Production of tyramine and histamine by bacteria isolated from Czech blue-veined cheese Niva

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Summary

Tyramine- and histamine-producing microorganisms were studied in 66 samples of blue-veined cheese Niva from retail shops. Bacteria from genera *Enterococcus* and *Lactobacillus* were isolated from cheese and isolates were identified and confirmed by biochemical tests and polymerase chain reaction (PCR) methods. Enterococci were genotypically identified as *Enterococcus faecalis* (n = 28), *Enterococcus casseliflavus* (n = 6), *Enterococcus faecium* (n = 1) and one strain was identified as *Enterococcus* spp. Forty-four isolates were identified as *Lactobacillus* spp. The identified isolates were grown in decarboxylase medium (20 g·l^{-1} of precursor amino acid) with a pH indicator and the results were confirmed by high performance liquid chromatography. Production of tyramine was demonstrated in 97% strains of *Enterococcus faecalis*, 33% strains of *Enterococcus casseliflavus*, 1 strain of *Enterococcus faecium* and 1 strain of *Enterococcus* spp. Among *Lactobacillus* spp., 22.7% of strains were found to be positive for tyramine production. The cultivation method showed a good correlation with the chemical analysis. The values for the tyramine production by strains of enterococci and lactobacilli were found in the range of 1 485–2 363 mg·l^{-1} and 903–1 870 mg·l^{-1}, respectively. No significant histamine production was detected for any of the isolated strains.

Keywords

biogenic amines; *Enterococcus*; *Lactobacillus*; decarboxylase; blue-veined cheese; HPLC

Biogenic amines (BA) are low-molecular-weight organic bases possessing biological activity. Some of them play important roles in many human and animal physiological functions, such as the regulation of body temperature, stomach volume, gastric pH and brain activity [1]. However, the consumption of food containing high concentrations of these compounds may cause toxic effects in susceptible individuals. The most notorious food-borne intoxications caused by BA are related to histamine and tyramine. Besides fish, cheese is the next most commonly implicated food item associated with histamine poisoning, whereas tyramine has been related to migraine and hypertensive crisis in patients treated with monoamine-oxidase inhibitor (MAOI) drugs [2]. BA are mainly generated by enzymatic decarboxylation of the corresponding amino acids by specific enzymes derived from microorganisms present in the foods [3, 4]. Among decarboxylase-positive microorganisms, many strains of *Enterobacteriaceae* and certain lactobacilli, pediococci and enterococci are particularly active [1]. Several methods to determine BA production have been described. Numerous procedures are based on the use of differential media with specific substrates and a pH indicator, or on the analysis by high performance liquid chromatography (HPLC) [5]. Recently, methods for the detection of genes encoding microbial decarboxylases responsible for the production of BA have become available [6].

High levels of BA are found in cheeses produced with cultural moulds, in particular in blue-veined cheeses, such as Roquefort, Gorgonzola, Stilton and Danish Blue [7, 8]. The Niva cheese is a typical Czech variant of an internal mould cheese, produced with the use of strains of *Penicillium roqueforti*. Niva cheese is produced from pasteurized cows' milk and is delicate and spicy, being similar in taste to the French ewes' milk cheese, Roquefort. Niva has obtained a Protected Geographical Indication status [9].

Data regarding the assessment of the factors that can influence BA production in Niva cheese

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are rather scarce [10, 11]. Therefore, the aim of this work was to determine decarboxylase activity of bacteria, in particular of enterococci and lactobacilli, that had been isolated from Niva cheese purchased from retail shops, and to test the production of tyramine and histamine in a decarboxylase medium.

MATERIALS AND METHODS

Samples and bacterial strains

Sixty-six cheese samples (300 ± 50 g) were collected from different retail shops in South Moravia, Czech Republic during a period of two months (April, May) 2008. Cheeses originated from three different producers. They were sampled within two days after the delivery to the market. Samples were transported to laboratory in sterile, cold (4 °C) containers and preserved at this temperature.

Two control strains of lactic acid bacteria (LAB) were provided by the Czech Collection of Microorganisms (CCM; Masaryk University, Brno, Czech Republic), namely, tyramine-producing strains *Lactobacillus brevis* CCM 1815 (ATCC 8287) and *Enterococcus faecalis* CCM 4224 (ATCC 29212). *Lactobacillus buchneri* DSM 5987, a histamine-producing strain, was purchased from German Collection of Microorganisms and Cell Cultures (DSMZ; Braunschweig, Germany).

