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# Content and distribution of biogenic amines in Dutch-type hard cheese

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**Abstract**

Content of biogenic amines (BA; histamine, tyramine, tryptamine, putrescine, 2-phenylethylamine, cadaverine, spermidine, spermine) and counts of microorganisms (total aerobic and facultative anaerobic mesophilic microorganisms, TAC; lactic acid bacteria, LAB; coliforms; total anaerobic microorganisms, TAN; yeasts + moulds), were evaluated within the ripening interval of 1–6 months in Dutch-type hard cheese, produced by two different producers (the cheeses designated as R30 and R45) using pasteurized milk and different starter cultures (designated as R30-FD and R30-CH in the case of the R30 cheeses). The cheeses from the two producers differed in their fat content (30% and 45% of fat in dry matter, respectively).

Substantially higher ($P < 0.01$) contents of tyramine and the sum of biogenic amines were found in the outer-part samples in comparison with the core ones in the R30 cheeses, which corresponded with higher ($P < 0.01$) TAN counts in this part of the cheese with presumably more favourable growth conditions.

Tyramine content increased linearly ($P < 0.01$) with increasing time of ripening both in R30-FD and R30-CH and R45 cheese, and concentration of this BA in the R45 cheese after 18 weeks of ripening (309.5 mg $kg^{-1}$) exceeded a toxicological limit. Also putrescine content and sum of all BA increased significantly ($P < 0.001$) in all cheeses within the ripening interval.

Tyramine, putrescine and phenylethylamine contents, and sum of all biogenic amines and sum of polyamines in R45 cheese (299.8, 60.8, 54.3, 419.0 and 63.2 mg $kg^{-1}$ of the fresh matter, respectively) were significantly higher ($P < 0.01$) after 22nd week of ripening in comparison with both R30 cheeses (15.7–41.2, 6.0–6.7, 1.0–2.0, 36.4–68.3 and 7.1–7.5 mg $kg^{-1}$, respectively). However, TAC, TAN and LAB counts tended to be higher ($P > 0.05$) in R30-FD and R30-CH samples than in R45 cheeses in this time interval. The microbiological analysis based only on basic groups of microorganisms was inconclusive regarding differences in BA content in the cheeses.


*Keywords:* Tyramine; Decarboxylase activity; Starter culture; Ripening cheese

## 1. Introduction

Biogenic amines (BA) are the low-molecular organic bases formed mainly by decarboxylation of amino acids (Silla-Santos, 1996). After fish, cheese is the next most commonly implicated food item associated with BA poisoning (Stratton, Hutkins, & Taylor, 1991). However, this regards not cheese in general, but mainly ripening and/or long-term stored hard and semi-hard cheese, where the degradation of proteins during ripening leads to the accumulation of free amino acids, which can be converted into amines due to the activity of bacterial decarboxylases (Innocente & D'Agostin, 2002). This usually poses no problem in healthy people, but in allergenic individuals or those being administered monoamine oxidase (MAO) inhibitors (indirect sympathomimetic drugs), negative health effects after intake of relatively low levels of BA (e.g. 6 mg of tyramine; Silla-Santos, 1996) can be expected. According to Millichap and Yee (2003) cheese is a leading diet factor triggering pediatric and adolescent migraine. Apart from

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vasoactive amines such as tyramine (hypertension, headache) or histamine (hypotension, headache, flushing, abdominal cramps; Stratton et al., 1991), growing attention has been devoted to the polyamines spermidine and spermine, and diamines putrescine and cadaverine, due to their ability to form stable carcinogenic N-nitroso compounds and to enhance the growth of chemically induced aberrant crypt foci in the intestine (Paulsen, Reistad, Eliassen, Sjaastad, & Alexander, 1997).

Presence of the microorganisms with the decarboxylase activity is one of the main factors determining BA content in a cheese. Therefore BA content in cheese produced from raw unpasteurized milk was often studied (Fernández-García, Tomillo, & Nunez, 2000; Innocente & D'Agostin, 2002; Novella-Rodrígues, Veciana-Nogués, Izquerdo-Pulido, & Vidal-Carou, 2003; Pinho, Ferreira, Mendes, Oliveira, & Ferreira, 2001). Region-specific cheeses produced from sheep or goat milk were included in several studies (Novella-Rodrígues, Veciana-Nogués, Trujillo-Mesa, & Vidal-Carou, 2002; Novella-Rodrígues et al., 2003; Ordónez, Ibánez, Torre, & Barcina, 1997; Pinho et al., 2001; Valsamaki, Michaelidou, & Polychroniadou, 2000). As far as biogenic amines in a cheese from a cows' milk are concerned, many more studies with semi-soft cheese (Schneller, Good, & Jenny, 1997) or Swiss-type hard cheese (Petridis & Steinhart, 1996a; Petridis & Steinhart, 1996b) were carried out in comparison with a Dutch-type hard cheese.

Microorganisms with a decarboxylase activity can be starter microorganisms (Fernández-García et al., 2000), non-starter lactic acid bacteria (NSLAB) or other spontaneous microflora (Roig-Sagués, Molina, & Hernández-Herrero, 2002). However, it is difficult to find a straight correlation between microorganisms counts and BA content in cheese, because amine producing abilities of different strains of various bacteria differ widely (Halasz, Barath, Simon-Sarkadi, & Holzapfel, 1994; Innocente & D'Agostin, 2002; Valsamaki et al., 2000).

Biogenic amines are often distributed within the cheese unevenly, and the results of the experiments regarding BA distribution in the cheese are inconsistent. E.g. for tyramine, often quantitatively the most important BA in cheese, Petridis and Steinhart (1996a) found higher concentrations at the edge of the Swiss-type hard cheese in comparison with the inner part, completely contrary to the results of Novella-Rodrígues et al. (2003).

