D'Agostin 2002). The content of histamine, tyramine, 2-phenylethylamine, and cadaverine in various types of cheeses have been reported at range from trace levels to 1,290, 1,800, 400, and 2,370 mg.kg^{-1}, respectively, while for polyamines, the content of putrescine, spermidine, and spermine in cheeses have been reported to reach 1,100, 100, and 50 mg.kg^{-1} (Komprda and Dohnal 2009).

The effect of various environmental conditions, the BA-producing microorganisms, and the genetic organization of the biosynthetic pathways involved in BA production has been reviewed recently by Linares et al. (2011). Particular factors affecting BA production in cheeses have been studied in detail in quantitatively important types of cheese, namely, Swiss-type (Petridis and Steinhart 1996), Dutch-type (Komprda et al. 2007), Gouda (Leuschner et al. 1998), or blue cheese (Rabie et al. 2011). Recently, BA/PA contents in typical French (Coton et al. 2012), Italian (Schirone et al. 2011), Portuguese (Pintado et al. 2008), Turkish (Ekici et al. 2010; Yildiz et al. 2010; Andic et al. 2011), or Czech (Standarová et al. 2010) cheeses have been reported.

The objective of the present study was to assess the safety of a Czech curd smear-ripened cheese, taking into account the following factors that could affect BA/PA content: the season in which the cheese was produced, cheese shape, storage temperature, and time of ripening/storage.

2 Materials and methods

2.1 Material

The subject of the study was a curd smear-ripened cheese (called “Olomouc curd cheese”). The smear was produced using a surface-growing culture of *Brevibacterium linens*. The cheeses were obtained from a regular commercial production schedule over three seasons: winter (December–February), spring (March–May), and summer (July–September).

Two types of curd were used as raw materials, both produced from pasteurized milk by microbial acidification (without rennet): “thermophilic” and “mesophilic curds.” The age of the product was designated as zero at this point. Curds (fat content 0.1 %) were brine salted (35 g of NaCl per 1 kg of curd) for 5 days after which the curds were formed into two shapes using two different mould configurations: discs (diameter, 45 mm; height, 11 mm; weight, 20 g) and bars (20×20 mm; length, 100 mm; weight, 31.3 g). Thermophilic and mesophilic curds were then mixed in the ratio of 2:1 and 1:2 for the discs and bars, respectively. During blending, the following ingredients were added: a culture of *B. linens* (BBL; suspension of 12.4 mL per 100 kg of curd), *Candida valida* (8.3 mL per 100 kg; pH adjustment for proper BBL growth), a mixture of *Pediococcus acidilactici*+dextrose+yeast extract+maltose (a total concentration of 200 g per 100 kg; the concentration of the individual components is confidential; product designation: Bioprotector, type Fargo 37; producer, Amarex Ltd, Prague, Czech Republic); acidity regulator Na_2HCO_3+$CaCO_3$ (0.8 % *w*/*w*).

Immediately after forming (age of the product 6 days), disc- and bar-shaped curd cheeses were placed on racks in a ripening chamber, rinsed with water for surface homogenization of the BBL culture, dried, and ventilated for two more days. These cheeses were considered mature after a further 3 days of ripening (total ripening time of 10–11 days).

The mature cheeses were wrapped in a polypropylene foil: five pieces per package and four pieces per package in the case of discs and bars, respectively. One half of the wrapped mature cheeses (within each of the winter, spring, and summer productions) was consequently stored at 5 °C and the other half at 20 °C. Cheese samples, stored under these conditions, were analyzed after 42 days (the minimum shelf-life) and after 66 days (the expiration date).

In summary, samples were taken at 0, 5, 6, 8, 10, 42, and 66 days. Independent replicate samples were taken at each sampling time for each cheese shape (disc and bar), and the mean of this parallel determination was used as a variable for statistical analysis.

2.2 Amine analysis

BAs and PAs were determined according to Komprda and Dohnal (2009). Cheese samples were homogenized using a minishaker MS2 IKA (IKA Werke GmbH, Staufen, Germany). Ten grams was weighed into an 85-mL plastic centrifuge tube, and 20 mL of 0.1 M HCl and 0.5 mL of an internal standard solution (1,7-diaminoheptane, concentration, 1 $mg.mL^{-1}$; Sigma-Aldrich, Prague, Czech Republic) were added. The sample was then extracted for 2 min using a disintegrator Heidolph Diax 900 (Heidolph Instruments, Germany). The suspension was centrifuged at 755×*g* for 10 min at 4 °C (Hettich Universal 32R; Hettich, Germany). The supernatant was filtered through paper filter (Filtrak, No. 390), and the solid residue was extracted for a second time as above. The combined extracts were made up to 50 mL with deionized water and filtered through a disposable nylon membrane filter 13 mm, 0.45 μm (Chromatography Research Supplies, Addison, USA).

One milliliter of the extract (standard) was mixed with 0.5 mL of saturated Na_2CO_3 (pH adjusted to 11.2) in a 4-mL amber vial for 1 min, to which 1 mL of the derivatizing agent (5-dimethylaminonaphthalene-1-sulfonyl chloride; 5 mg dissolved in 1 mL of 2-propanone; Sigma-Aldrich, St. Louis, USA) was added and the mixture shaken for 1 min. The vials were placed in a thermostat (EVATERM for 25 vials; volume, 4 ml; Labicom, Olomouc, Czech Republic), and derivatization was allowed to proceed for 1 h in the dark at 40 °C. The vial was shaken repeatedly for 15 min, and the mixture was allowed to rest for 15 min after the derivatization reaction was completed. Finally, 250 μL of a 10 mM ammonia solution was added. Amine derivatives were extracted in diethylether (3×1 mL), and the organic phase evaporated to dryness under nitrogen. The remaining solid residue was dissolved in 0.5 mL acetonitrile (high-performance liquid chromatography gradient grade, Sigma-Aldrich, Prague, Czech Republic). The solution was filtered through a 0.45-μm nylon membrane filter, and an aliquot was injected onto the chromatographic column Zorbax Eclipse XDB C18 (150×4.6 mm, particle size of 5 μm) with a guard column Meta Guard ODS-2 (30×4.6 mm; particle size, 5 μm; MetaChem Technologies, Torrance, USA).

