Tyramine Production by Enterococci from Various Foodstuffs: A Threat to the Consumers

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Abstract: Tyramine is the most frequent biogenic amine found in cheese and is also frequently found in other fermented foods and beverages. In total 273 different strains of enterococci from various foodstuffs were studied. A multiplex PCR was designed for the genotypic differentiation of various enterococci strains at species level and for determination of the presence of tyramine producing (*tyrdc*) gene. *E. faecalis* and *E. faecium* were found to be prominent strains in dairy and meat products while, *E. faecium* and *E. mundtii* were found to be ruling in case of fruits and vegetables. HPLC analysis was carried out for determination of levels of tyramine. 211 i.e. 86.8% of samples were containing about 1000–1500 mg/l of tyramine, while 10 i.e. 4.1% were found to have tyramine in the range 100–500 mg/l. Negative samples showed 0 mg/l of tyramine.

Keywords: *tyrdc* gene; tyramine; PCR, HPLC; enterococci

INTRODUCTION

Recent trends in the food safety are promoting an increasing search for trace compounds that can affect human health. Biogenic amines, the so called natural amines with physiological significance, belong to this group of substances (Karovičová & Kohajdová 2003). Biogenic amines, which are produced in a number of foods, are organic bases with an aliphatic, aromatic, or heterocyclic structure (Alvarez *et al.* 2006). Biogenic amines such as histamine, tyramine, putrescine, and others can be formed in food as a result of metabolic processes of microorganisms (Leszczynska *et al.* 2004). It is known that a diet rich in tyramine can cause increase in blood pressure and neuralgic pains, and that an imbalance in the level of tyramine is thought to underlie altered brain function in many pathological conditions, including dystoniasis, Parkinson's disease, schizophrenia, drug addiction, and mood disorders (Premont *et al.* 2001). In fermented foods, biogenic amines are mainly generated by decarboxylation of the corresponding amino acids through substrate specific enzymes of the microorganisms present in the food (Munoz *et al.* 2006). Bacteria of the genus *Enterococcus* are ubiquitous Gram positive, catalase-negative cocci that often occur in large numbers in vegetables, plant material, and foods, especially those of animal origin such as dairy products (Giraffa 2003). The lactic acid bacteria known to possess significant tyrosine decarboxylase activity in cheeses are enterococci (Giraffa *et al*. 1997).

The aim of the study was to look for the ability to produce tyramine in different species of enterococci originating from various foodstuffs.

MATERIALS AND METHODS

Bacterial strains. 273 of enterococci strains isolated from various foodstuffs and stored at –75°C in 20% glycerol medium originated from the strain collection of Department of Hygiene and Milk Technology (University of Veterinary and Pharmaceutical Sciences Brno).

Species specific identification method (PCR). Species specific identification of enterococci was

Table 1. List of primers used in this study

Designation	Primer sequence	Gene	Product size (bp)	Species	Reference
EFS 1 EFS 2	F: 5'-ACT TAT GTG ACT AAC TTA ACC -3' R: 5'-TAA TGG TGA ATC TTG GTT TGG-3'	*sod* A	360	*E. faecalis*	Jackson *et al.* (2004)
EFM 1 EFM2	F: 5'-GAA AAA ACA ATA GAA GAA TTA T-3' R: 5'- TGC TTT TTT GAA TTC TTC TTT A-3'	*sod* A	215	*E. faecium*	Jackson *et al.* (2004)
EDU 1 EDU 2	F: 5'-CCT ACT GAT ATT AAG ACA GCG-3' R: 5'-TAA TCC TAA GAT AGG TGT TTG-3'	*sod* A	295	*E. durans*	Jackson *et al.* (2004)
EHI 1 EHI 2	F: 5' CTT TCT GAT ATG GAT GCT GTC-3' R: 5' TAA ATT CTT CCT TAA ATG TTG-3'	*sod A*	187	*E. hirae*	Jackson *et al.* (2004)
ECAS 1 ECAS 2	F: 5'-TCC TGA ATT AGG TGA AAA AAC-3' R: 5'-GCT AGT TTA CCG TCT TTA ACG -3'	*sod* A	288	*E. casseliflavus*	Jackson *et al.*, (2004)
EMU 1 EMU 2	F: 5'-CAG ACA TGG ATG CTA TTC CAT CT-3' R: 5'-GCC ATG ATT TTC CAG AAG AAT-3'	*sod* A	98	*E. mundtii*	Jackson *et al.* (2004)
TD 2 TD 5	F: 5'-ACA TAG TCA ACC ATR TTG AA -3' R: 5'-CAA ATG GAA GAA GAA GTA GG -3'	*tyrdc*	>970		Coton & Coton (2005)
BSF 8 BSR1541(IC*)	F: 5'-AGA GTT TGA TCC TGG CTC AG-3' R: 5'-AAG GAG GTG ATC CAG CCG CA-3'	*16S rRNA*	>970		Coton & Coton (2005)

