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A new fear in wine: Isolation of *Staphylococcus epidermidis* histamine producer

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ABSTRACT

Biogenic amines (BAs) are undesirable compounds in all foods and beverages because they may induce food-borne intoxications when consumed at high concentrations. The aim of this study was to identify the organism responsible for the synthesis of histamine in a Tempranillo red wine from Ribera de Duero D.O. (Denomination of origin), Spain. Bacteria present in wines after malolactic fermentation were isolated, identified and typed. Four strains of the species *Oenococcus oeni* and two strains of *Staphylococcus epidermidis* were found. None of the *O. oeni* strains produced histamine, cadaverine or putrescine, but one of the *S. epidermidis* strains (Pa) was able to produce all three in synthetic medium and grape must, although not in wine. From the data obtained in this work, histamine present in Tempranillo wine B was produced by the *S. epidermidis* strain Pa. This is the first report in which the presence of *S. epidermidis* has been reported in wine and whose ability to produce histamine, putrescine and cadaverine has been demonstrated in grape must.

1. Introduction

Biogenic amines (BAs) are organic bases endowed with biological activity that can be present in a wide range of foods, such as cheese, fish products, meat products, fermented vegetables, soy products and alcoholic beverages like beer and wine (Halász, Baráth, Simon-Sarkadi, & Holzapfel, 1994; Smit, du Toit, & du Toit, 2008; Suzzi & Gardini, 2003; ten Brink, Damink, Joosten, & Huis in't Veld, 1990). They are undesirable in foods and beverages and their occurrence is of current research interest for toxicological reasons (high BAs concentrations cause physiological disorders like headaches, respiratory distress, hyper-hypotension and some allergic-like symptoms) (Anli & Bayram, 2009; Ladero, Calles-Enriquez, Fernandez, & Alvarez, 2010; Shalaby, 1996; Silla Santos, 1996), and, secondly, because they can be considered as a quality index that can inform about undesired microbial activity or inappropriate manufacturing practices (Nykänen & Suomalainen, 1983; Ruiz-Capillas & Jiménez-Colmenero, 2004).

BAs in foods are mainly generated by decarboxylation of the corresponding amino acids through substrate-specific decarboxylase enzymes present in microorganisms involved in food fermentation or contamination (Halász et al., 1994; Silla Santos, 1996). Although amino acid decarboxylases are not widely distributed among bacteria, several species of many genera are capable of decarboxylating one or more amino acids. However, the ability of microorganisms to decarboxylate amino acids is highly variable depending not only on the species, but also on the strain and the environmental conditions (E. Coton, Rollan, Bertrand, & Lonvaud-Funel, 1998; Marcobal, Martín-Álvarez, Polo, Muñoz, & Moreno-Arribas, 2006). Among BAs, histamine is the most important compound related to food-borne intoxications (Halász et al., 1994; Silla Santos, 1996), due to its strong biological activity (Chatonnet, 2009). It is well known that allergy-like food poisoning appears to be caused mainly by histamine, which is produced by the enzymatic decarboxylation of the amino acid histidine, normally present in food itself. Many different bacterial species of the *Enterobacteriaceae* family are known to possess histidine decarboxylase and they have the ability to produce histamine (Halász et al., 1994; Silla Santos, 1996). While histamine in raw fish is synthesized mostly by gram-negative enteric bacteria, in fermented products, such as cheese, fish sauce and alcoholic beverages, it is produced by gram-positive lactic acid bacteria (Silla Santos, 1996).

Wine is susceptible to the formation of BAs (Polo, Ferrer, Peña-Gallego, Hernández-Orte, & Pardo, 2010). Winemaking involves transforming the components of grape must into an end product whose quality depends in part on the metabolism of microorganisms present during fermentation (Cocolin, Heisey, & Mills, 2001).

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Sometimes these microorganisms cause depreciation of wine due to the formation of undesirable compounds which negatively affect wine aroma and flavours or are toxic to humans, as in the case of BAs (Nykänen & Suomalainen, 1983). The main BAs associated with wine are putrescine, histamine and tyramine (Bartowsky & Stockley, 2010; Pineda, Carrasco, Peña-Farfal, Henríquez-Aedo, & Aranda, 2012). Histamine is the most important, because it is not only the most toxic BA, but also its toxicity is enhanced by other BAs and ethanol present in this beverage. Thus, histidine methyl transferase, diamine oxidase and monoamine oxidase (enzymes involved in histamine detoxification in humans) are inhibited by agmatine, cadaverine, tyramine, phenylethylamine, tryptamine, putrescine and ethanol, thereby increasing the toxic effect of histamine (Ancín-Azpilicueta, González-Marco, & Jiménez-Moreno, 2008; Chu & Bjeldanes, 1982; Taylor & Eitenmiller, 1986). In addition, wines containing high histamine levels risk being rejected in markets with high quality and safety standards (Polo et al., 2010).

The main microorganisms responsible for histamine in wine are LAB, especially *Lactobacillus hilgardii*, *Lactobacillus mali, Oenococcus oeni* and *Pediococcus parvulus* (Costantini, Cersosimo, Del Prete, & Garcia-Moruno, 2006; Coton et al., 2010; Landete, Ferrer, & Pardo, 2005; Landete, Ferrer, & Pardo, 2007; Mazzoli et al., 2008). However, in recent years, several strains of *Enterococcus faecium* and enterobacteria, described as histamine producers, have been isolated from must and wine (García-Moruno & Muñoz, 2012; Pérez-Martín, Seseña, Izquierdo, & Palop, 2014). Although histamine can be formed at different moments of vinification, it increases mainly during malolactic fermentation (MLF) and aging (Aerny, 1985; Arena & Manca de Nadra, 2001; Lonvaud-Funel, 2001; Marcobal et al., 2006). *O. oeni* is one of the main species isolated at that stage of vinification (Davis, Wibowo, Lee, & Fleet, 1986; van Vuuren & Dicks, 1993), and thus many authors have considered it as the main species responsible for histamine production (Coton et al., 1998; Landete et al., 2005; López et al., 2009; Lucas, Claisse, & Lonvaud-Funel, 2008).