Amines were determined using a liquid chromatograph HP 1100 (Agilent Technologies, Wilmington, USA) consisting of a quaternary pump (G1311A), vacuum degasser (G1322A), and automatic sampler (G1313A). The separation was performed by gradient elution with H_2O/ACN (time 0–23 min: H_2O 35–0 %; ACN 65–100 %) at a flow rate of 0.8 $mL.min^{-1}$. Separated amines were identified using a photometric UV/VIS detector (G1314A) at 254 nm. BA/PA concentrations were expressed as milligram per kilogram of original (fresh) cheese. Tyramine, histamine, tryptamine, cadaverine, putrescine, spermidine, and spermine hydrochlorides (Sigma-Aldrich, Prague, Czech Republic) were used as standards.

The measured data were evaluated, including repeatability of the analytical process and recoveries according to Komprda et al. (2007).

2.3 Microbiological determinations

The groups of bacteria with a presumed ability to form biogenic amines were determined: lactic acid bacteria (LAB) using the de Man-Rogosa-Sharpe agar (Noack, Austria) after anaerobic cultivation (using CO_2 incubator with IR sensor, Direct Heat 321 model, Thermo Fisher Scientific, USA) at 30 °C for 72 h and (apart from LAB as a whole group) *Enterococcus* spp. on the Slanetz–Bartley medium with triphenyltetrazolium chloride (Noack) at 37 °C for 48 h under aerobic conditions.

2.4 Determination of dry matter and pH

Dry matter (DM) was determined as follows: An aliquot of the cheese sample was grated, and 3 g was weighed into an aluminum dish and dried to a constant weight in a drying chamber (Binder, Merck, Germany) at 102 °C. For the determination of pH, 10 g of the finely grated cheese was weighed into a 50-mL beaker, 50 mL of distilled water was added, and after 30 min, the pH value was measured in the water extract using a WTW pH 95 microprocessor apparatus (Fisher Scientific, Germany).

Dry matter and pH were measured in all cheese samples taken at days 0, 5, 6, 8, 10, 42, and 66.

2.5 Statistical analysis

A general linear model of the multiple-way analysis of the variance-ratio test (ANOVA of the main effects) was used for the mutual comparison of the effects of the main variability factors (season, cheese shape, storage temperature, and the cheese age) on the BA/PA content. The differences between single BAs/PAs and the counts of microorganisms, respectively, as influenced by individual variability factors were calculated separately using one-way analysis of the variance-ratio test with the *post hoc* Duncan's test.

The above analysis was complemented by calculation of the correlation matrix of all analyzed traits (chemical, microbiological, and physical); relationships were considered to be significant at $P<0.01$.

Regression analysis was used to evaluate the dependency of BA/PA contents on the age of the cheese, based on the set of all cheeses taken during the whole experiment.

3 Results

3.1 Comparison of the effects of the main variability factors on the content of amines

The values of the effects of the tested variability factors (season, cheese shape, storage temperature, and cheese age) assessed as the mean squares using general linear model of the variance-ratio test with ANOVA of the main effects are presented for particular amines in Table 1. The results shown in Table 1 represent percentages of that part of total variability that was explained by the above-mentioned factors.

Storage temperature (cheeses stored either at 20 °C or at 5 °C) accounted on average for 46 % of explained variability in BA/PA content, shape (disc or bar) explained 26 %, cheese age explained 15 %, and season (cheeses produced in winter, spring or summer) explained 13 %.

The main deviations from the above-mentioned average values (storage temperature, 46 %; shape, 26 %; cheese age, 15 %; season, 13 %) concerned histamine and the polyamines spermidine and spermine. Season+shape accounted for 91 % of explained variability in histamine content. In the case of spermidine and spermine, it was 84 and 81 % respectively.

Table 1 Extent of effects of the tested variability factors on content of biogenic amines and polyamines in curd cheeses

Amine	Variability factor (percentages of explained variability[a])			
	Season[b]	Shape[c]	Temperature[d]	Age[e]
Tyramine	5	17	61	16
Histamine	40	49	1	5
Tryptamine	4	9	70	17
2-Phenylethylamine	1	2	80	17
Cadaverine	2	0	78	18
Putrescine	6	0	65	28
Spermidine	22	61	7	8
Spermine	25	55	5	14

[a] Assessed from mean squares of particular factors; general linear model of the multiple-way analysis of the variance-ratio test; ANOVA of the main effects; $n=695$ for season, temperature, and the cheese age; $n=579$ for shape

[b] Winter, spring, and summer

[c] Disc and bar

[d] Cheese ripened/stored at either 20 or 5 °C

[e] Zero to 66 days

In the following sections, particular sets of cheeses are compared within individual factors irrespective of the other tested factors.

3.2 The effect of the storage temperature, including other physical characteristics (pH, NaCl)

Regarding the storage temperature, only mature cheeses ready for consumption (cheeses aged from 11 to 66 days), irrespective of the season and shape, were evaluated from this viewpoint in this part of the experiment.

Substantially higher ($P<0.05$) amounts of tyramine, tryptamine, 2-phenylethylamine, cadaverine, and putrescine, respectively, were formed in cheeses stored at 20 °C in comparison to 5 °C (Fig. 1).

