Production of Biogenic Amines by Enterococci

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Abstract: Enterococci were presented in all tested samples of raw cow milk (six samples) at the level 10^3–10^5 CFU/ml, fresh cheeses (five samples) at the level 10^2–10^6 CFU/g and semi-hard cheeses (five samples) at the level 10^3–10^5 CFU/g. All 33 isolated *Enterococcus* strains were screened for decarboxylase activity by usage differential growth medium and 20 of them possessed tyrosine decarboxylase activity. A collection of eight strains with the strongest decarboxylase activity were identified by species specific PCR as *E. faecium* (Z3, Z4, Br4 and 6/4D strains) and *E. faecalis* (Ž4, 3/3C and 4/1A strains). *Enterococcus* spp. Z1 strain was not identified at the species level by used methods, but the genus was confirmed by PCR method. Their tyrosin decarboxylase activity was confirmed by TLC and detection of *tdc* gene. Z1, Z3 and Z4 strains showed also histidine decarboxylase activity on the differential growth medium with histidine, but this activity was evaluated by TLC as a false positive reaction of medium.

Keywords: *Enterococcus*; decarboxylase; tyrosine; histidine; TLC; PCR

Enterococcus bacteria are ubiquitous microorganisms, but have a predominant habitat in the gastrointestinal tract of humans and animals. Their ubiquitous nature determines their frequent finding in foods as contaminants. In addition, the notable resistance of enterococci to adverse environmental conditions explains their ability to colonise different ecological niches, and may then serve as indicators of the sanitary quality of food, and their spreading within the food chain through contaminated animals and foods. Enterococci can also contaminate finished products, such as fermented foods, and for this reason their presence in many foods (such as cheeses and fermented sausages) can only be limited but not completely eliminated using traditional processing technologies. Enterococci play an important role in the dairy industry. They are used as starter lactic acid bacteria (LAB) and nonstarter lactic acid bacteria (NSLAB) in production of variety of cheeses to develop organoleptic characteristics during the cheese ripening, because they possess acidifying, proteolytic and lipolytic activities and ability to metabolise citrate. In addition they show probiotic and protective activities. On the other hand, enterococci have recently become recognised as serious nosocomial pathogens causing bacteraemia, endocarditis, urinary tract and other infections. Enterococci are low grade pathogens but their intrinsic resistance to many antibiotics and their acquisition of resistance to the few antibiotics available for treatment in clinical therapy, such as the glycopeptides, have led to difficulties and a search for new drugs and therapeutic options. Enterococci can cause food intoxication through production of biogenic amines and can be a reservoir for worrisome opportunistic infections and for virulence traits. Clearly, there is no consensus on the acceptance of their presence in foodstuffs and their role as primary pathogens is still a question mark (Franz *et al*. 1999; Giraffa 2002, 2007).

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Biogenic amines (BA) are natural antinutrition factors and are important from a hygienic point of view as they have been implicated as the causative agents in a number of food poisoning episodes, particularly histamine toxicity 'scombroid poisoning' and tyramine toxicity 'cheese reaction', and they are able to initiate various pharmacological reactions (Shalaby 1995, 1996). In spite of amines being considered as endogenous to food originating from plant matter, such as fruits and vegetables, BA are formed in other foods as a result of microbial action during aging and storage. The most important BA occurring in foods are histamine, putrescine, cadaverine, tyramine, tryptamine, p-phenylethylamine, spermine, and spermidine. Foods likely to contain BA include fish, fish products, meat products, eggs, cheeses, fermented vegetables and soy bean products, beers and wines. The factors which influence the formation of BA in foods include the availability of free amino acids, the presence of microorganisms that can decarboxylate amino acids, the favourable conditions of such microorganisms for the growth and production of their enzymes (Shalaby 1999). *Enterobacteriaceae* and certain LAB, especially enterococci and lactobacilli, are particularly active in the production of BA. These amine-producing microorganisms either may form part of the food associated population or may be introduced by contamination before, during or after processing of the food product. Therefore, microorganisms naturally present in raw materials, introduced throughout the processing or added as starter culture can critically influence BA production during the manufacture of fermented products (Bover-Cid *et al*. 2001).

The aim of this study was to estimate the numbers of enterococci in raw cow milk and several dairy products from different origin, to isolate enterococci strains, to identify them and to characterise their ability to produce tyramine and histamine by different methods.

