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Production of biogenic amines "in vitro" in relation to the growth phase by *Enterobacteriaceae* species isolated from traditional sausages

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ABSTRACT

Histidine, lysine, ornithine and tyrosine decarboxylase activities were tested in 79 strains of *Enterobacteriaceae* (41 of *Hafnia alvei*, 17 of *Serratia liquefaciens*, 5 of *Enterobacter cloacae*, 4 of *Citrobacter braakii*, 2 of *Proteus vulgaris*, 2 of *Proteus mirabilis*, 2 of *Providencia stuartii*, 2 of *Klebsiella terrigena*, 1 of *Rahnella aquatilis*, 1 of *Salmonella arizonae*, 1 of *Citrobacter youngae* and 1 of *Escherichia coli*) isolated from Botillo, a Spanish traditional sausage. In general, the strains were positive for all four activities, with the exception of two strains of *H. alvei* and the *E. coli* strain, which did not display histidine decarboxylase activity. The strains of *P. mirabilis* and *P. stuartii* did not exhibit any of the four activities tested.

Accumulation of putrescine and cadaverine was studied throughout growth of the 75 strains that displayed ornithine and lysine decarboxylase activities. Biogenic amines were produced particularly in the exponential phase, with maximum accumulation occurring after between 12 to 72 h, depending on the biogenic amine and microbial species considered. Maximum accumulation of putrescine varied greatly between species and within the same species, and ranged from 18 mg/l in the *R. aquatilis* strain to 7325 mg/l in a *H. alvei* strain. Maximum accumulation of cadaverine varied less than that of putrescine, and ranged from 30 mg/l in the *R. aquatilis* strain to 1935 mg/l in a *S. liquefaciens* strain.

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1. Introduction

Biogenic amines are basic nitrogen compounds usually formed by decarboxylation of precursor amino acids (Janz, Scheiber & Beutling, 1983; Halász, Baráth, Simon-Sarkadi & Holzapfel, 1994; Silla Santos, 1996).

Formation of biogenic amines in foods is important not only because of the unfavourable effects on flavour, but also as regards health. Biogenic amines affect blood pressure, and excessive quantities in food can trigger migraines, gastric and intestinal problems and allergic responses in sensitive people (Smith, 1980; Taylor, 1985, Stratton, Hutkins & Taylor, 1991). These substances are especially dangerous in people being treated with monoaminooxidase enzyme inhibitors (Stratton et al., 1991).

During ripening of meat products, the proteins undergo degradation processes; large peptides are first generated and then degraded into oligopeptides, and these are in turn degraded to free amino acids. The free amino acids are then catabolised, giving rise to different compounds such as ammonia, α-ketoacids, methylketones and amines.

In meat products, formation of biogenic amines is largely associated with the activity of microorganisms present in meat (Ten Brink, Damink, Joosten & Huis in't Veld, 1990; Shalaby, 1996; Paulsen & Bauer, 1997). In raw-cured sausages, microorganisms of the *Enterobacteriaceae* family have been revealed to have a high capacity for producing biogenic amines, particularly putrescine and cadaverine (Silla Santos, 1998; Bover-Cid, Hugas, Izquierdo-Pulido & Vidal-Carou, 2001; Durlu-Özkaya, Ayhan & Vural, 2001).

Ripening of sausages provides conditions that are very favourable for the production of biogenic amines, due to the active growth of microbial populations, acidification and proteolysis. Different measures have been taken with the aim of preventing or minimizing formation of biogenic amines during the manufacture of raw-cured sausages, such as improved hygiene in production plants, the use of starter cultures formed by lactic acid bacteria with acidifying capacity, and the use of certain preservatives (Buncic et al., 1993; Bover-Cid, Hugas, Izquierdo-Pulido & Vidal-Carou, 2000, 2001; Bover-Cid, Hugas, Izquierdo-Pulido & Vidal-Carou, 2000; Maijala, Eerola, Aho & Hirn, 1993; Suzzi & Gardini, 2003; Komprda et al., 2004; Lu et al., 2010). Although such practices usually reduce the production of biogenic amines, they do not totally prevent the production, and moreover, the increased proteolysis that results from the use of starter cultures may actually increase the availability of amino acids precursors.