NaCl concentration in cheeses changed substantially during the experiment; however, because only one initial NaCl concentration (35 g per 1 kg of the curd) was used, an effect of NaCl on BA production was not studied in a more detail. The pH was positively correlated ($P<0.01$) with the content of tyramine ($r=0.44$), 2-phenylethylamine ($r=0.38$), cadaverine ($n=0.41$), and putrescine ($r=0.49$).

3.3 Cheese shape

The content of each of the eight amines tested in the disc- and bar-shaped cheeses (irrespective of a season, storage temperature, and the cheese age; $n=293$ and 286 for rings and bars, respectively) is compared in Fig. 2. The content of tyramine, histamine, phenylethylamine, spermidine, and spermine, respectively, was higher ($P<$

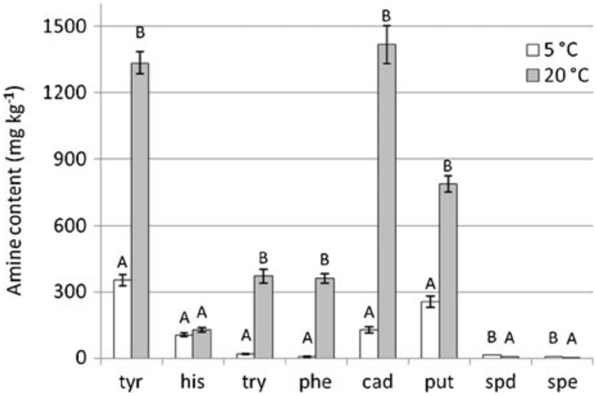


Fig. 1 Biogenic amine/polyamine content in curd cheeses stored either at 5 °C (n=184) or 20 °C (n=186). Mature cheeses (age from 11th to 66th day), irrespective of the season, were evaluated; one-way analysis of the variance ratio test. *tyr* tyramine, *his* histamine, *try* tryptamine, *phe* 2-phenylethylamine, *cad* cadaverine, *put* putrescine, *spd* spermidine, *spe* spermine. Different letters (*A and B*) within a given amine differ at $P<0.05$

0.05) in the discs. In particular, this difference was significant in the case of tyramine. An exception to this was tryptamine, which had a higher ($P<0.05$) content in the bar-shaped cheeses.

3.4 Effect of the cheese age

3.4.1 Time dependence of amine content in cheeses

Similarly to Section 3.1, the results of this section were obtained from data based on the total set of cheeses (irrespective of a season, shape, and storage temperature) within the whole time range from 0 to 66 days.

The content of most amines (except histamine and spermidine) increased (P< 0.001) with increasing age of the cheese. All dependences were linear; addition of a quadratic term to the regression equation was not significant in any of the tested amines. Time dependences of the three quantitatively most significant biogenic amines are presented in Fig. 3.

Although the highest absolute values were found for tyramine, the content of cadaverine increased most rapidly of all the amines tested: nearly 15 mg.kg^{-1}.day^{-1}.

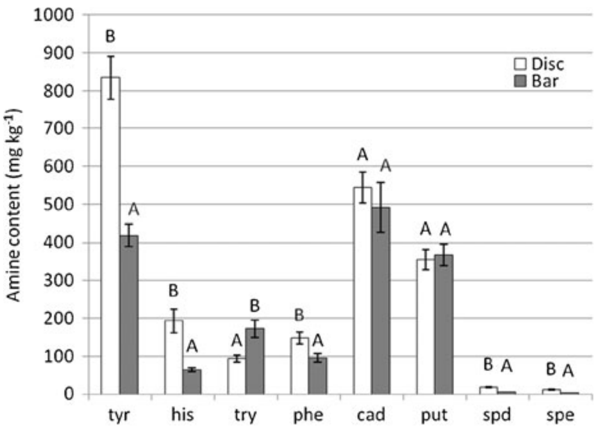


Fig. 2 Content of biogenic amines/polyamines in curd cheeses produced in the form of discs or bars. The sets of all disc- and bar-shaped cheeses, respectively, were evaluated irrespective of a season, storage temperature, and age of the cheese; one-way analysis of the variance ratio test, n=293 and 286 for discs and bars, respectively. Different letters (*A and B*) within a given amine differ at $P<0.05$

Fig. 3 Dependence of quantitatively most important biogenic amines on the age of the cheese; regressions calculated based on the set of all analyzed cheeses irrespective of a season (winter, spring, and summer), shape (discs and bars), and storage temperature (5 and 20 °C); n=695

On the other hand, histamine content did not change at all in the present experiment (P=0.71, R^2=0.02).

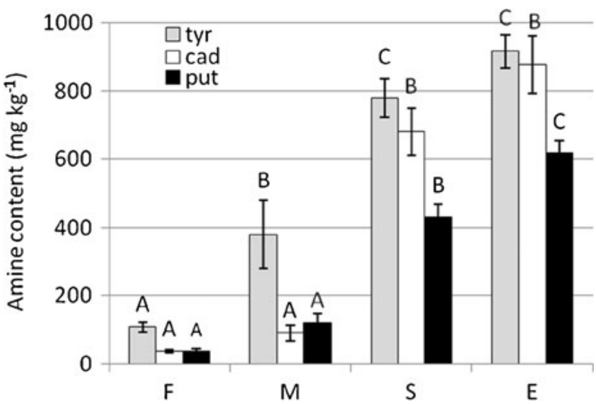
The sum total of the five biogenic amines tested (Y; mg.kg^{-1}) increased with the increasing age of the cheese (X; days) according to the equation $Y = 159 + 35.9X$ ($R^2 = 0.22$; $P < 0.001$). Corresponding regressions for the sum of the three polyamines and the sum of all eight amines tested were as follows: $Y = 28.5 + 9.38X$ ($R^2 = 0.27$; $P < 0.001$) and $Y = 28.5 + 9.38X$ ($R^2 = 0.27$; $P < 0.001$), respectively.