MATERIALS AND METHODS

Screening of enterococci counts and their isolation. Samples were taken from raw cow milk (6 samples), fresh cheeses (5 samples) and semi-hard cheeses (5 samples), diluted in a sterile saline solution (for milk) or citrate buffer (used only for the preparation of the cheese first dilution) and 100 µl plated on Slanetz-Bartley medium (Oxoid, Great Britain). The plates were incubated at 37°C for 48 h aerobically. All suspect colonies were at first counted and then some of them were selected at random from plates and the isolates were purified by repeated streaking onto Slanetz-Bartley medium. Strains were cultivated in BHI broth at 37°C for 18 h aerobically.

Identification of isolates. The collection of 47 isolated strains was tested for catalase activity and Gram stained according to standard procedures. Identification of the strains was conducted according to cell morphology; CO_2 production from glucose; and the growth at 10, 30, 37 and 45°C; in the presence of 1.2 mol/l NaCl; and in BHI adjusted to pH 4.5 and 9.6, respectively, as described by Axelsson (1998) and Stiles and Holzapfel (1997). Isolates with decarboxylase activity were identified using the ENCOCCUStest (Pliva-Lachema, a.s., Brno, Czech Republic), by the production of pyrolidonyl arylamidase (PYRAtest, Pliva-Lachema, a.s., Brno, Czech Republic), the motility and the production of pigment as well. Genus specific confirmation was done using PCR method with detection of *tuf* gene according to Cupáková *et al.* (2005). Species specific identification was based on detection of *sod*A gene by duplex PCR using primers specific for *Enterococcus faecium* or *E. faecalis* as published by Jackson *et al.* (2004). DNA for genotypic identification was extracted by GenElute^{TM} Bacterial Genomic Kit (Sigma Aldrich, USA).

Screening of decarboxylase activity. All freshly grown *Enterococcus* strains were streaked onto improved medium with tyrosine (TDAM) or histidine (HDAM) as published by Bover-Cid and Holzapfel (1999). Strains with purple colour of medium around colonies possessed decarboxylase activity (positive tyrosine, tdc^+; or histidine, hdc^+ decarboxylase activity). Production of BA was detected also by thin-layer chromatography (TLC) according to Landeta *et al.* (2007). Tyrosine decarboxylase activity was besides these two methods confirmed by PCR method as published by Marcobal *et al.* (2005).

RESULTS AND DISCUSSION

Enterococci were determined in all tested 16 samples. Their counts in raw milk (six samples) were at the level 10^3–10^5 CFU/ml. *Enterococcus* spp. is well

known for its resistance to the heat treatment and to other physico-chemical parameters (salt content, pH), its occurrence in raw milk, fresh and semi-hard cheeses, pasteurised and non-pasteurised products, is common (Flint 2002; Giraffa 2003; Jurkovič *et al*. 2006). This was proved in this study as well, in fresh cheeses (five samples) counts of enterococci were at the level 10^2–10^6 CFU/g and in semi-hard cheeses (five samples) they were at the level 10^3–10^5 CFU/g.

All 33 isolates preliminary identified as enterococci were screened for histidine (*hdc* activity) and tyrosine (*tdc* activity) decarboxylase activity using improved agar growth media TDAM and HDAM. The *tdc* activity was observed within 20 strains, and three of them possessed also *hdc* activity. On the basis of evaluation of the purple colour intensity of TDAM and HDAM, the collection of eight strains with the highest decarboxylase activity was selected for other experiments and identified on the species level. Results are shown in Table 1. Differential growth media, as TDAM and HDAM, allow an easy, cheap, and rapid preliminary selection of strains possessing amino acid decarboxylase activity (Marcobal *et al.* 2006). However, the detection of BA producing bacteria by conventional culture techniques is often tedious and unreliable, exhibiting disadvantages such as lack of speed, appearance of false positive/negative results, low sensibility, requirements for costly and sophisticated equipment or that only one BA is detected (Landeta *et al.* 2007). The problem of false positive and negative reactions of growth media for detection decarboxylase has been persisting despite different improvements and modifications of these media and was described by many authors (Bover-Cid & Holzapfel 1999; Suzzi & Gardini 2003; Marcobal *et al.* 2006). Histidine decarboxylase activity of Z1, Z3 and Z4 strains observed on the HDAM was not during other experiments stable and consequently it was confirmed as false positive reaction of medium by use of TLC method. All eight strains were *tdc*$^+$; four of them (Z3, Z4, Br4 and 6/4D) were identified as *E. faecium* and three of them (Ž4, 3/3C and 4/1A) as *E. faecalis*. *Enterococcus* spp. Z1 strain was not species identified by used methods (ENCOCCUStest and PCR with detection of *sod*A gene with specific primers for *E. faecalis* and *E. faecium*), but the genus was confirmed by PCR method with detection of *tuf* gene. Enterococci are probably irrelevant in cheese-related histamine toxicity. However, it was reported that *E. faecalis* produced 2-phenylethylamine and also substantial amounts of tyramine in skim milk, which is the main raw material of fermented dairy products (O'Brien *et al.* 2004). Significant part of Terrincho sheep cheese microflora form enterococci, they contribute to the increased production of tyramine, cadaverine, putrescine and other BA according to the amount of free amino acids (Pintado *et al.* 2008). *Enterococcus* spp. isolates with tyramine production were detected also in dutch type semi-hard cheese, especially by *E. durans, E. faecalis* and *E. casseliflavus* (Komprda *et al.* 2008).