The objectives of the present study were threefold. Firstly, we evaluated the distribution of BA content in the inner and the edge part of a hard Dutch-type cheese produced from pasteurized milk ripening under the foil. Secondly, we measured the BA content within the ripening interval of 1–6 months in the above type of cheese produced from two different producers, each of them using a different starter culture, but both operating in high-ranking hygienic conditions. Finally, we determined total counts of microorganisms, lactic acid bacteria, coliforms, total anaerobic counts and yeasts and moulds in all above-mentioned samples of cheeses.

## 2. Materials and methods

### 2.1. Material

Biogenic amine content and counts of microorganisms were evaluated during ripening of Dutch-type hard cheeses produced from pasteurized milk. Cheeses from two different producers differed in the fat content (30% or 45% of fat in dry matter, DM, respectively) and were designated as R30 (producer 1) and R45 (producer 2). Actual dry matter contents and fat contents of the cheese samples were determined in our laboratory according to the Czech standards ČSN ISO 5534 and ČSN ISO 1735, respectively.

The experimental design was influenced by the production schedule of the respective producers. Two batches of R30 cheese and one batch of R45 cheese were produced using three respective microbial cultures. All cultures contained the same bacterial species (Table 1; the relative proportions of each species in a given starter culture were subject of a trade secret of the particular producers). All cultures were dosed in the same amount; 10 g of the freeze-dried culture was used for 500 L of a bulk starter, 25 L of the bulk starter was then added to 48,000 L of milk for the cheese production. Cheeses were pressed to the blocks of final weight of 12 kg and wrapped into the plastic foil. The blocks ripened under the foil at the temperature of 10 °C.

Three blocks within each batch of the cheese (Table 1) were randomly chosen, and from these blocks the samples were taken after 4, 8, 18, 22 and 26 weeks of ripening (both batches of the R30 cheeses), and after 4, 8, 14, 18 and 22 weeks (R45 cheeses), respectively. Again, the differences in the sampling intervals between both producers followed from the above-mentioned different production schedules.

Table 1
Characteristics of the cheese samples used for the biogenic amine determinations and microbiological analyses

| Producer | Commercial producer 1 | | | | Commercial producer 2 |
|---|---|---|---|---|---|
| Fat[a] (%) | 15.8 | | 16.5 | | 26.0 |
| Dry matter[b] (%) | 51.5 | | 50.8 | | 57.3 |
| Fat in DM (%) | 30.7 | | 32.5 | | 45.4 |
| Microbial culture[c] | Flora Danica Normal[d] | | CH-N-11[d] | | Probat 505[e] |
| Batch | R30-FD | | R30-CH | | R45 |
| Ripening temperature (°C) | 10 | | 10 | | 10 |
| Part of the cheese | Core | Edge | Core | Edge | Core |

[a] Determined in our laboratory according to the Czech standard ČSN ISO 1735.
[b] Determined in our laboratory according to the Czech standard ČSN ISO 5534.
[c] Each starter culture contained the same bacterial species: *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *diacetilactis*, *Leuconostoc mesenteroides* subsp. *cremoris*; the cultures differed in relative content of the species.
[d] Chr. Hansen (Nienburg/Weser, Germany).
[e] Danisco (Copenhagen, Denmark).

When taking the sample, the particular block was cross-cut and a 200 g inner (core) part and a 200 g outer (adjacent to the foil) part of a slice was taken in the case of R30 cheese. In the case of R45 samples, it was a producers' demand to take only a 200 g core part of the slice. Subsamples for microbiological, chemical (biogenic amines) and other (dry matter, total solids, fat, NaCl) analyses were taken from the same part of a slice each time.

The sample weighing 100 g was homogenized in a Moulinex blender (Moulinex, Groupe SEB Czech Republic, Prague) and stored in the dark-glass powder bottles at −18 °C until the biogenic amine analyses. Microbiological analysis was performed immediately after refrigerated transportation of the sample from the producer to the laboratory.

It is necessary to underline that the experiment was carried out in operational conditions and the producers agreed to perform it only once.

### 2.2. Biogenic amine analysis

The sample was thawed overnight, 10 g (±1 mg) was weighed into the 85 mL test tube, 0.5 mL of an internal standard (1,7-diaminoheptane; concentration 1 mg $mL^{-1}$) was added and the sample was extracted for 2 min with 20 mL of 0.1 M hydrochloric acid (HCl) using a disintegrator Heidolph Diax 900 (Heidolph Instruments GmbH, Kelheim, Germany). Suspension was centrifuged at 755*g* 10 min at 4 °C (Hettich Universal 32R; Hettich International, Kirchlegen, Germany). The supernatant was filtered through paper filter and the solid residue was extracted for the 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). In order to compare the extraction efficiency, an aliquot (10 g) of the same cheese sample with the same internal standard was extracted for 2 min with 15 mL of 5% trichloracetic acid by the same procedure as mentioned above.

An aliquot of either extract was derivatized by dansyl chloride (5-dimethylaminonaphthalene-1-sulfonyl chloride, DCl). The derivatizing agent was prepared by dissolving of 5 mg of dansyl chloride in 1 mL of 2-propanone (Sigma–Aldrich, St. Louis, USA). The derivatization proceeded as follows: 1 mL of an extract (or standard) was mixed with 0.5 mL of saturated $Na_2CO_3$ (pH adjusted to 11.2), 1 mL of the derivatizing agent was added and the mixture was shaken for 1 min (MS2 Minishaker IKA; IKA Werke GmbH, Staufen, Germany). Derivatization proceeded for 1 h in dark at 40 °C, and the amine derivatives were extracted by diethylether (3 × 1 mL). The organic phase was evaporated to dryness under the nitrogen and the solid residue was dissolved in 0.5 mL of acetonitrile (ACN). The solution was filtered through the nylon membrane filter 0.45 μm and injected onto the chromatographic column.