3.4.2 Comparison of the particular production phases

Apart from the time regressions of biogenic amine and polyamine contents, the concentrations in the cheeses of the three most quantitatively important amines (tyramine, cadaverine, and putrescine) were separately compared for the four distinct production phases: after drying (8 days), mature cheese (10 days), end of the minimum shelf-life (42 days), and end of the expiration date (66 days). The results are presented in Fig. 4.

The tyramine content in the mature (M) cheeses reached a value of 379 mg.kg^{-1}. By the end of the expiration date (E), the content of this amine exceeded 900 mg.kg^{-1}. Putrescine reached values of 119 and 617 mg.kg^{-1} in the M- and E-cheeses, respectively (Fig. 4). The sum total of the three polyamines in the M and E samples was on average 139 and 446 mg.kg^{-1}.

Fig. 4 Content of the three quantitatively most important biogenic amines in curd cheese in four production phases (irrespective of shape and season). *tyr* tyramine, *cad* cadaverine, *put* putrescine, *F* cheese after the drying was finished (8 days), *M* mature cheese (10 days), *S* end of minimum shelf-life (42 days), *E* expiration date (66 days). One-way analysis of the variance ratio test, n=45 (F)–108 (S). Different letters (*A–D*) within a given amine differ at P<0.05



3.5 Effect of season

As can be seen in Fig. 5, the trend of the differences in BA/PA content between cheeses produced in different seasons of the year was ambiguous. As far as the quantitatively most important amines are concerned, both tyramine and cadaverine content tended to be lower (P=0.065 and P=0.082, respectively) in the summer in comparison to the winter cheeses. On the other hand, the content of putrescine was higher (P<0.05) in cheeses produced in summer than in winter.

Moreover, both tyramine and histamine contents were unequivocally higher (P< 0.05) in the spring curd cheeses as compared to the summer ones.

The above ambiguity was complemented by the fact that differences in the content of the sum of BAs (1,704 vs. 1,475 vs. 1,391 mg.kg^{-1}; P>0.05) nor of the sum of all amines (1,986 vs. 1,936 vs. 1,788 mg.kg^{-1}; P>0.05) were established between cheeses produced in winter, spring, and summer.

3.6 Counts of the tested microorganisms

The counts of LAB and enterococci in cheeses at the end of the minimum shelf-life and at the end of the expiration date after storage at 5 and 20 °C, respectively, are shown in Table 2.

When the data from Table 2 were averaged for total sets of cheeses stored at 5 and 20 °C, respectively (irrespective of other variability factors), both LAB counts (8.46 vs. 7.80 log cfu.g^{-1}) and counts of enterococci (6.33 vs. 6.02 log cfu.g^{-1}) were significantly higher (P<0.05) in cheeses stored at 5 °C. As far as an effect of the season is concerned, counts of LAB tended to be lower (8.11 vs. 8.21 log cfu g^{-1}; P= 0.11) and enterococci counts were significantly lower (5.83 vs. 6.49 log cfu.g^{-1}; P< 0.05) in the summer cheeses than in the winter samples.

No significant relationship of any amine to the LAB counts was found in the present experiment (P>0.05). Contrary to LAB as a whole group, counts of enterococci (part of the LAB) correlated positively (P<0.01) with the content of most amines, with the exception of histamine, spermidine, and spermine. Based on the

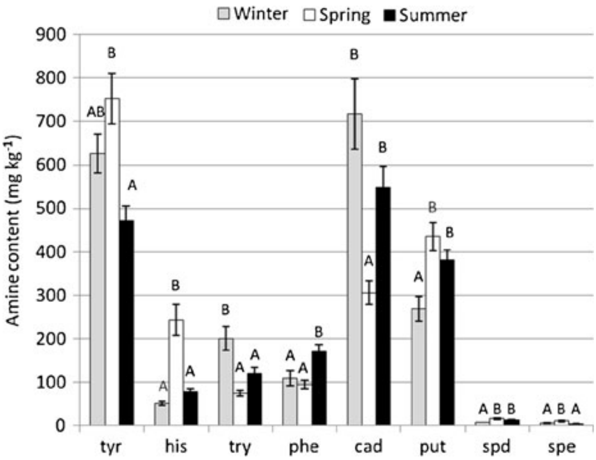


Fig. 5 Biogenic amine/polyamine content in curd cheeses produced in the winter, spring, and summer season, respectively. *tyr* tyramine, *his* histamine, *try* tryptamine, *phe* 2-phenylethylamine, *cad* cadaverine, *put* putrescine, *spd* spermidine, *spe* spermine. One-way analysis of the variance ratio test, n=229 (winter), 268 (spring), 198 (summer). Different letters (*A and B*) within a given amine differ at P<0.05

Table 2 Counts of lactic acid bacteria (LAB) and enterococci in cheeses at the end of the minimum shelf-life (42 days) and at the end of the expiration date (66 days) after storage at 5 and 20 °C, respectively