As molecular methods are fast, reliable and culture-independent, they represent an interesting

Table 1. Characterisation enterococci with decarboxylase activity

Strain	Source	Activity		Species identification
		tdc	*hdc*	
Z1	semi-hard cheese	+	+*	*Enterococcus* spp.
Z3	semi-hard cheese	+	+*	*E. faecium*
Z4	semi-hard cheese	+	+*	*E. faecium*
Br4	fresh cheese	+	–	*E. faecium*
Ž4	fresh cheese	+	–	*E. faecalis*
3/3C	raw cow milk	+	–	*E. faecalis*
4/1A	raw cow milk	+	–	*E. faecalis*
6/4D	semi-hard cheese	+	–	*E. faecium*

+ positive decarboxylase activity; – negative decarboxylase activity; *consequently revealed as false positive reaction of HDAM medium

alternative for the detection of bacteria producing BA. Since the BA are produced by the decarboxylation of a precursor amino acid by the enzymatic action of an amino acid decarboxylase, it is possible to develop molecular detection methods targeting the genes coding for these amino acid decarboxylase enzymes (Marcobal *et al*. 2006). In this study the detection of *tdc* gene by PCR method according to Marcobal *et al*. (2005) was used. A primer set previously described, P1-rev (5'-CCRTARTCNGGNATAGCRAARTCNGTRTG-3') and P2-for (5'-GAYATNATNGGNATNGGNYTN-GAYCARG-3'), was used to amplify a 924 bp final PCR product. In all of eight strains it was able to detect the corresponding a 924 bp *tdc* amplicon except for negative controls (*Lc. lactis* subsp. *lactis* NIZO B643, *Lc. lactis* subsp. *cremoris* NIZO B33, *Lbc. paracasei* 171R2) (Figure 1). This set of primers for detection *tdc* gene was used in the study of De Las Rivas *et al*. (2008) as well. They also tested another pair of primers for *tdc* gene (TDC-F and TDC-R) and obtained results were the same. Simultaneous detection of genes that encode different amino acid decarboxylases was previously described by many authors (Coton & Coton 2005; Burdychova & Komprda 2007; Komprda *et al.* 2008; Landete *et al*. 2007) and was usually designed as multiplex PCR with different kind of primers.

TLC methods constitute a simple solution to the reports describing false-positive or negative reactions in routine screening procedures generally involving the use of a differential medium containing a pH indicator. TLC methods have many advantages, including simplicity of operation and cost effectiveness since many samples can be analysed on a single plate with use of a low amount of solvent. This method can be easily adopted by microbiology laboratories as a quality tool for the analysis by BA production by bacteria (Marcobal *et al.* 2006) and was successfully used for detection of BA by many other authors (Roig-Sagués *et al*. 1997; Shalaby 1999; Pessione *et al*. 2005). In our study only tyramine was detected by this method in all eight strains except for negative controls (*Lc. lactis* subsp. *cremoris* NIZO B33, *Lbc. paracasei* 171R2) – Figures 2 and 3.