Microbiological analysis

After transfer to the laboratory, samples (10 g) were homogenized in a stomacher with 90 ml of sterile physiological saline solution prewarmed to 45 °C, and serial decimal dilutions were plated on selective media. The following groups of bacteria were determined:

- lactobacilli on De Man-Rogosa-Sharpe agar (MRS; Oxoid, Cambridge, United Kingdom) incubated under microaerobic conditions at 30 °C for 72 h and
- enterococci on Slanetz-Bartley Agar (Oxoid) incubated at 37 °C for 48 h.

The various microbial groups were identified using conventional tests, including microscopy, catalase, oxidase and pyrrolidonylarylamidase tests. Pure cultures were confirmed to the genus level by polymerase chain reaction (PCR) method for the genus *Enterococcus* [12] and *Lactobacillus* [13]. Species-specific PCR [14] was used for the identification of *Enterococcus* species (*E. faecalis, E. faecium, E. durans, E. hirae, E. malodoratus, E. casseliflavus, E. avium, E. gallinarum* and *E. mundtii*).

Determination of histidine and tyrosine decarboxylase activity of microorganisms by a cultivation method

For the genera *Enterococcus* and *Lactobacillus*, a decarboxylase medium described by Joosten & Northolt (JNM; [15]) was used, modified by the addition of 0.005% pyridoxal-5-phosphate monohydrate (Sigma-Aldrich, Schnelldorf, Germany). The test medium containing the precursor amino acids L-histidine dihydrochloride and L-tyrosine *di*-sodium salt, (Sigma-Aldrich) was composed of: 0.5% tryptone (Carl Roth, Karlsruhe, Germany), 0.5% yeast extract (Bio-Rad, Marnes-la-Coquette, France), 0.05% Tween 80 (HiMedia, Mumbai, India), 0.5% NaCl, 0.1% glucose, 0.02% $MgSO_4.7\,H_2O$, 0.005% $MnSO_4.H_2O$, 0.004% $FeSO_4.7\,H_2O$, 0.01% $CaCO_3$ (all chemicals Penta, Chrudim, Czech Republic), 2% agar (Oxoid) and 0.006% bromocresol purple dye (Carl Roth) as a pH indicator. The concentration of each of the supplementing amino acids was 2%. After sterilization by autoclaving for 10 min at 121 °C, pH was adjusted to the value of 5.5 ± 0.1.

For inoculation, a 24-h pure culture of bacteria obtained from the plates of Slanetz-Bartley agar or MRS agar was used. The amino acid-containing broths were inoculated with the adjusted suspensions to cell concentration of 10^6 CFU·ml^{-1}. All isolates were inoculated into tubes containing the decarboxylase medium with or without the tested amino acid. The medium without amino acid was used as a negative control. The inoculated media were incubated for 48 h at a temperature optimal for the individual bacteria: under microaerobic conditions at 30 °C for lactobacilli, and aerobically at 37 °C for enterococci. A positive result was indicated by a change in colour of the indicator to purple, as a result of a shift in pH. Cultures with a positive reaction were sedimented by centrifugation, separated by microfiltration (pore size, 0.45 μm), and analysed for biogenic amines.

Analysis of biogenic amines

Tyramine and histamine were determined as dansyl derivatives. Their quantitative determination was carried out using reverse-phase high performance liquid chromatography (RP-HPLC) with fluorescence and photodiode array (PDA) detection, modified for the determination of BA in the specific matrix. The mobile phase composition and gradient programme were used according to the method of Paulsen et al. [16].

HPLC conditions

Liquid chromatograph: Alliance 2695 with photodiode array 2996 detector and fluorescence

2475 detector (Waters, Milford, Massachusetts, USA); chromatographic column: Zorbax Eclipse XDB C18, 150 × 4.6 mm, 5 μm with 30 × 4.6 mm, 5 μm precolumn (Agilent, Palo Alto, California, USA); gradient elution mobile phase A: 0.1 mol·l^{-1} acetic acid : acetonitrile : methanol (90 : 5 : 5), mobile phase B: 0.1 mol·l^{-1} acetic acid : acetonitrile : methanol (10 : 45 : 45); flow 1 ml·min^{-1}; injection volume 10 μl; fluorescence detection at $\lambda_{ex}/\lambda_{em}$ = = 330/500 nm; UV detection at 254 nm. Samples were analysed in duplicates. Identification was carried out using an external standard.