Season	Cheese shape	T^a (°C)	Counts of microorganisms (mean±SEM) (log cfu.g^{-1})			
			Minimum shelf-life (42 days)		Expiration date (66 days)	
			LAB	*Enterococcus* spp.	LAB	*Enterococcus* spp.
Winter	Disc	5	7.78^{B}±0.10	7.04^{F}±0.09	8.24^{DEF}±0.05	6.43^{D}±0.07
		20	7.83^{B}±0.12	5.78^{BC}±0.11	7.59^{C}±0.07	6.39^{D}±0.02
	Bar	5	8.90^{D}±0.09	7.43^{G}±0.10	8.66^{G}±0.13	7.86^{F}±0.10
		20	8.97^{D}±0.09	7.03^{FG}±0.05	8.07^{DE}±0.12	7.39^{E}±0.06
Spring	Disc	5	7.80^{B}±0.07	5.97^{BCD}±0.06	7.91^{CD}±0.06	5.17^{B}±0.13
		20	7.72^{AB}±0.04	6.31^{DE}±0.04	7.20^{B}±0.04	5.66^{C}±0.11
	Bar	5	7.86^{B}±0.02	5.50^{A}±0.09	8.01^{DE}±0.04	4.90^{B}±0.06
		20	7.38^{A}±0.04	5.65^{AB}±0.07	6.94^{AB}±0.06	4.32^{A}±0.05
Summer	Disc	5	8.38^{C}±0.03	6.26^{DE}±0.05	8.30^{EF}±0.05	6.15^{D}±0.10
		20	7.56^{AB}±0.05	6.10^{CD}±0.08	6.82^{A}±0.10	5.23^{B}±0.13
	Bar	5	8.71^{CD}±0.10	6.66^{EF}±0.07	8.57^{FG}±0.04	6.43^{D}±0.03
		20	7.73^{AB}±0.07	6.58^{E}±0.05	7.95^{CDE}±0.10	6.16^{D}±0.10

A–G means marked with different letters in columns differ at $P<0.05$; one-way analysis of the variance-ratio test with the post hoc Duncan's test; $n=16$

[a] Storage temperature

values of the coefficient of correlation, counts of *Enterococcus* spp. explained approximately one third of variability in the content of tyramine, tryptamine, and cadaverine, and one quarter of variability in the content of 2-phenylethylamine and putrescine, respectively.

4 Discussion

4.1 The comparison of effects of the main variability factors on amines content

Similarly to our results (storage temperature accounted in average for 46 % of explained variability in BA/PA content), Pinho et al. (2001), Standarová et al. (2010), and Buňková et al. (2010) respectively identified storage temperature as an important factor influencing BA content in cheeses.

Shape (disc or bar) explained 26 % of variability in amines content in the present study, in agreement with the data of Petridis and Steinhart (1996), who reported a significant effect of shape on tyramine, putrescine, and cadaverine content in the Swiss-type cheese.

In the case of spermidine and spermine, season and shape and not temperature were the decisive external factors affecting the content of these polyamines in cheeses in the present study (84 and 81 % of the explained variability). This was mainly due to the fact that spermidine and spermine content did not significantly change during ripening and storage, irrespective of the storage temperature. These PAs are predominantly synthetized in a mammalian cell and enter a product directly from the raw materials (Novella-Rodrígues et al. 2003).

4.2 The effect of the storage temperature, including other physical characteristics (pH, NaCl)

The finding that substantially higher ($P<0.05$) amounts of tyramine, tryptamine, 2-phenylethylamine, cadaverine, and putrescine, respectively, were detected in cheeses stored at 20 °C as compared to 5 °C (Fig. 1) was expected, considering the general suggestion that the temperature control is the most efficient way to prevent BA formation (Stratton et al. 1991; Linares et al. 2011). Similar results have also been reported by Standarová et al. (2010) in the same type of cheese as in the present study. However, the authors (Standarová et al. 2010) also found higher spermidine and spermine contents in the curd cheeses stored at 20 °C in comparison to 5 °C, which was not the case in the present study.

As far as the relationship between the pH values and amines contents in cheeses are concerned, pH of the medium is one of the key factors involved in BA production (Linares et al. 2011). However, since formation of biogenic amines can increase the pH of the medium (Fernández et al. 2007), the positive correlations between pH and amines content found in the present study could mean that the higher pH values were the consequence and not the cause of the BAs synthesis.

Despite the fact that NaCl concentration in the cheeses changed markedly between days 0 and 66 in the present study, it was not considered a variability factor for BAs formation because all raw materials (curds) were salted using the same initial concentration. It can only be stated that the literature regarding the effect of NaCl on BA production are contradictory. NaCl addition to milk can prevent BA formation according to Linares et al. (2011). Similarly, Santos et al. (2003) reported that NaCl decreased spermidine, putrescine, histamine, and tyramine levels in milk treated with a culture containing *Lactococcus lactis* ssp. *cremoris* and *L. lactis* ssp. *lactis*. On the other hand, two strains of *L. lactis* ssp. *lactis* produced tyramine only in broth with the highest NaCl concentration in an experiment of Buňková et al. (2011). Aliakbarlu et al. (2011) suggested a positive effect of a low, but a negative effect of a high salt concentration on BA formation.

4.3 Cheese shape

An effect of cheese shape on the BA/PA content has scarcely been mentioned in the available literature. The only relevant data were reported by Petridis and Steinhart (1996), who found substantially higher histamine, tyramine, putrescine, and cadaverine content, respectively, in the round-loaf Swiss-type cheeses than in the square-block counterparts. These findings are in agreement with the results of the present study as far as histamine and tyramine are concerned.

These authors (Petridis and Steinhart 1996) did not mention any possible reason for the above differences. The physical traits measured in the present study have not provided an explanation either: mean DM content, NaCl content, and pH values were 37.4 and 37.2 % ($P<0.05$), 5.2 and 5.0 % ($P>0.05$), and 6.99 and 6.70 ($P<0.05$) in the discs and bars, respectively. Despite the significant differences in the case of DM and pH, the absolute values were so similar that no effect on the counts of the amine-forming microorganisms can be presumed. Consequently, neither counts of LAB (7.86 vs. 8.17 log cfu.g^{-1} in discs and bars, respectively; $P<0.05$) nor the enterococci counts (5.82 vs. 5.91 log cfu.g^{-1}; $P>0.05$) were able to explain the shape-associated differences in amine contents in the present study.