Complete inhibition of biogenic amine formation during production of sausages, without any adverse effects, is desirable. However, production of biogenic amines is an extremely complex phenomenon that depends on several variables such as the growth kinetics of the microorganisms and their proteolytic and decarboxylase activities. In

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order to design strategies for specific inhibition of the production of these compounds, it is essential to obtain information about the potential production of biogenic amines by the microorganisms present in fermented meat products and about the growth phase during which maximum production takes place.

In a previous study (Lorenzo, Martínez, Franco & Carballo, 2008), appreciable concentrations of biogenic amines, particularly putrescine and cadaverine, were observed in Spanish traditional sausages, and the *Enterobacteriaceae* present in these meat products seems to be responsible for these amine formation.

Studies on the production of biogenic amines by *Enterobacteriaceae* strains are scarce, and existing studies are generally qualitative (Silla Santos, 1998) or only quantify the amount of amines produced by the strains after a specific incubation period (Bover-Cid et al., 2001; Durlu-Özkaya et al., 2001; Pircher, Bauer & Paulsen, 2007). To date, no studies of the production and accumulation of biogenic amines throughout growth of the *Enterobacteriaceae* species have been published.

The objectives of the present study, which forms part of a wider research project involving improvement of the quality of traditional sausages, were: (i) to evaluate the ability of strains of *Enterobacteriaceae* isolated from traditional sausages to produce biogenic amines "in vitro", (ii) to study the production of these biogenic amines throughout the different stages of the growth of the strains, and (iii) to determine the maximum quantity of each amine accumulated in broth culture of each strain.

2. Materials and methods

2.1. Bacterial strains and preparation of inocula

In this study, 79 strains of *Enterobacteriaceae* (41 of *Hafnia alvei*, 17 of *Serratia liquefaciens*, 5 of *Enterobacter cloacae*, 4 of *Citrobacter braakii*, 2 of *Proteus vulgaris*, 2 of *Proteus mirabilis*, 2 of *Providencia stuartii*, 2 of *Klebsiella terrigena*, 1 of *Rahnella aquatilis*, 1 of *Salmonella arizonae*, 1 of *Citrobacter youngae* and 1 of *Escherichia coli*) were used. The strains were isolated from a traditional Spanish sausage (Botillo) at the end of the manufacturing process. Botillo is manufactured in NW of Spain from pieces of ribs, vertebrae and other pork bones with their fleshy parts, skin and lard. The meat is mixed with salt, sweet and spicy paprika, garlic and marjoram and is left to stand for 48 h and then stuffed into pork caecum. It then undergoes a heating-smoking process for 7 days, and afterwards it is left to dry and ripen for a period from 15 days to 3 months. The *Enterobacteriaceae* strains were initially identified by classical methods (García Fontán, Lorenzo, Martínez, Franco & Carballo, 2007) and their identity was confirmed with the API™ 20E system (bioMérieux, Marcy-l'Etoile, France). Strains were stored at −80 °C in nutrient broth (Oxoid Ltd., Basingstoke, Hampshire, UK), with 20% glycerol as a cryoprotective agent. Before use, the strains were reactivated by incubation in nutrient broth at 37 °C.

In order to prepare the inocula used in the quantitative analysis, samples of nutrient broth cultures were collected after 15 h of incubation, centrifuged at 12,000×*g* and the cells (sediment) were washed by resuspension in a solution of 0.85 % NaCl and centrifugation at 12,000×*g* (three times). Finally, the cells were suspended in the 0.85 % NaCl solution to provide inocula containing 5×10^8 CFU/ml.