F – Forward primer, R – Reverse primer, bp – base pair, IC* – Internal control

carried out using the PCR method based on the detection of genus specific section of the *sod* A gene encoding the enzyme manganese-dependent superoxide dismutase (Jackson *et al.* 2004) (Table 1).

Methods used for the tyramine detection

Method based on tyrdc gene detection (PCR). DNA was extracted from bacterial cultures by boiling procedure. A multiplex PCR was designed for the production of tyramine by the presence of *tyrdc* gene. Primers used for the study are listed in Table 1. The amplification program for multiplex PCR was similar to that used by Coton and Coton (2005). All multiplex experiments were performed in a Peltier Thermal Cycler PTC-200 (MJ Research, USA) using Qiagen Multiplex PCR kit (Qiagen, Hilden, Germany) in the total volume of 25 µl. PCR products were detected by electrophoresis in 2% (w/v) agarose gels in 0.5 × TBE buffer at 120 V for 45 min and visualised with ethidium bromide staining.

Cultivation method of tyramine production. Phenotypic identification of tyramine production was carried out by in modificated decarboxylase medium acording Maijala (1993). The medium used for identification contained 2.09% Casein Yeast Magnesium Broth (Hi Media, India), 0.01% $CaCO_3$ (Merck, Darmstadt, Germany), 0.20% L-tyrosine hydrochloride (Sigma-Aldrich, U.S.A.), 0.006% Bromocresol purple (P-Lab, Prague, Czech Republic). Medium was autoclaved for 15 min at 121°C and pH adjusted to 5.3. Strains were subcultured on Slatnez-Bartley medium (Hi Media, India) and incubated at 37°C for 24 hours. 1 ml of culture suspension with 1.5 McFarland density was inoculated in 1 ml of the decarboxylase medium broth. This was incubated at 20°C and 5°C for 24 h, 48 h, 72 h and 6 days. A purple colored suspension was considered to be positive reaction and a yellow colored to be negative. All strains were tested in duplicates.

Determination of tyramine by HPLC. Tyramine was determined by high performance liquid chromatography (HPLC) method with percolumn derivatisation. The aliquote 0.5 ml of suspension

was derivatised using dansylchloride (5-dimethylaminonaphthalene-1-sulfonylchloride) (Paulsen *et al.* 1997). Analyses were carried out on a liquid chromatograph Alliance 2695 (Waters, USA) with 2475 fluorescence detector. For the separation the chromatographic column Zorbax Eclipse XDB C8, 150 × 4.6 nm, 5 µm (Agilent, USA) was used. Gradient elution was performed, mobile phase A consisted of 0.1M acetic acid: acetonitrile (90:10), mobile phase B of 0.1 M acetic acid:acetonitrile: methanol (45:45), mobile-phase flow rate was 1 ml/min, column temperature 35°C, injection volume was 10 µl. The eluted dansyl derivates were detected by measuring the fluorescence at 330 nm and 500 nm, as excitation and emission lengths. The evaluation were carried out using the Empower software (Waters, USA), the external standard method was used.