4.4 Effect of the cheese age

4.4.1 Time dependence of amine contents in cheeses

The rapid increase in the cadaverine content in the curd cheeses in the present study (Fig. 3) can be compared to the data of Standarová et al. (2010), who reported an increase in the rate of cadaverine accumulation of 12 and 55 mg.kg^{-1}.day^{-1} in smear-ripened curd cheeses stored at 5 and 20 °C. High cadaverine content in a fermented food product usually suggests low hygienic standards in treating the raw materials and during manufacture (Latorre-Moratalla et al. 2008).

As far as tyramine is concerned, the rate of increase in the curd cheeses in the present study (13.5 mg.kg^{-1}.day^{-1}; Fig. 3) was in agreement with the data of Standarová et al. (2010) for the same type of cheese: 3 and 22 mg.kg^{-1}.day^{-1} in cheeses stored at 5 and 20 °C. For a comparison, tyramine content increased more rapidly in curd cheeses evaluated in the present study than in the Dutch-type cheese (0.7 mg.kg^{-1}.day^{-1}; Komprda et al. 2008a), brined Feta cheese (2 mg.kg^{-1}.day^{-1}; Valsamaki et al. 2000) or a semihard, salty, and herb-added Turkish cheese Otlu (0.8 mg.kg^{-1}.day^{-1}; Ekici et al. 2010). On the other hand, the increase in the rate of tyramine accumulation in the smear-ripened curd cheeses is comparable to a blue-vein cheese (12 mg.kg^{-1}.day^{-1}; Komprda et al. 2008b).

4.4.2 Comparison of the particular production phases

The level of tyramine detected in the present study in the mature cheeses (Fig. 4) was 30 % higher than the level reported by Standarová et al. (2010) for the same type of cheese at the corresponding age. On the other hand, cadaverine content, an indicator of low hygienic standards during cheese manufacture (Latorre-Moratalla et al. 2008), was more than four times lower in the present study in comparison to the corresponding samples reported by Standarová et al. (2010), suggesting higher hygienic standards of the curd cheese production in the present study.

When taking into account the more conservative (lower) value of tyramine levels tolerated by high risky consumers (50–100 mg according to Latorre-Moratalla et al. 2008), a 100-g portion of the curd cheese at the end of the expiration date in the present study (more than 90 mg of tyramine; Fig. 4) would be considered unsafe, especially for patients treated with the MAOI drugs, even with a new generation of these drugs.

Putrescine contents found in the present study in the M- and E-cheeses, respectively (Fig. 4) would be safe, including even the latter value, regarding the no-observed-adverse-effect-level for putrescine (180 mg.kg^{-1} body weight.day^{-1}) suggested by Til et al. (1997).

The values of the sum total of the three polyamines in the M and E samples that were established in the present study at the mean level of 139 and 446 mg.kg^{-1}, respectively, are still not possible to evaluate from the viewpoint of either a recommended daily intake (growth and development of the digestive system; wound healing) or a limit whose exceeding would have deleterious effects in cancer patients (Komprda et al. 2008a).

4.5 Effect of season

The only data regarding the seasonal variation in BA formation in cheeses found in the available literature was reported by Gaya et al. (2005) for ewes' milk. The authors found that the highest levels of histamine, tryptamine, and tyramine in cheeses were produced in spring, winter, and spring, respectively. These results are in agreement with the data shown in Fig. 5 of the present study.

4.6 Counts of the tested microorganisms

As far as the counts of microorganisms in the curd cheeses are concerned, the two main issues for a discussion follow from the results of the present study: firstly, higher counts of LAB, including enterococci in the majority of samples stored at the refrigerated temperature as compared to the room temperature, and secondly, a lack of correlation between BAs content and LAB counts (with a positive relationship between BAs content and enterococci counts at the same time).

At 5 °C, which is far from the optimal growth conditions for LAB (including enterococci), these bacteria might be in a dormant state where they neither grew nor died: the mean LAB and enterococci counts did not change significantly ($P>0.05$) between day 10 (end of ripening; 8.34 and 6.11 log cfu.g^{-1}, respectively) and day 66 (the expiration date; 8.26 and 6.14 log cfu.g^{-1}, respectively). On the other hand, at 20 °C, LAB (including enterococci) could be metabolically active during the storage period but were no more recovered under the conditions used, especially if they have already reached the stationary phase during the ripening period: the mean LAB and enterococci counts after 66 days were 7.41 and 5.85 log cfu.g^{-1}, respectively.

Despite this, the results of the present study are contrary to the only available data directly comparing the growth of LAB in cheeses stored at refrigerated and ambient room temperature, respectively: Perveen et al. (2011) reported a higher increase in LAB counts in cream cheese stored at 21 °C as compared to 4 °C; however, the storage period was much shorter in their study (28 days) than in the present study (42 and 66 days).

The fact that no significant relationship of any amine to the LAB counts was found in the present study is in full agreement with the data of Yildiz et al. (2010), who reported identical results in the Turkish acid curd cheese Civil. Microbiological analysis did not provide any conclusive data regarding differences in BA content in Dutch-type hard cheese in a study of Komprda et al. (2007). On the other hand, Pintado et al. (2008) found a significant correlation between lactococci counts and cadaverine and tyramine content in the Portuguese Terrincho cheese. Ladero et al. (2008), using real-time quantitative PCR for quantification of histamine-producing LAB, reported a good relationship between the cycle threshold value (C_t; the number of cycles at which the signal exceeds a threshold for detection of DNA-based fluorescence when plotting fluorescence against the number of cycles on a logarithmic scale) and content of histamine in randomly purchased commercial cheeses.