2.2. Preliminary qualitative tests for biogenic amine production

As a preliminary test of the capacity of the bacterial strains to produce biogenic amines, the method described by Joosten and Northolt (1987) was used. The culture medium used contained tryptone (0.5%), yeast extract (0.5%), NaCl (0.5%), glucose (0.1%), Tween 80 (0.05%), $MgSO_47H_2O$ (0.02%), $CaCO_3$ (0.01%), $MnSO_44H_2O$ (0.005%), $FeSO_47H_2O$ (0.004%), bacteriological agar (2%), and purple bromocresol (0.006%) as pH indicator. The precursor amino acids of each biogenic amine (histidine, lysine, ornithine, and tyrosine) were added individually to the culture medium to a final concentration of 2%. The final pH was adjusted to 5.5 ± 0.1, the medium was sterilized and distributed in Petri dishes. Plates of the culture medium containing each one of the precursor amino acids were streaked, in order to obtain individual colonies, with each bacterial strain. The plates were incubated at 37 °C and examined after 12, 24, 48, 72 and 120 h of incubation; a positive result was manifested by the appearance of a purple halo around the colonies.

2.3. Quantitative analysis of the biogenic amines produced throughout growth of the bacterial strains

Firstly, in order to determine the growth curves for the different microbial strains used, tubes containing 5 ml aliquots of culture medium described by Joosten and Northolt (1987), without agar or bromocresol purple, were each inoculated with 0.1 mL of a solution (0.85 g NaCl/l) containing 5×10^7 CFU of the strain. The tubes, with a final concentration of 10^7 CFU/ml, were incubated at 37 °C. At intervals of 3 h, until 96 h of incubation, one tube was removed and the optical density (OD) was measured in a DINKO 8500 II spectrophotometer (DINKO Instruments, Barcelona, Spain).

In order to quantify the production of each biogenic amine throughout growth of the different bacterial strains, in each bacterial strain, and for each individual precursor amino acid, 20 tubes (5 ml each) of the culture medium (Joosten & Northolt, 1987) containing 2% of the corresponding individual precursor amino acid were each inoculated with 0.1 ml of a solution (0.85 g NaCl/l) containing 5×10^7 CFU. The tubes, with a final concentration of 10^7 CFU/ml, were incubated at 37 °C. For each bacterial strain and each precursor amino acid, two different tubes were taken after 0, 2, 4, 8, 12, 16, 24, 48 and 72 h of incubation. The OD was measured in one tube, and the corresponding biogenic amine was determined in the other. Firstly, 1 ml of 2 N HCl was added to the tube in order to stop microbial growth and decarboxylation. The content of the tube was then placed in a 25 ml volumetric flask, 1 ml of 1.7 diaminoheptane (internal standard) was added, and the final volume was made up with a 0.6 N $HClO_4$ solution. An aliquot (0.5 ml) of the mixture was then immediately placed in a tube, and 100 µl of 2 N NaOH (to make the solution more alkaline), 150 µl of a saturated solution of $NaHCO_3$ and 1 ml of dansyl chloride, were added consecutively. The tube was shaken gently, and placed in a water bath at 40 °C for 45 min. In order to remove residues of dansyl chloride, 50 µl of ammonia were then added and the mixture was left to stand for 30 min. Finally, the volume was made up to 2.5 ml with acetonitrile and the mixture was filtered (0.25 µm).

Separation, identification and quantification of the biogenic amines were carried out by HPLC, following the procedure described by Eerola, Hinkannen, Lindfors and Hirvi (1993), with a Spectra System chromatograph (Thermo Finnigan, San José, CA, USA) equipped with a SCM 1000 degasser, a P4000 pump, an AS 3000 automatic injector and a Photodiode Array UV6000LP detector. Separation of the different biogenic amines was carried out on a reverse phase C18 mod Kromasil 100 column (15 cm, 4 mm ID) (Teknokroma S. Coop. C. Ltda., San Cugat del Vallés, Barcelona, Spain). The temperature of the column was 40 ± 1 °C and the wavelength of the detector 254 nm. The chromatographic conditions used are described in Table 1; a solution of 0.1 M ammonium acetate was used as eluent A, and acetonitrile as eluent B.

A standard solution containing appropriate amounts of agmatine, tryptamine, 2-phenylethylamine, putrescine, cadaverine, histamine, tyramine, spermidine, spermine and 1,7-diaminoheptane (as internal standard) was used to quantify the biogenic amines present in the samples.

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