The method was validated using the EffiValidation 3 software (Effichem, Lysice, Czech Republic). For the individual BA, levels of determination of the method were 1.2 mg·l^{-1} for histamine and 1.8 mg·l^{-1} for tyramine. The repeatability of the analytical process ranged from 4.8% for tyramine to 8% for histamine. The recovery rate of the method ranged from 87.5% for histamine to 103% for tyramine.

Statistical evaluation

Means of the two measurements were used in statistical evaluation. All the statistical tests were performed using statistical software STAT Plus (Veterinary Research Institute Brno, Czech Republic). Before the actual testing, Box-Cox transformation was applied to the data. Further, the data were processed by Student's *t*-test, and Tukey's test of significance of differences.

RESULTS AND DISCUSSION

Biogenic amine production has been most extensively studied with respect to histamine and tyramine, which are probably the most important BA of bacterial origin in food, due to their toxicological effects. In this study, BA production in blue-veined cheese Niva associated with species of the genera *Enterococcus* and *Lactobacillus* was investigated.

Identification of bacterial strains

A total of 140 strains isolated of the cheese samples were identified using biochemical analysis and genus-specific PCR methods. Presently, the standard method for identification of enterococci is a phenotypic characterization, primarily using biochemical tests. The tests are usually performed in test tubes and may require significant amounts of time for preparation and interpretation of results. Furthermore, this methods suffers from a low throughput since 10 or more tests may be necessary for the differentiation of the species [14]. Therefore, we used molecular methods for identification purposes. Primers used were derived from the type strains and had been tested for their exclusivity and inclusivity, and had been found species-specific with isolates from different samples of food and clinical origins [14]. On the whole, 45 isolates of enterococci were obtained and identified to the species level by PCR: 28 strains of *E. faecalis* (78%), 1 strain of *E. faecium* (3%), 6 strains of *E. casseliflavus* (16%) and one strain of *Enterococcus* spp. (3%; Tab. 1). *E. faecalis* was found to be the most abundant species. One strain of the genus *Enterococcus* could not be assigned to any species. Of the 95 isolates from MRS media, 44 were identified as *Lactobacillus* spp. by PCR.

The results obtained correspond with data published earlier for cheeses produced in different countries. The most commonly reported enterococci in cheese were *E. faecalis*, *E. faecium*, *E. durans* and *E. casseliflavus*, followed by *E. hirae* and *E. gallinarum*. In Caprino cheese, a traditional cheese produced in Italy, *E. faecalis* and *E. faecium* were the most frequently isolated species, followed by *E. durans, E. hirae* and *E. gallinarum* [17]. Rea et al. [18] studied the effect of strains of enterococci (*E. faecalis, E. faecium, E. durans* and *E. casseliflavus*) on tyramine contents in Cheddar cheese during cheese manufacture and ripening. Similarly Morandi et al. [19] isolated from Italian raw milk cheeses four species of the genus *Enterococcus*: *E. faecalis, E. faecium, E. durans* and *E. hirae. E. faecalis* and *E. faecium* were the dominant enterococcal species. *E. durans, E. faecalis* and *E. casseliflavus* were identified in isolates from a Dutch-type semi-hard cheese [20].

Decarboxylase activity of the bacteria

The identified species were inoculated to specific decarboxylase media supplemented with tyrosine or histidine. A modified JNM decarboxylase medium supplemented with pyridoxal-5-phosphate as a cofactor for the decarboxylase reaction was used. This modification had been successfully used by Bover-Cid & Holzapfel [20], providing an improved detection of BA-producing microbial strains.