Komprda et al. (2008a) tried to explain an often-missing straight correlation between BA content and counts of the cheese-associated LAB by a possibility that BA decarboxylase activity is not an ability of bacterial genera or species, but a function of particular strains within a given species. From the factors tested in the present study, a discrepancy between the amines content and LAB counts was probably affected to a large degree by the storage temperature (see above); therefore, the LAB strains possessing decarboxylase activities, which were likely in a minority among all the LAB strains, could be inhibited more at 5 °C and manifested themselves only at the higher temperature.

However, a conventional microbiological analysis using selective media was not able to test the above hypothesis; a detailed analysis of the amine-producing bacterial strains using quantitative PCR (Ladero et al., 2008) was not an objective of the present study.

The positive correlations ($P<0.01$) of the enterococcal counts with the contents of the majority of amines in the present study correspond to the fact that enterococci are considered to be established producers of biogenic amines, especially tyramine (Linares et al. 2009). Ladero et al. (2012) recently suggested that tyramine and putrescine biosynthesis is a species-level characteristic in enterococci. Tyrosine-decarboxylase-positive enterococci seemed to be responsible for the high concentration of tyramine also in a blue-vein cheese in an experiment of Ladero et al. (2010).

However, Rea et al. (2004), who investigated the effect of six strains of enterococci on tyramine production in cheddar cheese, did not find any relationship between the ability of the strains to produce tyramine in broth and in cheese nor an explanation for this result.

5 Conclusions

Storage temperature and the cheese age accounted for a decisive part of the explained variability in BA/PA content in the curd cheeses.

No significant relationship of any amine to the LAB counts was found in the present study; on the other hand, counts of *Enterococcus* spp. explained approximately one third of variability in the content of tyramine, tryptamine, and cadaverine and one quarter of variability in the content of 2-phenylethylamine and putrescine, respectively.

The content of tyramine, cadaverine, and putrescine (quantitatively the most important amines in the present study) increased during production/storage by 13.5, 14.6, and 9.7 mg.kg^{-1}.day^{-1}. Histamine content did not change significantly during the whole study.

Tyramine content in the mature cheeses (11 days old) was on average bellow 380 mg.kg^{-1}. Its content at the end of the expiration date exceeded on average 900 mg.kg^{-1}; a 100-g portion of such a cheese would be considered unsafe, especially for patients treated with the MAOI drugs.

The sum of all amines (sum of BAs+PAs) did not differ between cheeses produced in winter, spring, and summer. However, amines content was on average 25 % higher in the disc-shaped cheeses as compared to the bars. Neither differences in the counts of LAB and enterococci nor the variations in dry matter content, NaCl content, and pH, respectively, were able to explain the differences in amines content due to the shape of the cheese.

The content of tyramine, cadaverine, and putrescine in the cheeses stored at 20 °C was on average nearly four, 11, and more than three times higher, respectively in comparison with the cheeses stored at 5 °C.

High-risky consumers (intolerant people, patients consuming MAO inhibitors) are recommended to store this type of cheese at refrigerated temperature, no longer than 2 weeks and to favor the bar-shaped products.

Acknowledgment The experiment was in part financially supported by the Internal Grant Agency of Mendel University in Brno, project no. TP 10/2010. The authors are also very grateful to dipl. ing. Zdeněk Pospíšil for providing the cheese samples and technical assistance.

References

Aliakbarlu J, Alizadeh M, Razavi-Rohani SM, Agh N (2011) Biogenic amines in Iranian white brine cheese: modelling and optimisation of processing factors. Int J Dairy Technol 64:417–424

Andic S, Tuncturk Y, Gencellep H (2011) The effect of different packaging methods on the formation of biogenic amines and organic acids in Kashar cheese. J Dairy Sci 94:1668–1678

Buňková L, Buňka F, Mantlová G, Čablová A, Sedláček I, Švec P, Pachlová V, Kráčmar S (2010) The effect of ripening and storage conditions on the distribution of tyramine, putrescine and cadaverine in Edam-cheese. Food Microbiol 27:880–888

Buňková L, Buňka F, Pollaková E, Podešvová T, Dráb V (2011) The effect of lactose, NaCl and an aero/anaerobic environment on the tyrosine decarboxylase activity of *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis*. Int J Food Microbiol 147:112–119

Coton M, Delbes-Paus C, Irlinger F, Desmasures N, Le Fleche A, Stahl V, Montel MC, Coton E (2012) Diversity and assessment of potential risk factors of Gram-negative isolates associated with French cheeses. Food Microbiol 29:88–98

EC (2005) Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbial criteria for foodstuffs. Offic J E U L338:1–26

Eerola S, Roig-Sagués A-X, Lilleberg L, Aalto H (1997) Biogenic amines in dry sausages during shelf-life storage. Z Lebensmitt Untersuch Forsch A 205:351–355

Ekici K, Tarakci Z, Alemdar S, Alisarli M (2010) Influence of starter cultures on the accumulation of histamine and tyramine in vacuum-packaged Otlu (Herby) cheese during ripening. Asian J Chem 22:6133–6139

Fernández M, Linares DM, Rodríguez A, Alvarez MA (2007) Factors affecting tyramine production in *Enterococcus durans* IPLA 655. Appl Microbiol Biotechnol 73:1400–1406

Gaya P, Sánches C, Nuñez M, Fernández-García E (2005) Proteolysis during ripening of Manchego cheese made from raw or pasteurized ewes' milk. Seasonal variation. J Dairy Res 72:287–295

Innocente N, D'Agostin P (2002) Formation of biogenic amines in a typical semihard Italian cheese. J Food Prot 65:1498–1501

Komprda T, Dohnal V (2009) Amines. In: Toldrá F, Nollet L (eds) Handbook of dairy food analysis. Taylor & Francis, Boca Raton

