Journal of Dairy Research (2006) **73** 318–321. *© Proprietors of Journal of Dairy Research 2006*
doi:10.1017/S0022029906001750 *Printed in the United Kingdom*

Early PCR detection of tyramine-producing bacteria during cheese production

María Fernández, Ana Belén Flórez, Daniel M Linares, Baltasar Mayo and Miguel A Alvarez*

Instituto de Productos Lácteos de Asturias (CSIC), Carretera de Infiesto s/n, 33300 Villaviciosa, Asturias, Spain

Received 27 May 2005 and accepted for publication 17 December 2005

Biogenic amines (BA) are toxic substances that appear in foods and beverages. Tyramine is the most abundant BA in cheeses. A PCR method was developed to detect the presence of tyramine-producing bacteria during cheese manufacture and ripening. Six different batches of a farmhouse blue cheese were analysed by PCR. Tyramine concentrations were also determined by HPLC. The PCR method was able to anticipate tyramine accumulation in the cheeses; the presence of tyramine-producing microorganisms in the early stages of manufacture correlated well with a high concentration of BA in mature cheese samples.

Keywords: Biogenic amines, tyrosine decarboxylase, tyramine, PCR detection, cheese.

Biogenic amines (BA) are organic bases of low molecular weight that are produced during the normal metabolism of microorganisms, plants and animals. These compounds have important physiological properties (Shalaby, 1996), but a number of toxic psychoactive and vasoactive effects have been associated with the consumption of foods containing high concentrations. Psychoactive BA affect the nervous system by acting on neural transmitters, while vasoactive BA alter blood pressure. These problems are more severe in persons who, because of genetic reasons or medical treatment, have low monoamine oxidase activity (Bodmer et al. 1999); this enzyme is responsible for BA detoxification. Histamine poisoning (scombroid poisoning) and tyramine toxicity (cheese reaction) are two examples of food-borne diseases caused by excess BA consumption (Shalaby, 1996).

Biogenic amines are frequently found in a wide range of foods and drinks, although their concentrations vary extensively (both between and within foods) (Silla-Santos, 1996). Foods likely to contain high levels are dairy products, fish and fish products, meat and meat products, fermented vegetables and soy products, and alcoholic beverages such as wine and beer (Silla-Santos, 1996; Lonvaud-Funel, 2001; Suzzi & Gardini, 2003).

Biogenic amines are formed by the decarboxylation of the corresponding amino acid. This decarboxylation depends on the presence of microorganisms containing specific decarboxylase activity as well as the availability of the substrate amino acid. Other variables such as temperature, pH, a_w or NaCl concentration may be involved in the synthesis and/or activity of these microbial enzymes (Suzzi & Gardini, 2003). Microorganisms involved in BA production were once thought of only as contaminants, but in fermented foods these microorganisms are frequently associated with the raw material and, in some instances, may even participate in the fermentation process (Joosten & Northolt, 1987; Rodríguez-Jerez et al. 1994).

From a microbial point of view, cheese is a complex habitat which is rather similar in all the studied cheese made with raw milk. LAB are predominant with Lactococci being the most numerous microorganisms during all the ripening stages. Lactobacilli reach significant densities although lower than those of lactococci. In Cabrales cheese, at the end of the ripening period lactobacilli and lactococci reach densities of 10^6 cfu/g and 10^8 cfu/g respectively (Flórez et al. 2005). Besides the probable presence of decarboxylase-positive microorganisms (some strains of *Lactobacillus, Lactococcus, Enterococcus*), cheese is an ideal substrate for BA formation since the microorganism decarboxylase activity is favoured by the environmental conditions of ripening. In addition, the manufacturing process involves proteolysis and free amino acid release (Novella-Rodríguez et al. 2003a). In cheese, the BA that appears with the greatest frequency and in the highest concentration is tyramine, although considerable variation is seen between different cheese types (Novella-Rodríguez et al. 2003a). Tyramine is formed by the decarboxylation of tyrosine, an amino acid that can be present in very high concentrations in cheese.