Tab. 1 presents the qualitative and quantitative data on the production of BA by the LAB strains, as well as the number of isolates and control strains with tyramine- and histamine-producing abilities. Tyramine-producing activity was observed at 1, 27, 2 and 1 strain, being 100, 97, 33 and 100% for *E. faecium, E. faecalis, E. casseliflavus* and *Enterococcus* spp., respectively. This is consistent with previous findings, when the Dutch-type semi-hard cheese strains of *E. durans*, *E. faecalis* and *E. cas-*

seliflavus were identified as tyrosine decarboxylase-positive strains [20]. From Tab. 1 is evident that almost all identified species were active in the production of tyramine, the highest activities being found in *E. faecalis* and *E. faecium*. About two thirds of *E. casseliflavus* strains were found to be lacking the ability to decarboxylate tyrosine. This is not surprising since it has been previously reported that *E. casseliflavus* did not produce tyramine in Cheddar cheese [18]. The production of histamine was not recorded in any of our isolates of enterococci. This corresponds with the previously reported findings [21, 22].

Nearly one half (46%) from 95 isolates suspected to be lactobacilli, was confirmed as *Lactobacillus* spp. Among these 44 strains, 10 strains (22.7%) were positive for a tyramine-producing ability. Although lactobacilli have been reported to show strong histidine decarboxylase activity and amine-producing capacity in cheese [15], no significant histamine production was observed in our study. This was a different result than that of the study of Roig-Sagués et al. [23], in which the Spanish cheese isolates tested for histidine- and tyrosine-decarboxylase activity exhibited the production of histamine by *L. buchneri* and tyramine by *L. brevis*.

The obtained results on BA production in the synthetic medium confirm that the ability to produce amines may be strain-dependent rather than being related to specific species [24]. However, in our study, this feature seems to be more common among strains of *E. faecalis* with regard to the production of tyramine (Tab. 1). The results demonstrate a pronounced intra-species as well as inter-species variability in amine formation capacity. The ability to produce tyramine was significantly ($P < 0.05$) higher in strains of *E. faecalis* than in those of *E. casseliflavus*. Significantly ($P < 0.01$) more strains (86.1%) of enterococci (31 strains from 36 identified) showed tyramine-producing activity, whereas only 22.7% (10 strains from 44 identified) strains of lactobacilli did so. These results are in agreement with those published by Rea et al. [18].

Analysis of biogenic amines

Amine-producing ability was confirmed quantitatively by RP-HPLC analysis (Fig. 1). After the cultivation, tyramine and histamine contents were determined in the liquid medium. The values observed for tyramine exceeded 1 000 $mg \cdot l^{-1}$ in most strains of the *Enterococcus* and *Lactobacillus* genera (Tab. 1). The determined values are consistent with those published by Pircher et al. [25] in cheese isolates (591 $mg \cdot l^{-1}$ for histamine and 2 597 $mg \cdot l^{-1}$ for tyramine). Similarly, Rea et al. [18] reported the production of 1 052 $mg \cdot l^{-1}$ of tyramine by isolates from Cheddar cheese.

A decarboxylase medium used in the present study has been reported to facilitate an improved detection of BA-producing microbial strains. However, the procedure may have some limitations in terms of sensitivity leading to contradictory results [21]. In some reports, occurrence of either false positive or false negative results was described. The former were associated with the production of other, non-BA alkaline compounds, and the latter were due to the fermentation of sugars that reduced the pH of the medium [5]. For this reason, BA production has to be confirmed by the chemical analysis. However, compared to the chromato-

Tab. 1. Biogenic amine production by lactobacilli and enterococci in decarboxylase media supplemented with tyrosine and histidine, respectively.

Strains	Isolates total	*n*	Number of decarboxylase-positive strains		BA concentration in the cultivation medium[a] [$mg \cdot l^{-1}$]	
			L-Tyrosine	L-Histidine	Tyramine	Histamine
E. faecalis		28	27	0	27 (1 846–2 363)	ND
E. faecium		1	1	0	1 (1 485)	ND
E. casseliflavus		6	2	0	2 (1 956–2 016)	ND
Enterococcus spp.		1	1	0	1 (2 047)	ND
Enterococci total	45	36	31	0		
Lactobacillus spp.	95	44	10	0	10 (903–1 870)	ND
E. faecalis CCM 4224 [b]			1	0	1 (2 129)	ND
L. brevis CCM 1815 [b]			1	0	1 (1 179)	ND
L. buchneri DSM 5987 [b]			0	1	ND	1 (1 593)

a – number of decarboxylase-positive strains by RP-HPLC and the concentration range of biogenic amines produced, b – control strains for the production of BA. *n* – number of strains identified by PCR, ND – not detected (limits of determination: tyramine – LOD = 1.8 $mg \cdot l^{-1}$, histamine – LOD =1.2 $mg \cdot l^{-1}$).