Biogenic amines were separated using a liquid chromatograph HP 1100 (Agilent Technologies, Wilmington, USA) consisting of a quarternary pump (G1311A), vacuum degasser (G1322A), automatic sampler (G1313A), UV/Vis detector with the variable wave-length (G1314A) and a fluorescence detector (G1321A). Separation after DCl derivatization was carried out by gradient elution with $H_2O$/ACN (time 0–23 min: $H_2O$ 35–0%, ACN 65–100%) on the Zorbax Eclipse XDB C18 column (150 mm × 4.6 mm, particle size 5 μm) with the guard column Meta Guard ODS-2 (30 mm × 4.6 mm, particle size 5 μm) at the flow rate 0.8 mL $min^{-1}$ using photometric UV/Vis detector at 254 nm.

Because of the several steps of the sample preparation, concentration of biogenic amines in the sample ($c_x$, in mg $kg^{-1}$) was corrected based on the method of internal standard, Eq. (1), as follows:

$$c_x = c_{IS} \times A_x / A_{IS} \times RF_x, \quad (1)$$

where $c_{IS}$ is the concentration of an internal standard (in mg $kg^{-1}$), $A_{IS}$ is the peak area of the internal standard (in area units), $A_x$ is the peak area of the biogenic amine (in area units) and $RF_x$, Eq. (2), is a response factor of the amine:

$$RF_x = c_{xr}/c_{ISr} \times A_{ISr}/A_{xr}, \quad (2)$$

where $c_{xr}$ is concentration of biogenic amine in the reference sample (mg $kg^{-1}$), $c_{ISr}$ is the concentration of an internal standard added to the reference sample (mg $kg^{-1}$), $A_{ISr}$ is the peak area of the internal standard in the reference sample (in area units) and $A_{xr}$ is the peak area of the biogenic amine in the reference sample (in area units).

The repeatability of the analytical process (expressed as a relative standard deviation, RSD) was determined by injecting a mixture of the biogenic amine standards after derivatization 10 times and injecting five extracts of the selected cheese sample with a low BA content (cheese R30 taken 4th week of ripening) after derivatization, respectively.

Recoveries were evaluated using repeatedly (five times) a real cheese sample with added mixture of BA standards with the concentration level of 2 mg $kg^{-1}$. Recovery ($R$) was calculated, Eq. (3), as:

$$\% R = [(CF - CU)/CA] \times 100, \quad (3)$$

where CU is concentration in the original sample, CA concentration of the added analyte and CF is concentration in the spiked sample (all concentrations in mg $kg^{-1}$). Concentrations of the particular amines in the real samples were not corrected for a respective recovery.

The separated amines (histamine, tyramine, tryptamine, putrescine, 2-phenylethylamine, cadaverine, spermidine, spermine) were identified by comparison of the retention times of the particular biogenic amine standards (all the amine standards were used as the respective hydrochlorides; supplied by Sigma–Aldrich), and their concentrations after DCl derivatization were expressed in mg $kg^{-1}$ of original (fresh) cheese.

### 2.3. Microbiological determination

Twenty grams of the cheese were taken aseptically from the slice (same part in each respective sampling), homogenized in the stomacher with 180 mL of distilled water, and serial decimal dilutions were prepared. The following groups of microorganisms were determined: total counts of aerobic and facultative anaerobic mesophilic microorganisms (TAC) on Plate Count Agar (PCA; Biokar Diagnostics, La Chapelle sue Erdre, France) after 72 h at 30 °C; lactic acid bacteria (LAB) on De Man-Rogosa-Sharpe medium (MRS; Biokar) after 72 h at 37 °C; coliforms on Violet Red Bile Agar (Biokar) after 72 h at 37 °C; total anaerobic counts (TAN) on PCA (Biokar) after 120 h at 30 °C (1 mL of the sample was overlaid in the test tube by the 10 cm agar column); yeasts + moulds on glucose–yeast extract-chloramphenicol agar (Noack & Co GmbH, Vienna, Austria) after 120 h at 25 °C.

### 2.4. Statistical evaluation

All microbiological and chemical traits were measured in duplicate in each part (core, edge, where applicable) of each block within each batch of the cheese. Means of these two measurements, representing a particular part of the block, were used in statistical evaluation.

The Unistat package, version 4.53 (Unistat Ltd., London, England) was used for calculation of the basic statistical characteristics, regressions (including significance testing of the linear and quadratic terms, respectively), differences between the core and the edge part of the cheese, and between cheeses from the two different producers, respectively, in biogenic amine content and microbial counts, and for calculation of the correlations between particular traits. As far as the differences between cheeses from two different producers are concerned, due to the dependence on the production schedule of the respective producers, it was not possible within the experimental design to assess which percent of total variability in BA content has been accounted for by the individual variability factors: starter culture, fat content, general hygienic conditions (these independent variables were not controllable and changed in the respective factorial designs). Therefore only one-way classification of the variance-ratio test (including Duncan's multiple range test) was used. On the other hand, percentage of total variability explained by an effect of starter culture, part of the cheese and time of ripening, respectively, was calculated for biogenic amine content and counts of microorganisms in R30 cheeses (producer 1) using multivariate analysis.

## 3. Results and discussion

### 3.1. Analytical method

The means of compositional values and other characteristics of the cheese samples are presented in Table 1.

The data characterizing repeatability of the chromatographic system (instrument), repeatability of the whole analytical process (method) and recovery of the biogenic amines are presented in Table 2. In full agreement with the data of Moret and Conte (1996) and Vale and Glória (1997), we received much better results regarding repeatability and recovery using HCl for BA extraction from the cheese as compared to trichloroacetic acid. Therefore, all presented results are based on HCl extraction of biogenic amines. Moret and Conte (1996) reported recovery of BA from Grana cheese at the range of 3% (putrescine)–79% (phenylethylamine), while Vale and Glória (1997) reported ranges of 71.4% (spermidine)–95.4% (histamine) from Parmesan cheese spiked with 4.0 mg biogenic amines per 100 g sample. Because we spiked the samples with BAs at the level of 2 mg $kg^{-1}$, our results are fully comparable with the data of Vale and Glória (1997) regarding the reliability of the analytical method.