Current tyramine detection methods are mainly based on expensive time-consuming techniques such as HPLC

*For correspondence; e-mail: maag@ipla.csic.es

https://doi.org/10.1017/S0022029906001750 Published online by Cambridge University Press

Table 1 Tyrosine and tyramine concentrations of samples during cheese manufacture at 3, 7, 15, 30, 60 and 90 days of ripening

Values are means ± SD for $n=6$

	tyrosine mg/kg	tyramine mg/kg
raw milk	117·69 ± 12·48	0
Curd	148·25 ± 50·27	0
3 d	167·89 ± 61·23	0
7 d	128·8 ± 10·23	0
15 d	90·9 ± 21·77	8·04 ± 6·91
30 d	612·99 ± 47·34	87·18 ± 57·02
60 d	1491·72 ± 318·59	334·81 ± 261·3
90 d	2191·42 ± 613·98	1041·81 ± 926·76
pasteurized milk	101·99	0
pasteurized cheese	2770·44	0

analysis, capillary electrophoresis or the use of enzyme sensor arrays (Lange et al. 2002; Lange & Wittmann, 2002). The use of molecular tools for the early and rapid detection of BA-producing microorganisms in foods would be very useful for predicting the accumulation of these compounds. The comparison of the tyrosine decarboxylase gene clusters from different producing microorganisms revealed a high similarity either in gene sequence or organization. Moreover, a clear relationship between the presence of the tyrosine decarboxylase gene (*tdcA*) and the ability to produce tyramine was also determined. Based on these results, a PCR method for the detection of tyramine-producing strains was proposed (Fernández et al. 2004). The aim of this study was to use the PCR method that amplifies an internal fragment of *tdcA* (Fernández et al. 2004), in order to detect tyramine-producing strains during cheese manufacture. With this knowledge it may be possible to take steps to prevent tyramine accumulation.

Materials and Methods

Cheese samples

Cabrales, a traditional, Spanish blue-veined cheese made with raw milk without starters, was used as a model in this study. Samples of six independent batches were taken at different manufacturing and ripening points, i.e., from raw milk, curd and cheese at 3, 7, 15, 30, 60 and 90 days of ripening. As negative controls, commercial pasteurised milk and a cheese elaborated with pasteurised milk were used.

Sample preparation and PCR analysis

Five grams of each sample were homogenized mechanically in 40 ml sodium citrate (20 g/l) in a Stomacher Lab-Blender 400 (Seward Medical, London, UK) for 1 min. DNA was extracted from the homogenate following the method of Ogier et al. (2002), and 20 ng were used in each PCR reaction. The oligonucleotides used and the PCR reaction conditions were as previously described

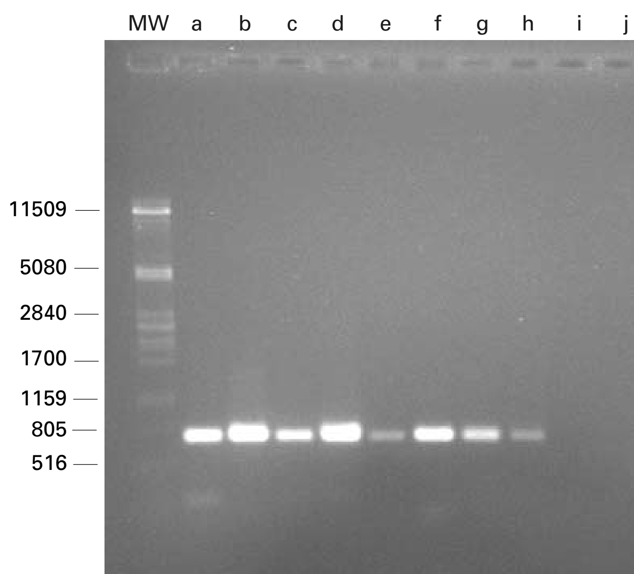

Fig. 1. PCR detection of tyramine-producing bacteria. Lane a raw milk; b curd; c, d, e, f, g and h represent cheeses at 3, 7, 15, 30, 60 and 90 days of ripening respectively; i pasteurized milk; j pasteurized milk cheese.

(Fernández et al. 2004). The amplicons were separated and visualized on 0·7% agarose gels as described by Sambrook et al. (1989).

Nucleotide sequence analysis

The PCR products were purified using the GenElute™ PCR Clean-Up Kit (Sigma-Aldrich St. Louis, MO, USA) according to the manufacturer's instructions. Sequencing was performed using an ABI Prism 373 A Strech automated sequencer. All sequences were analysed using CLONE software (v. 5) and compared with those in the SwissProt library (February 2004 release) and GenBank database using the TFASTA software (Pearson & Lipman, 1988).

Analytical procedure

Amine quantification was performed by reverse-phase (RP)-HPLC using a Waters liquid chromatograph controlled by Millenium 32 software (Waters, Milford, MA, USA). One gram of each sample was homogenized for 2 min at 20 000 rpm using an Ultra-Turrax homogenizer (OMNI International, Watersburry, USA), and the BAs extracted and quantified following the protocol described by Krause et al. (1995).