Komprda T, Smělá D, Novická K, Kalhotka L, Šustová K, Pechová P (2007) Content and distribution of biogenic amines in Dutch-type hard cheese. Food Chem 102:129–137

Komprda T, Burdychová R, Dohnal V, Cwiková O, Sládková P (2008a) Some factors influencing biogenic amines and polyamines content in Dutch-type semi-hard cheese. Eur Food Res Technol 227:29–36

Komprda T, Dohnal V, Závodníková R (2008b) Contents of some biologically active amines in a Czech blue-vein cheese. Czech J Food Sci 26:428–440

Ladero V, Linares DM, Fernández M, Alvarez M (2008) Real time quantitative PCR detection of histamine-producing lactic acid bacteria in cheese: relation with histamine content. Food Res Int 41:1015–1019

Ladero V, Fernández M, Cuesta I, Álvarez MA (2010) Quantitative detection and identification of tyramine-producing enterococci and lactobacilli in cheese by multiplex qPCR. Food Microbiol 27:933–939

Ladero V, Fernández M, Calles-Enríquez M, Sánchez-Llana E, Cañedo E, Cruz Martín M, Alvarez M (2012) Is the production of the biogenic amines tyramine and putrescine a species-level trait in enterococci? Food Microbiol 30:132–138

Latorre-Moratalla ML, Veciana-Nogués T, Bover-Cid S, Garriga M, Aymerich T, Zanardi E, Ianieri A, Fraqueza MJ, Patarata L, Drosinos EH, Lauková A, Talon R, Vidal-Carou MC (2008) Biogenic amines in traditional fermented sausages produced in selected European countries. Food Chem 107:912–921

Leuschner RGK, Kurihara R, Hammes WP (1998) Effect of enhanced proteolysis on formation of biogenic amines by lactobacilli during Gouda cheese ripening. Int J Food Microbiol 44:15–20

Linares DM, Fernández M, Cruz Martín M, Álvarez MA (2009) Tyramine biosynthesis in *Enterococcus durans* is transcriptionally regulated by the extracellular pH and tyrosine concentration. Microbial Biotech 2:625–633

Linares DM, Cruz Martín M, Ladero V, Álvarez MA, Fernández M (2011) Biogenic amines in dairy products. Crit Rev Food Sci Nutr 51:691–703

McCabe-Sellers BJ, Staggs CG, Bogle ML (2006) Tyramine in foods and monoamine oxidase inhibitor drugs: a crossroad where medicine, nutrition, pharmacy, and food industry converge. J Food Comp Anal 19:S58–S65

Novella-Rodrígues S, Veciana-Nogués MT, Izquerdo-Pulido M, Vidal-Carou MC (2003) Distribution of biogenic amines and polyamines in cheese. J Food Sci 68:750–755

Perveen K, Alabdulkarim B, Arzoo S (2011) Effect of temperature on shelf life, chemical and microbial properties of cream cheese. Afr J Biotechnol 10:16929–16936

Petridis KD, Steinhart H (1996) Biogene Amine in der Hartkäseproduktion: I. Einfluß verschiedener Parameter auf den Amingehalt im Endprodukt am Beispiel von Emmentaler Käse. Deut Lebensm Rdsch 92:114–120

Pinho O, Ferreira IMPLVO, Mendes E, Oliveira BM, Ferreira M (2001) Effect of temperature on evolution of free amino acid and biogenic amine contents during storage of Azeitão cheese. Food Chem 75:287–291

Pintado AIE, Pinho O, Ferreira IMPLVO, Pintado MME, Gomes AMP, Malcata FX (2008) Microbiological, biochemical and biogenic amine profiles of Terrincho cheese manufactured in several dairy farms. Int Dairy J 18:631–640

Rabie MA, Siliha HI, El-Saidy SM, El-Badawy AA, Malcata FX (2011) Effect of gamma-irradiation upon biogenic amine formation in blue cheese during storage. Int Dairy J 21:373–376

Rea MC, Franz CMAP, Holzapfel WH, Cogan TM (2004) Development of enterococci and production of tyramine during the manufacture and ripening of Cheddar cheese. Irish J Agric Food Res 43:147–258

Santos WC, Souza MR, Cerqueira MMOP, Gloria MBA (2003) Bioactive amines formation in milk by Lactococcus in the presence or not of rennet and NaCl at 20 and 32 °C. Food Chem 81:595–606

Schirone M, Tofalo R, Mazzone G, Corsetti A, Suzzi G (2011) Biogenic amine content and microbiological profile of Pecorino di Farindola cheese. Food Microbiol 28:128–136

Standarová E, Vorlová L, Kordiovská P, Janštová B, Dračková M, Borkovcová I (2010) Biogenic amine production in Olomouc curd cheese (Olomoucké tvarůžky) at various storage conditions. Acta Vet Brno 79:147–156

Stratton JE, Hutkins RV, Taylor SL (1991) Biogenic amines in cheese and other fermented foods. A review. J Food Prot 54:460–470

Til HP, Falke HE, Prinsen MK, Willems MI (1997) Acute and subacute toxicity of tyramine, spermidine, putrescine and cadaverine in rats. Food Chem Toxicol 35:337–348

Valsamaki K, Michaelidou A, Polychroniadou A (2000) Biogenic amine production in Feta cheese. Food Chem 71:259–266

Walter F, Ulrich S, Stein J (2004) Molecular mechanisms of the chemopreventive effects of resveratrol and its analogs in colorectal cancer: key role of polyamines? J Nutr 134:3219–3222

Yildiz F, Yetisemiyen A, Senel E, Durlu-Özkaya F, Öztekin S, Sanli E (2010) Some properties of Civil cheese: a type of traditional Turkish cheese. Int J Dairy Technol 63:575–580