Moret and Conte (1996) underline strict control of pH in the extraction phase in order to obtain reproducible recoveries. These authors reported optimum pH to be 11.5 (with a remark that pH 10, optimum value for tyramine recommended by other authors, is unsuitable for the other amines). Our results are somewhat different in this regard. We used pH 11.2, but this shift to the lower pH did not increase tyramine recovery. In fact, tyramine recovery was the lowest among all tested biogenic amines (Table 2); on the other hand, we found higher recoveries of the other amines at pH 11.2 in comparison with the data of Moret and Conte (1996) for this pH value.

### 3.2. Biogenic amine distribution within the cheese

The differences between the inner and the outer part were tested using the cheeses from both batches of producer 1 (R30-FD and R30-CH, respectively, see Table 1). Higher tyramine content in the outer-part in comparison with the core was found in the samples both after 4 weeks

Table 2
Repeatability (expressed as a relative standard deviation, RSD) of the determination, and recovery of the biogenic amines determined in the cheese samples

| Biogenic amine | Instrument[a] (RSD, %) | Method[b] (RSD, %) | Recovery[c] (%) |
|---|---|---|---|
| Tryptamine | 0.3 | 1.2 | 94.9 |
| Phenylethylamine | 0.4 | 6.8 | 90.3 |
| Histamine | 0.3 | 5.8 | 93.6 |
| Tyramine | 1.6 | 5.0 | 59.8 |
| Putrescine | 0.3 | 3.0 | 65.1 |
| Cadaverine | 0.3 | 2.9 | 74.6 |
| Spermidine | 0.3 | 1.8 | 63.3 |
| Spermine | 0.2 | 5.4 | 66.6 |

[a] Mixture of the biogenic amine standards after derivatization injected 10 times.
[b] Five extracts of the same cheese sample with a low BA content after derivatization was used.
[c] Real cheese sample spiked with the mixture of BA standards at the concentration level of 2 mg $kg^{-1}$, measured five times.

of ripening (0 vs. 1 mg $kg^{-1}$ and 1 vs. 4 mg $kg^{-1}$ in R30-FD and R30-CH cheese, respectively, $P > 0.05$), 8 weeks (3 vs. 13 mg $kg^{-1}$ and 11 vs. 50 mg $kg^{-1}$, $P < 0.01$), 18 weeks (32 vs. 95 mg $kg^{-1}$ and 35 vs. 115 mg $kg^{-1}$, $P < 0.01$), and 22 weeks of ripening (16 vs. 35 mg $kg^{-1}$ and 41 vs. 147 mg $kg^{-1}$, $P < 0.05$). Similar results were found regarding sum of total biogenic amines (9 vs. 11 mg $kg^{-1}$ and 14 vs. 28 mg $kg^{-1}$, $P < 0.05$, 4 weeks; 27 vs. 51 mg $kg^{-1}$ and 31 vs. 88 mg $kg^{-1}$, $P < 0.01$, 8 weeks; 61 vs. 139 mg $kg^{-1}$ and 50 vs. 169 mg $kg^{-1}$, $P < 0.05$, 18 weeks; 36 vs. 52 mg $kg^{-1}$ and 68 vs. 204 mg $kg^{-1}$, $P < 0.05$, 22 weeks of ripening for R30-FD and R30-CH cheese, respectively). Substantially higher ($P < 0.01$) content of tyramine and the sum of biogenic amines was found in the outer-part samples of both batches of the R30 cheese also at the end of ripening (26 weeks; Fig. 1).

Petridis and Steinhart (1996a) similarly reported an increase in histamine and tyramine content in the blocks of a Swiss-type cheese from the core outwards; the outermost segment differed significantly in this aspect from all other inner segments. However, the quoted authors used the cheese ripened under the rind and considered their results rather surprising due to the supposed (but not measured) lower water activity ($a_W$) in the edge in comparison with the inner parts. The authors explained their results by the good growth conditions for the aerobic and aerotolerant microorganisms (enterococci, coliforms) in the outer part of the cheese. On the other hand, in the present experiment, the cheese was ripened in the foil. We did not measure the water activity, but relatively high $a_W$ values under the foil could be reasonably supposed together with favourable conditions for the growth of anaerobes. Anaerobic counts, but also TAC and LAB were higher ($P < 0.01$) in the outer-part samples as compared to the core (Fig. 2) in our experiment.

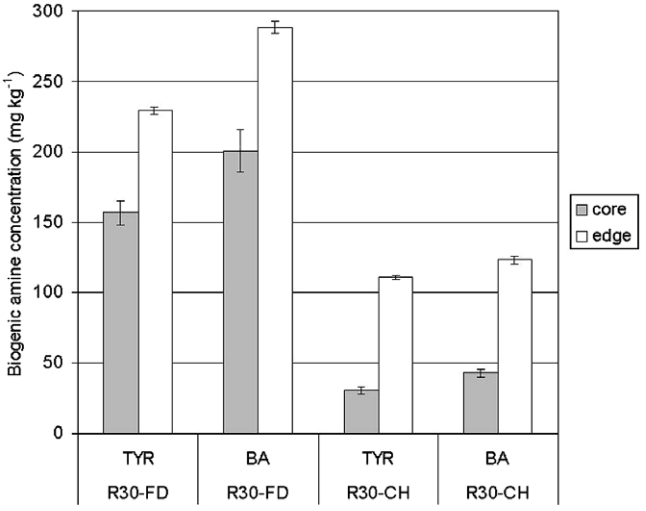


Fig. 1. Comparison of tyramine (TYR) and total biogenic amine (BA) content in the core and edge samples of two batches of cheese with 30% of fat in dry matter (R30-FD, R30-CH, see Table 1) at the end of ripening (26 weeks); mean ± standard error of the mean, $n = 3$.