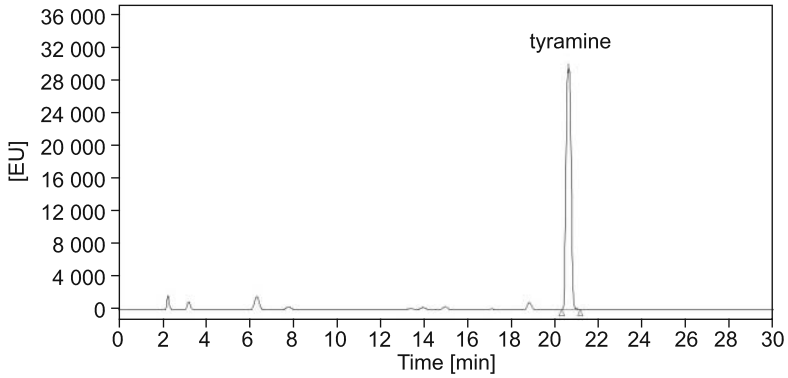

Fig. 1. An HPLC chromatogram of the decarboxylase medium of a tyramine-producing strain. EU – arbitrary units.

graphic analysis, the microbiological analysis using a decarboxylase medium facilitates pre-selection of strains with the decarboxylase activity. In our study, a good correlation was found between the microbiological method and determination of tyramine and histamine by HPLC (Tab. 1).

Although there is no legislation regarding the tyramine contents in cheese, the reduction of its concentration in food is recommended. HPLC and the cultivation method are techniques that can be useful for this aim: HPLC is necessary for the determination of the concentration of tyramine in the sample, while the cultivation method is useful as a screening method for large numbers of samples for the presence of tyramine-producing bacteria.

Application to food

The positive result for biogenic amine production in laboratory media does not automatically imply similar behaviour of bacterial strains in a food product. It must be taken into account that food is a complex system with a wide number of factors influencing microbial growth and activity.

It can be assumed that isolates which are capable of producing amines at low concentrations in the medium which is rich in nutrients and contains bacterial cultures at high densities, need not produce significant amounts of amines in the food samples [25]. Enterococci may be identified as bacteria that have a high ability to form tyramine in isolates from food. Despite low counts of enterococci in cheeses, higher levels of tyramine can be detected. We observed such relation in our previous work with Niva cheese [11], when a considerably high content of tyramine (298 $mg \cdot kg^{-1}$) was found only in samples with increased counts (10^4 $CFU \cdot g^{-1}$) of enterococci. In the present study, among 44 isolates of lactobacilli, the significant formation of tyramine was detected in 10 isolates. Although these bacteria may achieve high counts during cheese fermentation, it is difficult to find a direct correlation between biogenic amine contents and counts of the above mentioned bacteria, because the ability of different strains to produce biogenic amines may differ widely [4].

Cheese belongs to the most important tyramine and histamine sources in the diet. It is necessary to protect the consumer's health by preventing the production of high amounts of BA. With this goal, producers must control the kind of microorganisms that multiply during ripening, and must attempt to reduce the amounts of contaminating bacteria such as enterococci and some amine-producing lactobacilli. Their presence in cheese may result from raw milk contamination or post-pasteurization contamination from the cheese-making equipment. The proper hygiene conditions during cheese manufacture and ripening is the way to minimize the formation of biogenic amines in cheese.

CONCLUSIONS

The modified decarboxylase medium used for culture detection of bacteria producing high levels of tyramine was shown to be a good indicator medium, as no contradictory results were obtained. Good correlation was obtained between the results of this microbiological method and amine determination by HPLC.

The results obtained in this study confirm that the ability to produce of amines may differ not only among species of the same genus but also within the strains of the same species. No direct relationship between the amount of the produced tyramine and their bacterial producer can be drawn, as long as different bacteria can produce different amount of tyrosine decarboxylase.

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