RESULTS AND DISCUSSION

Out of 273 total samples analysed 187 originated from various milk and dairy products, 44 from meat and meat products, and 42 were found to be of vegetable and fruit origin. By species specific PCR 4 different species of enterococci were identified from milk and milk products, 5 different species from meat and meat products and also, 5 from vegetable and fruit origin. Of all the origin *E. faecalis* and *E. faecium* were found to be the dominant species in milk and milk products. The results obtained were in agreement with that published by Giraffa (2003). There was a slight contradiction with meat and meat products. Here *E. faecium* followed by *E. faecalis* was found to be dominant. *E. faecium* and *E. mundtii* were found to be the most prominent strains from vegetable and fruit origin. The results were in contradiction to that of Galvez *et al.* (2007) which showed *E. faecalis* and *E. faecium* ruled the fruit and vegetable origin. In all the samples other species were found but in low numbers. Detailed information regarding genotypic and phenotypic analysis is shown in Table 2. Here, from total 273 strains analysed, 243 i.e. 90.8% on PCR analysis showed the presence of *tyrdc* gene (gene responsible for tyramine production in enterococci). Similar pattern of results were found with incubating the samples in modified Maijala's medium. 20 i.e. 7.3% of the strains were found not to decarboxylate tyrosine. There were about 10 i.e. 3.6% of the strains which showed contradictory results in terms of phenotypic and genotypic analysis. Also,

Table 2. Results of genotypic and phenotypic analysis

Source	Strain	Total number identified (%)	Tyramine (%)		Contradictory (%)
			positive	negative	
Milk and milk products	*E. faecalis*	101 (54.01)	93 (49.7)	4 (2.1)	4 (2.1)
	E. faecium	82 (43.85)	79 (42.2)	1 (0.53)	2 (1.06)
	E. casseliflavus	3 (1.6)	2 (1.06)	1 (0.53)	0
	E. durans	1 (0.53)	1(0.53)	0	0
Meat products	*E. faecium*	22 (50)	20 (45.4)	2 (4.5)	0
	E. faecalis	14 (31.81)	12 (27.2)	2 (4.5)	0
	E. mundtii	5 (11.36)	4 (9.0)	1 (2.2)	0
	E. casseliflavus	2 (4.54)	1 (2.2)	1 (2.2)	0
	E. hirae	1 (2.27)	1 (2.2)	0	0
Vegetables and fruits	*E. faecium*	16 (38.09)	15 (35.7)	0	1(2.3)
	E. mundtii	12 (28.57)	6 (14.2)	6 (14.2)	0
	E. casseliflavus	9 (21.42)	5 (11.9)	2 (4.76)	2 (4.76)
	E. faecalis	4 (9.52)	4 (9.52)	0	0
	E. hirae	1 (2.38)	0	0	1 (2.3)
Total		273	243 (90.8 %)	20 (7.3 %)	10 (3.6 %)

from the Table 2 it is evident that almost all the species identified were active in the production of tyramine with *E. faecalis* and *E. faecium* to be the highest. The results of phenotypic analysis were confirmed by performing HPLC. The results showed that 211 i.e. 86.8% of the samples were containing about 1000–1500 mg/l of tyramine, while 10 i.e. 4.1% were found to have tyramine in the range of 100–500 mg/l. The results are threatening if compared to the legal limits set by the Nutritional Codex of the Slovak Republic which says the maximum tolerable limit of tyramine in cheese in 200 mg/kg (Karovičová & Kohajdová 2003). The European Union has set the regulations for levels of histamine. Regulations for tyramine are yet to come. Negative sample showed 0 mg/l of tyramine.

CONCLUSION

Enterococci because of their ability to help in ripening and flavor development in cheese production have been used as a starter culture for various combinations of European cheeses. The present study shows that along with *E. faecalis* and *E. faecium* there are various other species of enterococci responsible for the production of tyramine. Thus, estimation of the levels of tyramine at various stages of production is necessary not only from the point of view of toxicity but also as indicators of freshness or spoilage of food.

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