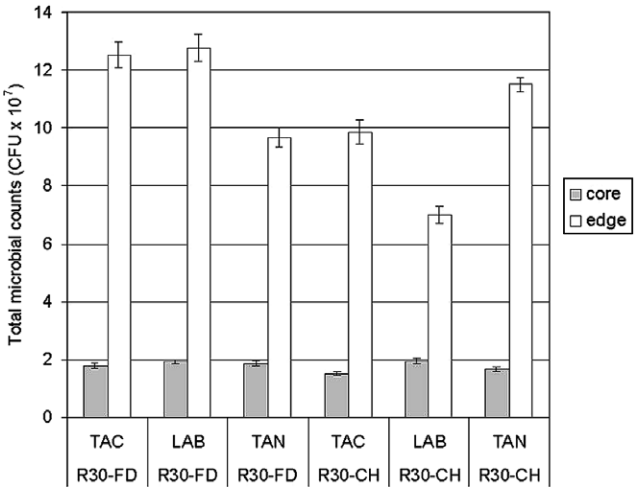


Fig. 2. Total aerobic + facultative anaerobic counts (TAC), lactic acid bacteria (LAB) and total anaerobic counts (TAN) in the core and edge samples of cheese with 30% of fat in dry matter (R30-FD, R30-CH, see Table 1) at the end of ripening (26 weeks); mean ± standard error of the mean, $n = 3$; CFU – colony-forming units.

Contrary to our results, Novella-Rodrígues et al. (2003) found higher tyramine content (but lower tryptamine content) inside the hard-ripened raw-milk goat cheese as compared to the edge. The authors admit they were not able to explain the differences in distribution between tyramine and tryptamine, but mentioned unspecified different external and internal microenvironmental conditions, and possible differences of tyramine and tryptamine producers regarding $O_2$ requirements.

In the present experiment, histamine content was higher ($P < 0.01$) in the outer part of the R30-CH cheese, but only tended to be higher ($P > 0.05$) in the outer part in the case of the R30-FD cheeses. Similarly, content of phenylethylamine and putrescine tended to be higher in the outer part of both the R30-FD and R30-CH cheese after 26 weeks of ripening, but due to the high variability the statistical significance was not established ($P > 0.05$).

Effect of the part of cheese on the content of quantitatively important biogenic amines and counts of microorganisms in the R30 cheeses was compared with an effect of starter culture and time of ripening, using multivariate analysis (Table 3). Regarding part of the cheese, there was great difference between histamine, tyramine and sum of total biogenic amines on the one hand (significant effect, $P < 0.01$; 14–29% of total variability, i.e. 21–48% of the explained variability), and putrescine and sum of total polyamines on the other hand (negligible effect, $P > 0.05$). Part of the cheese also accounted for 29–52% of total variability of microbial counts (70–76% of the explained component of variability). On the other hand, contrary to microbial counts, content of all quantitatively important BA was significantly ($P < 0.01$) influenced by the ripening interval, which accounted for 21–51% of total variability of these traits (dependence of BA content on the time of ripening, see Section 3.3).

Table 3
Effect (expressed as a percentage of total variability) of starter culture, part of the cheese and time of ripening on content of quantitatively important biogenic amines (original measured values in mg $kg^{-1}$) and counts of microorganisms (original measured values in cfu $\times 10^7$/g) in the R30 cheese

| Trait | Variability factors (% of total variability, significance) | | | | | | Unexplained variability (% of total variability) |
|---|---|---|---|---|---|---|---|
| | Starter culture[a] | | Part of the cheese[b] | | Time of ripening[c] | | |
| | % | P | % | P | % | P | |
| Histamine | 10 | <0.01 | 29 | <0.001 | 21 | <0.01 | 40 |
| Tyramine | 0 | >0.05 | 14 | <0.01 | 51 | <0.001 | 35 |
| Putrescine | 15 | <0.01 | 1 | >0.05 | 32 | <0.01 | 52 |
| Sum of all biogenic amines | 0 | >0.05 | 16 | <0.01 | 44 | <0.001 | 40 |
| Sum of polyamines[d] | 18 | <0.01 | 1 | >0.05 | 28 | <0.01 | 53 |
| Total aerobic counts | 0 | >0.05 | 44 | <0.001 | 13 | >0.05 | 43 |
| Lactic acid bacteria | 0 | >0.05 | 29 | <0.001 | 12 | >0.05 | 59 |
| Total anaerobes | 0 | >0.05 | 52 | <0.001 | 15 | <0.05 | 33 |

[a] Flora Danica Normal and CH-N-11 see Table 1.
[b] Core, edge.
[c] 4, 8, 18, 22, 26 weeks.
[d] Putrescine + cadaverine + spermidine + spermine.

Although BA distribution within the cheese could be evaluated only in the R30 samples, similar trends (higher tyramine, histamine and putrescine concentrations in the outer parts of the cheese) are possible to suppose also in the R45 samples, based on our preliminary (unpublished) results regarding BA distribution in cheeses of the same (Dutch) type from two producers (different than in the present experiment).

The polyamines spermidine and spermine are not formed by the microbial action in the cheese, but pass from the milk to the product, and therefore the differences in distribution cannot be expected. This was confirmed both by the data of Novella-Rodrígues et al. (2003) and by our results. However, the contents of both spermidine (0.2–0.4) and spermine (0.1–0.7 mg $kg^{-1}$) were negligible in the R30-FD and R30-CH cheese ripened 26 weeks in our experiment.

It follows from the comparison of the literature data and the results presented here, that the distribution of biogenic amines within the cheese is influenced by many different factors, including the kind of cheese, the pertinent microbial species (both starter and adventitious), and production and ripening conditions.