Results and discussion

In vitro amplification of nucleic acids by PCR has become a powerful tool for the detection of microorganisms in food samples. The technique offers the advantages of

https://doi.org/10.1017/S0022029906001750 Published online by Cambridge University Press

speed, specificity, sensitivity and simplicity. This study was initiated to evaluate the efficacy and usefulness of PCR for detecting the presence of tyramine-producing strains during cheese manufacture. Cabrales, a blue cheese previously described as having high tyramine concentrations (Roig-Sagués et al. 2002) was selected as a model.

Tyramine was detected by HPLC in cheese samples that had ripened for 15, 30, 60 and 90 d (Table 1). However, this technique detected no tyramine in the raw milk, curd, 3 or 7-day ripened cheese, pasteurized milk or pasteurized milk cheese (Table 1). Nevertheless, a band of the expected size (720 bp) corresponding to an internal fragment of the *tdcA* gene was detected by PCR in every sample type in every one of the six independent Cabrales cheese production processes (Fig. 1). The sequence of these PCR fragments revealed homology with *tdcA* gene sequences in the databases consulted (data not shown), showing the specificity of the reaction. In fact, this sequence is so highly conserved that it is not possible to distinguish *tdcA* genes from different species (Fernández et al. 2004).

No PCR products were formed with the pasteurized milk and pasteurized milk cheese. It has been previously shown that most of the tyramine producer microorganisms do not survive the pasteurization treatment and therefore, this process reduces the biogenic amine content in cheeses (Novella-Rodríguez et al. 2004). After pasteurization, DNA from dead tyramine producers would not be detected by PCR because DNA can easily be damaged by (i) the heat treatment, (ii) the bacterial nucleases and (iii) the nucleases present in milk.

Several authors have described the time of ripening as a critical factor in BA accumulation. The proteolysis of cheese increases over the ripening period, yielding free amino acids that can then be decarboxylated by microbial enzymes to produce BA (Novella-Rodríguez et al. 2003b). Fernández-García et al. (2000) showed the influence of proteinase activity on tyramine accumulation in Manchego cheese, and Leuschner et al. (1998) reported the effect of enhanced proteolysis on BA accumulation in Gouda cheese. Accordingly, the concentration of tyrosine (the substrate of the reaction), which was low in the original milk and at the beginning of the ripening period, explains the absence of tyramine in these samples (Table 1). A rise in the tyramine concentration was observed as ripening progressed (Table 1). The differences in the results between the HPLC and PCR methods show that PCR could be used to detect the presence of tyramine-forming bacteria in the early stages of manufacture – even before tyramine is synthesized. The wide range of tyramine accumulation seen among the different blue cheese samples (Table 1) has been reported by other authors (Roig-Sagués et al. 2002), but it is important to note that PCR products were obtained in all of them. The differences between the cheese samples in terms of the concentrations of these products (Fig. 1) cannot be directly related to the tyramine concentration of the samples since conventional PCR is not a quantitative tool. However, these results open up the possibility of developing quantitative real time PCR methods.

Pasteurization of the original milk (Novella-Rodríguez et al. 2003b) and especially the analysis of starter culture composition should be considered if tyramine accumulation at the end of cheese ripening is to be avoided. This would offer a healthier product to consumers. Indeed, the absence of amino acid decarboxylase activity is now included in the selection criteria for the industrial preparation of malolactic starters used in winemaking (Lonvaud-Funel, 2001). In addition to these preventive measures, the proposed PCR method allows the detection of tyramine-producing bacteria at any point in the fermentation. PCR positive results during the first stages of elaboration would strongly recommended an analysis of the tyramine concentration in the final product. Furthermore, the frequent presence of tyramine-producing microorganisms in the early stages of manufacture of a given cheese made with raw milk, would strongly recommend the use of pasteurized milk. Lastly, the rapid detection of these microorganisms would be particularly useful for guaranteeing the production of tyramine-free cheeses for people with low levels of monoamine oxidase (Bodmer et al. 1999).

This work was supported by European Union research grant no. QRLT-2002-02388. DM Linares was the recipient of a fellowship from the Spanish Ministry of Education and Science. M Fernández is the beneficiary of 13P CSIC contracts financed by the European Social Fund.