Identification of bacteria responsible for histamine production in wine is of great interest for winemakers, as this knowledge is fundamental for preventing the synthesis of this amine (García-Moruno & Muñoz, 2012).

The aim of this study was to identify the microorganism/s responsible for the histamine production observed in spoiled Spanish Tempranillo wine, quantify their ability to produce this amine, and determine in which moment of the vinification this BA could have been produced (grape must or wine).

2. Materials and methods

2.1. Origin of the study wines

Two wine samples (A and B) containing different histamine concentrations were obtained from commercial Tempranillo red wines produced in the Ribera del Duero D.O. region (Spain). These wines had a pH value of 3.8, total SO_2 35 mg/L, free SO_2 15 mg/L, and histamine contents of 1 mg/L (sample A) and 11.2 mg/L (sample B). They had been produced in parallel in the same cellar, from Tempranillo grapes in 25,000 L wooden vats. Both had been inoculated with the commercial starter culture Viniferm OE 104 (Agrovin), consisting in non-histamine-producing *Oenococcus oeni* bacteria, in order to accomplish MLF. When wine from vat B was detected as containing high amounts of histamine, weeks after the end of MLF, sterile collected samples (250 mL each) of wine B and another wine from the same cellar with low histamine content (wine A) were sent to our laboratory.

2.2. Isolation of bacteria and general bacterial growth conditions

Decimal dilutions (in NaCl 0.9%) of wines were spread in duplicate on MRS (Scharlab, Barcelona, Spain) plates supplemented with 0.5% L-cysteine and on MLO plates (Maicas, Natividad, Ferrer, & Pardo, 2000). Actistab 0.15 g/L (Gist-Brocades, 50% natamycin) was added to both media, to inhibit the growth of yeast and fungi. MRS and MLO plates were incubated aerobically at 28 °C for seven days.

2.3. Identification of isolates by amplified ribosomal DNA restriction analysis (16S-ARDRA)

To identify LAB, the 16S rDNA gene was amplified by PCR following the protocol described by Rodas, Ferrer, and Pardo (2003). DNA template for PCR was obtained from a cell suspension made by dissolving one colony in 10 μL of sterile milliU water (Millipore) (Rodas et al., 2003). Universal primers pA and pH described by Edwards, Rogall, Blöcker, Emde, and Böttger (1989) were used. Amplified DNA was digested with the restriction enzyme *Mse*I (Fermentas). 16S-ARDRA digestion products were resolved by electrophoresis in 1.2% (w/v) SeaKem LE agarose (FMC, USA) in 0.5 x TBE (45 mM Tris–HCl, 45 mM boric acid and 1 mM EDTA pH 8.0), using 1 Kb plus ladder (Invitrogen, BRL) as a molecular size standard. Agarose gels were stained with ethidium bromide (0.5 μg mL^{-1}) and images were digitized with the GelPrinter Plus system (TDI).

2.4. Identification of isolates by 16S rDNA sequence analysis

16S rDNA gene was amplified as described for the 16S-ARDRA technique. Amplification product was purified using UltraClean PCR clean-up kit (MoBio) and automatically sequenced at the University of Valencia's Central Service for Experimental Research (SCSIE). Sequence searches were performed in the GenBank DNA database of the National Centre for Biotechnology Information (NCBI) using the BLAST algorithm (Altschul, Gish, Miller, Myers, & Lipman, 1990).

2.5. Typing of isolates by random amplification of polymorphic DNA (RAPD)

DNA templates were obtained from cell suspensions in milliU water (Millipore) (Rodas et al., 2003). Four primers were used separately in this study, M13 (Zapparoli, Reguant, Bordons, Torriani, & Dellaglio, 2000), COC (Cocconcelli, Porro, Galandini, & Senini, 1995), 17R (Rodas, Ferrer, & Pardo, 2005), and OPL5 (Acedo-Félix & Pérez-Martínez, 2003). DNA amplifications were carried out in 50 μL PCR mixtures containing 1 μM primer, 200 μM dNTPs, 10 mM Tris–HCl (pH 8.8), 50 mM KCl, 0.1% Triton X-100, 2 mM $MgCl_2$, 1 U of Dynazyme™ II DNA Polymerase (Finnzymes) and 1 μL template DNA prepared as described above (Rodas et al., 2003). PCR was performed as described by Rodas et al. (2005) in a Techne thermal cycler PTC-100TM (MJ Research, Watertown, USA).

Amplified RAPD bands were resolved by electrophoresis in 1.2% (w/v) SeaKem LE agarose (FMC, USA) in 0.5 x TBE (45 mM Tris–HCl, 45 mM boric acid and 1 mM EDTA pH 8.0) gels and a 1 Kb plus ladder was used as molecular size standard. Images were stained and digitized as described in the previous section.

2.6. RAPD reproducibility study

A reproducibility study for each primer used for typing was carried out on 10% isolates and two iterations of the entire procedure to determine the minimum percentage of similarity necessary for strain discrimination. Two separate cultures of each isolate were grown and a total of two reactions per strain were carried out.