As already mentioned above, microbiological traits (TAC, LAB and TAN) corresponded with the distribution of the biogenic amines within the cheese in the present experiment (compare Figs. 1 and 2). The same was true regarding counts of yeasts + molds: $5.5 \times 10^1$ vs. $2.6 \times 10^4$ cfu $g^{-1}$ in the inner and outer part of the R30-FD cheese ripened 26 weeks (differences significant at $P < 0.01$), and $8.5 \times 10^1$ vs. $3.9 \times 10^3$ cfu $g^{-1}$ in the corresponding parts of the R30-CH cheese ($P < 0.05$). Coliforms were not present in any R30 cheese after 26 weeks of ripening.

### 3.3. Dependence of biogenic amine content on the time of ripening

Tyramine content in the core samples increased linearly ($P < 0.01$; inclusion of the quadratic term was not significant) with increasing time of ripening both in R30-FD and R30-CH and in R45 cheese. However, the regression gradient was substantially higher in the case of R45 cheese (Fig. 3). Also total biogenic amine content in the inner parts of the cheeses ($Y$; mg $kg^{-1}$ of the fresh matter) increased significantly ($P < 0.01$) with increasing time of ripening ($X$, days) in R30-CH ($Y = 0.25X + 13.9$, $R^2 = 0.63$), R30-FD ($Y = 0.88X - 28.6$, $R^2 = 0.55$) and R45 ($Y = 2.95X - 78.2$, $R^2 = 0.71$) samples. In the case of putrescine, the significant increase ($P < 0.01$) was found only in R30-FD ($Y = 0.11X - 2.6$, $R^2 = 0.62$) and R45 ($Y = 0.34X - 16.9$, $R^2 = 0.77$) core samples.

On the other hand, spermine content ($Y$; mg $kg^{-1}$) decreased both in R30-FD core samples ($Y = 2.2 - 0.01X$; $R^2 = 0.83$, $P < 0.01$) and in R45 cheese ($Y = 1.8 - 0.01X$; $R^2 = 0.43$, $P < 0.05$).

Petridis and Steinhart (1996b) reported most marked increase (among all biogenic amines tested) of histidine,

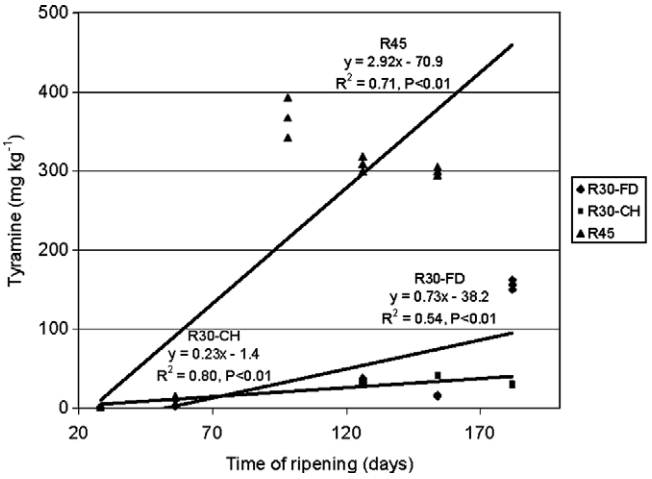


Fig. 3. Development of tyramine content in the core part of the R30-FD, R30-CH and R45 cheese (see Table 1) with the time of ripening; $n = 15$ (3 blocks × 5 ripening intervals; 28, 56, 126, 154, 182 and 28, 56, 98, 126, 154 days in R30 and R45 cheeses, respectively).

tyramine, cadaverine and putrescine in Swiss-type cheese stored 5 months at 5 °C. At this time interval, the content of the above amines was 250, 320, 170 and 160 mg $kg^{-1}$, respectively. From these figures, only tyramine content is comparable with our data (300 mg $kg^{-1}$ in R45 cheese at 22 weeks; Table 4). Histamine content in R45 cheese at 22 weeks of storage (2 mg $kg^{-1}$) was by two orders of magnitude lower in comparison with the data of Petridis and Steinhart (1996b). The possible reason of the low histamine content in our experiment can be the fact, that neither starter culture contained *Lactobacillus casei*, an acknowledged (Petridis & Steinhart, 1996b) strong histamine producer.

As a potent vasoactive substance, tyramine is considered to be the most toxic biogenic amine, toxicological levels being above 100 mg $kg^{-1}$ of the consumed food (Silla-Santos, 1996). From this viewpoint, R45 cheese (but not R30), should be approached with caution when ripened more than 60 days. In the time interval from the 18th week of ripening, this product (R45) contained also higher levels of putrescine (Table 4), a known tyramine toxicity potentiator (Bover-Cid, Izquierdo-Pulido, & Vidal-Carou, 2000). However, the sum of all biogenic amines after 22 weeks of ripening (Table 3), was well under the safety limit of 900 mg $kg^{-1}$ for the sum of tyramine + histamine + putrescine + cadaverine according to Valsamaki et al. (2000). On the other hand, considering that toxic dose for patients receiving non-selective MAO inhibitors was suggested to be 6 mg of tyramine (Novella-Rodrígues et al., 2003), already 20 g portion of R45 cheese, 60 days old, could be dangerous for these patients.

### 3.4. Comparison of biogenic amine content in cheese from two different producers

According to Stratton et al. (1991) BA formation is related to raw milk quality. However, no differences between producers can be inferred from raw milk quality in the present experiment, because both producers used raw milk of the same quality, meeting the requirements of the Czech Veterinary Law for total counts of aerobic and facultative anaerobic mesophilic microorganisms ($\leqslant$100,000 $ml^{-1}$) and for somatic cell counts ($\leqslant$400,000 $ml^{-1}$).