References

Bodmer S, Imark C & Kneubühl M 1999 Biogenic amines in food: Histamine and food processing. *Inflammation Research* **48** 296–300

Fernández M, Linares DM & Alvarez MA 2004 Sequencing of the tyrosine decarboxylase cluster of *Lactococcus lactis* IPLA 655 and the development of a PCR method for detecting tyrosine decarboxylating lactic acid bacteria. *Journal of Food Protection* **67** 2521–2529

Fernández-García E, Tomillo J & Nuñez M 2000 Formation of biogenic amines in raw milk Hispanico cheese manufactured with proteinases and different levels of starter culture. *Journal of Food Protection* **63** 1551–1555

Flórez AB, López-Díaz TM, Álvarez P & Mayo B 2005 Microbial characterisation of the traditional Spanish blue-veined Cabrales cheese: identification of dominant lactic acid bacteria *European Food Research and Technology* DOI 10.1007/s00217-005-0230-8

Joosten HMLJ & Northolt MD 1987 Conditions allowing the formation of biogenic amines in cheese. 1. Decarboxylative properties of some non-starter bacteria. *Netherlands Milk and Dairy Journal* **41** 259–280

Krause I, Bockhardt A, Neckemann H, Henle T & Klostermeyer H 1995 Simultaneous determination of amino acids and biogenic amines by reversed-phase high performance liquid chromatography of the dabsyl derivatives. *Journal of Chromatography A* **715** 67–79

Lange J, Thomas K & Wittmann C 2002 Comparison of a capillary electrophoresis method with high-performance liquid chromatography for the determination of biogenic amines in various food samples. *Journal of Chromatography B* **779** 229–39

Lange J & Wittmann C 2002 Enzyme sensor array for the determination of biogenic amines in food samples. *Analytical and Bioanalytical Chemistry* **372** 276–283

Leuschner RGK, Kurihara R & Hammes WP 1998 Effect of enhanced proteolysis on formation of biogenic amines by lactobacilli during

https://doi.org/10.1017/S0022029906001750 Published online by Cambridge University Press

Gouda cheese ripening. *International Journal of Food Microbiology* **44** 15–20

Lonvaud-Funel A 2001 Biogenic amines in wines: role of lactic acid bacteria. *FEMS Microbiology Letters* **199** 9–13

Novella-Rodríguez S, Veciana-Nogués MT, Izquierdo-Pulido M & Vidal-Carou MC 2003a Distribution of biogenic amines and polyamines in cheese. *Journal of Food Science* **68** 750–755

Novella-Rodríguez S, Veciana-Nogués MT, Trujillo-Mesa AJ & Vidal-Carou MC 2003b Profile of biogenic amines in goat cheese made from pasteurized and pressurized milks. *Journal of Food Science* **67** 2940–2944

Novella-Rodríguez S, Veciana-Nogués MT, Roig-Sagués A, Trujillo-Mesa A & Vidal-Carou MC 2004 Evaluation of biogenic amines and microbial counts throughout the ripening of goat cheeses from pasteurized and raw milk. *Journal of Dairy Research* **71** 245–252

Ogier JC, Son O, Gruss A, Tailliez P & Delacroix-Buchet A 2002 Identification of the bacterial microflora in dairy products by temporal temperature gradient gel electrophoresis. *Applied and Environmental Microbiology* **68** 3691–3701

Pearson WR & Lipman DJ 1988 Improved tools for biological sequence comparison. *Proceedings of the National Academy of Sciences USA* **85** 2444–2448

Rodríguez-Jerez JJ, Mora-Ventura MT, López-Sabater EI & Hernández-Herrero MM 1994 Histidine, lysine and ornithine decarboxylase bacteria in Spanish salted semi-preserved anchovies. *Journal of Food Protection* **57** 784–788

Roig-Sagués AX, Molina AP & Hernández-Herrero MM 2002 Histamine and tyramine-forming microorganisms in Spanish traditional cheeses. *European Food Research and Technology* **215** 96–100

Sambrook H, Fritsch EF & Maniatis T 1989 Molecular cloning a laboratory manual 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Shalaby AR 1996 Significance of biogenic amines to food safety and human health. *Food Research International* **29** 675–690

Silla-Santos MH 1996 Biogenic amines and their importance in food. *International Journal of Food Microbiology* **29** 213–231

Suzzi G & Gardini F 2003 Biogenic amines in dry fermented sausages: a review. *International Journal of Food Microbiology* **15** 41–54

https://doi.org/10.1017/S0022029906001750 Published online by Cambridge University Press