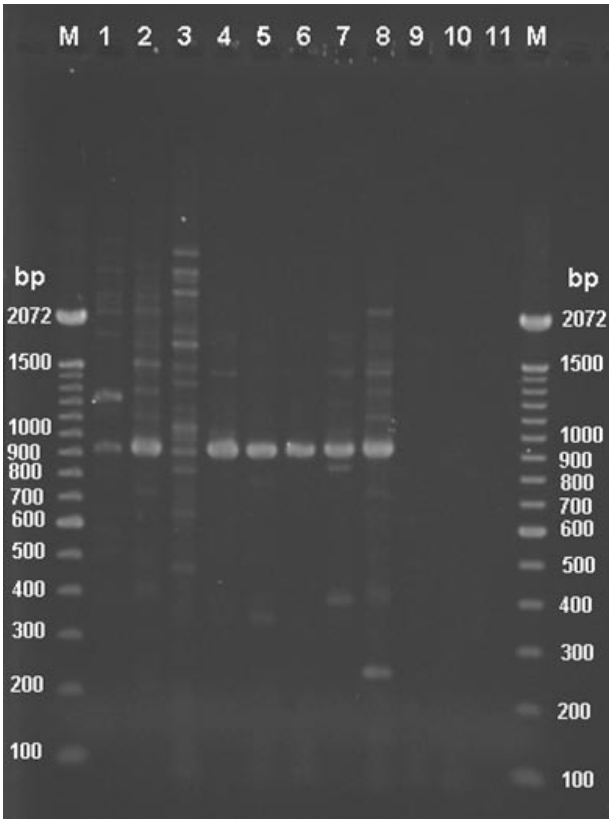

Figure 1. Detection of *tdc* gene: M – 100 bp DNA marker, 1 – Z1; 2 – Z3; 3 – Z4; 4 – Ž4; 5 – Br4; 6 – 3/3C; 7 – 4/1A; 8 – 6/4D; 9 – *Lc. lactis* subsp. *lactis* NIZO B643; 10 – *Lc. lactis* subsp. *cremoris* NIZO B33; 11 – *Lbc. paracasei* 171R2

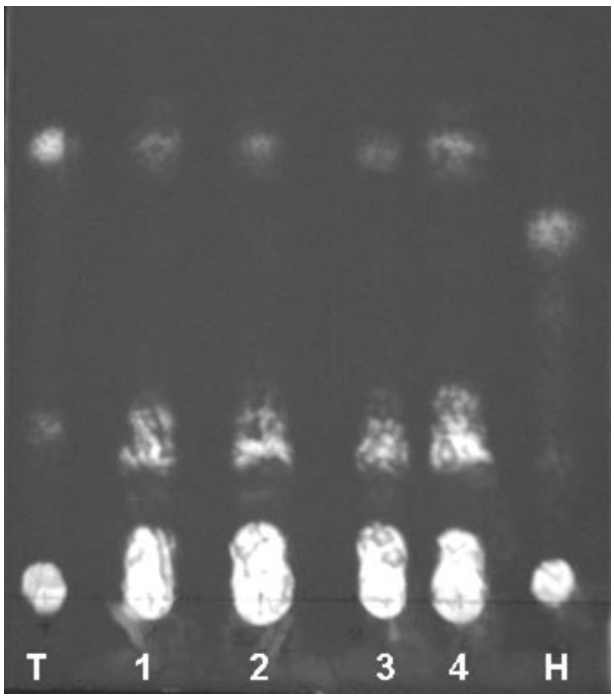

Figure 2. Detection of tyramine and histamine by TLC: T – tyramin;, H – histamine; 1 – Z1, 2 – Z3; 3 – Z4,; 4 – Ž4

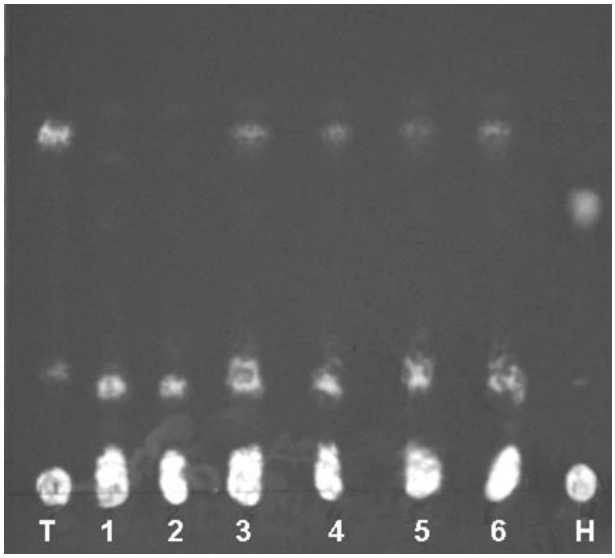

Figure 3. Detection of tyramine and histamine by TLC: T – tyramine; H – histamine; 1 – *Lc. lactis* subsp. *cremoris* NIZO B33; 2 – *Lbc. paracasei* 171R2; 3 – Br4; 4 – 3/3C; 5 – 4/1A; 6 – 6/4D

CONCLUSIONS

Enterococci as potential producers of biogenic amines were presented in all tested samples of cow raw milk, fresh and semi-hard cheeses. All isolated *Enterococcus* strains were screened for decarboxylase activity by usage differential growth medium, 20 strains from 33 strains possessed tyrosine decarboxylase activity. A collection of eight strains with the strongest decarboxylase activity were identified by species specific PCR method and their tyrosin decarboxylase activity was confirmed by TLC and detection of *tdc* gene. Results obtained by screening easy method based on differential growth media, as TDAM and HDAM, should be confirmed by use of molecular or instrumental methods (e.g. TLC or HPLC).

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