Comparison of biogenic amines content in core samples of R30 (R30-FD and R30-CH) and R45 cheese in particular ripening intervals is presented in Table 4, including the microbiological traits. Development of the counts of total aerobic + facultative anaerobic mesophilic microorganisms and lactic acid bacteria during ripening of R30 and R45 cheeses is presented in Fig. 4.

Quantitatively, most important biogenic amine was tyramine. Tyramine content, and also content of phenylethylamine, putrescine, sum of total biogenic amines and sum of polyamines after 18 and 22 weeks of ripening was higher ($P < 0.01$) in R45 cheeses in comparison with both R30-FD and R30-CH core samples (Table 3). Tyramine

Table 4
Comparison of biogenic amines content (mg $kg^{-1}$) and microorganisms counts in core samples of three batches of Dutch-type hard cheese ripened for varying times (mean ± standard error of the mean; $n = 3$)

| Trait | Ripening time | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 8 weeks | | | 18 weeks | | | 22 weeks | | |
| | R30-FD[a] | R30-CH[a] | R45[a] | R30-FD[a] | R30-CH[a] | R45[a] | R30-FD[a] | R30-CH[a] | R45[a] |
| Tryptamine | ND | ND | 0.4 ± 0.0 | 1.3 ± 0.3 | ND | 1.2 ± 0.4 | 1.8 ± 0.8 | 0.6 ± 0.5 | ND |
| Phenylethylamine | 1.3 ± 0.8 | ND | 0.6 ± 0.1 | $2.0^{A}$ ± 0.4 | $1.5^{A}$ ± 0.2 | $43.5^{B}$ ± 3.9 | $1.0^{A}$ ± 0.1 | $2.0^{A}$ ± 0.1 | $54.3^{B}$ ± 1.3 |
| Histamine | 14.3 ± 1.7 | 16.3 ± 3.4 | 0.5 ± 0.1 | 9.6 ± 2.3 | 10.4 ± 0.6 | 2.8 ± 0.4 | 10.7 ± 5.9 | 17.1 ± 0.1 | 1.8 ± 0.4 |
| Tyramine | $2.9^{A}$ ± 0.2 | $11.4^{B}$ ± 0.5 | $14.4^{C}$ ± 0.4 | $34.5^{A}$ ± 2.9 | $34.7^{A}$ ± 0.2 | $309.5^{B}$ ± 9.2 | $15.7^{A}$ ± 0.4 | $41.2^{B}$ ± 1.2 | $299.8^{C}$ ± 4.8 |
| Putrescine | $6.1^{B}$ ± 0.9 | $1.2^{A}$ ± 0.2 | $0.3^{A}$ ± 0.1 | $13.1^{A}$ ± 0.9 | $2.1^{A}$ ± 0.5 | $26.6^{B}$ ± 2.0 | $6.0^{A}$ ± 1.0 | $6.7^{A}$ ± 0.1 | $60.8^{B}$ ± 2.2 |
| Cadaverine | 1.4 ± 0.5 | 0.6 ± 0.1 | 0.9 ± 0.1 | 0.9 ± 0.1 | 0.6 ± 0.0 | 0.9 ± 0.1 | $0.8^{A}$ ± 0.1 | $0.7^{A}$ ± 0.1 | $2.0^{B}$ ± 0.1 |
| Spermidine | 0.3 ± 0.1 | 0.4 ± 0.1 | 0.4 ± 0.1 | 0.2 ± 0.0 | 0.3 ± 0.0 | 0.2 ± 0.0 | 0.3 ± 0.0 | ND | 0.3 ± 0.1 |
| Spermine | 1.1 ± 0.4 | 0.6 ± 0.2 | 0.2 ± 0.0 | 0.3 ± 0.0 | ND | 0.5 ± 0.2 | 0.1 ± 0.0 | 0.2 ± 0.0 | 0.2 ± 0.1 |
| Sum of biogenic amines | 27.4 ± 4.9 | 30.5 ± 4.2 | 17.9 ± 0.4 | $61.9^{A}$ ± 7.1 | $49.5^{A}$ ± 1.2 | $385.2^{B}$ ± 11.1 | $36.4^{A}$ ± 3.4 | $68.3^{A}$ ± 1.6 | $419.0^{B}$ ± 6.1 |
| Sum of polyamines[b] | 8.9 ± 2.1 | 2.8 ± 0.6 | 1.8 ± 0.1 | $14.5^{A}$ ± 0.9 | $3.0^{A}$ ± 0.5 | $28.1^{B}$ ± 2.2 | $7.1^{A}$ ± 1.1 | $7.5^{A}$ ± 0.1 | $63.2^{B}$ ± 2.5 |
| Total aerobic counts (cfu × $10^7$) | 1.1 ± 0.2 | 1.5 ± 0.3 | 1.4 ± 0.3 | 1.6 ± 0.3 | 2.4 ± 0.5 | 2.8 ± 0.5 | 1.7 ± 0.3 | 1.5 ± 0.2 | 0.8 ± 0.1 |
| Lactic acid bacteria (cfu × $10^7$) | 0.8 ± 0.2 | 1.5 ± 0.5 | 0.7 ± 0.1 | 1.8 ± 0.6 | 3.6 ± 1.1 | 2.5 ± 0.8 | 1.8 ± 0.6 | 2.4 ± 0.6 | 0.5 ± 0.1 |
| Total anaerobes (cfu × $10^7$) | 0.9 ± 0.2 | 1.3 ± 0.3 | 0.8 ± 0.1 | 1.7 ± 0.4 | 2.0 ± 0.4 | 4.0 ± 0.6 | 1.4 ± 0.3 | 1.6 ± 0.3 | 0.4 ± 0.1 |
| Coliforms (cfu × $10^3$) | ND | ND | 0.3 ± 0.1 | ND | ND | ND | ND | ND | ND |
| Yeasts + moulds (cfu × $10^4$) | ND | ND | 0.8 ± 1.6 | ND | ND | 11.3 ± 2.2 | ND | ND | 14.8 ± 3.5 |

ND – not detected.
[A,B,C] means with different superscripts within the given time interval differ at $P < 0.01$.
[a] Cheese characteristics see Table 1.
[b] Putrescine + cadaverine + spermidine + spermine.

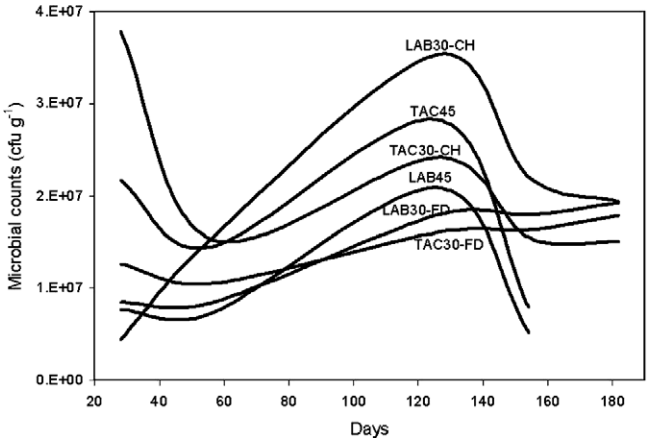


Fig. 4. Development of the counts of total aerobic + facultative anaerobic mesophilic microorganisms (TAC) and lactic acid bacteria (LAB) in the core parts of the R30-FD, R30-CH and R45 cheese (see Table 1) during ripening; $n = 15$ (3 blocks × 5 ripening intervals; 28, 56, 126, 154, 182 and 28, 56, 98, 126, 154 days in R30 and R45 cheeses, respectively).

content in R45 cheese was higher ($P < 0.01$) already in the samples after 8 weeks of ripening.

As far as a comparison of both R30 batches is concerned, tyramine content was higher ($P < 0.01$) in R30-CH samples than in R30-FD cheeses after 8 and 22 (but not after 18) weeks of ripening (Table 4). On the other hand, putrescine content in R30-FD after 8 weeks of ripening was higher ($P < 0.01$) in comparison with the R30-CH cheese.

Contrary to the evaluation of the core and edge samples of the cheeses from the same producer (Figs. 1 and 2), biogenic amine content did not correspond with the microbiological traits during ripening as far as a comparison of R30 and R45 cheeses is concerned. This is apparent from Table 4 (TAC, TAN and LAB counts tended, $P > 0.05$, to be higher in R30 cheeses in comparison with R45 ones) and from comparison of Figs. 3 and 4. This inconsistency of the microbiological and chemical (biogenic amine content) traits is a confirmation (viewed from another angle) of the significant differences in biogenic amine concentration in the cheeses with comparable microbiological profile found in the experiment of Schneller et al. (1997). According to Innocente and D'Agostin (2002) it is not possible to find a straight correlation (under the conditions similar to the present experiment) between microbial counts and biogenic amine content. This is likely due to the fact, that the amine-producing microorganisms in cheese are adventitious rather than the constituents of the starter culture, and abilities of these various bacteria differ widely according to total counts, synergism between different species, level of proteolysis, salt content, pH and ripening temperature (Valsamaki et al., 2000). Similar conclusions can be drawn from the data of Gennaro, Gianotti, Marengo, Pattono, and Turi (2003), who reported that the conditions for minimizing the formation of biogenic amines in cheese were different for each amine due to the fact that different bacteria and metabolic pathways were involved.

On the other hand, total aerobic counts (cfu $g^{-1}$) were positively correlated ($P < 0.01$) with histamine ($r = 0.45$), putrescine ($r = 0.40$) and total biogenic amine ($r = 0.60$) content (in mg $kg^{-1}$ of fresh matter) in R30 cheese (all core and edge samples of both R30-FD and R30-CH cheeses taken together as a one set); and with tyramine content in both R30 ($r = 0.59$; $P < 0.01$) and R45 ($r = 0.52$; $P < 0.05$) samples in the present experiment. The relationship between content of the above biogenic amines (mg $kg^{-1}$) and lactic acid bacteria (cfu $g^{-1}$) was similar: $r = 0.53$, 0.62, 0.46 and 0.65 for histamine, tyramine, putrescine and sum of biogenic amines, respectively ($P < 0.01$). Similarly to our results, LAB counts and tyramine content were correlated closely in the Swiss-type cheese in the experiment of Petridis and Steinhart (1996b). According to these authors, *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*, which were also constituents of both starter cultures used in production of R30 cheese in the present experiment (Table 1), should be considered important tyramine producers.

## 4. Conclusions

Higher counts of anaerobic bacteria found in the outer parts of the cheeses (more favourable growth conditions in this part of the cheese, which ripened in the foil), appeared to be a likely reason for substantially higher contents of quantitatively most important biogenic amines, histamine and tyramine, in comparison with the core part of the cheeses.

In three cheeses, R30-FD, R30-CH and R45, the effect of time of ripening on biogenic amine content was demonstrated. Tyramine content in all cheeses increased linearly in the course of ripening, and the concentration of this biogenic amine (more than 300 mg $kg^{-1}$) in the R45 cheeses after 18 weeks of ripening substantially exceeded an established toxicological limit for tyramine (100 mg $kg^{-1}$ of the consumed food). Individuals suffering from food intolerances and food allergies, and patients receiving monoamin-oxidase inhibitors, should avoid consumption of such ripened cheese.

Determination of bacterial counts on the one hand, and biogenic amine content on the other hand, gave the contradictory results, when R30 and R45 cheeses were mutually compared. Therefore, the microbiological analysis based only on basic groups of microorganisms did not provide any conclusive clue regarding differences in biogenic amine content in the cheeses.

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