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# Are *Enterococcus* populations present during malolactic fermentation of red wine safe?

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ABSTRACT

The aim of this study was the genetic characterisation and safety evaluation of 129 *Enterococcus* isolates obtained from wine undergoing malolactic fermentation. Genetic characterisation by randomly amplified polymorphic DNA-PCR displayed 23 genotypes. 25 isolates representative of all genotypes were identified as *Enterococcus faecium* by species-specific PCR and assayed for antibiotic resistance, presence of virulence genes and aminobiogenic capacity, both in decarboxylase medium and wine. The aminobiogenic capacity in wine was analysed in presence (assay 1) and absence (assay 2) of *Oenococcus oeni* CECT 7621. Resistance to tetracycline, cotrimoxazol, vancomycin and teicoplanin was exhibited by 96% of the strains, but none of them harboured the assayed virulence genes. All of the strains harboured the tyrosine decarboxylase (*tdc*) gene, while 44% were positive for tyramine in decarboxylase medium. Only five out of 25 strains survived in wine after seven days of incubation, and when concentrations of biogenic amines in wines were determined by HPLC, only those wines in which the five surviving strains occurred contained biogenic amines. Histamine, putrescine and cadaverine were detected in wines from both assays, although concentrations were higher in assay 2. Tyramine and phenylethylamine were detected only in absence of *O. oeni*. This research contributes for the knowledge of safety aspects of enterococci related to winemaking.



## 1. Introduction

Malolactic fermentation (MLF) is a bacterial process that usually occurs after completion of alcoholic fermentation by yeast in the winemaking process. Lactic acid bacteria commonly found in wine during MLF belong to the *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Oenococcus* genera. The presence of *Enterococcus* isolates, although not frequent, has also been reported in red wine undergoing MLF (Capozzi et al., 2011) and at the surface of grape berries at harvest (Renouf et al., 2005) and must grapes (Marcobal et al., 2004). In all cases, the strains isolated belonged to *Enterococcus (E.) faecium* species.

The *Enterococcus* genus is important in environmental, food and clinical microbiology. The function of these bacteria in foods is controversial. Some authors affirm that they may play a beneficial role in the production of various traditional fermented foods, such as cheeses, sausages and olives (Foulquié-Moreno et al., 2006) and, therefore, they may be successfully used as starter cultures and probiotics. Other studies associate their presence to spoilage of foods, especially meats, and to human disease (Franz et al., 2011).

Although enterococci are generally not considered as food-borne pathogens, they are capable of disseminating virulence or antibiotic resistance genes through the food chain (Kojima et al., 2010).

Another matter of concern with respect to the safety of enterococci is their aminobiogenic capacity. Biogenic amines (BA) are natural compounds of low molecular weight formed by the decarboxylation of amino acids, with the participation of substrate-specific enzymes from diverse microorganisms. These compounds are considered a threat for human health and their toxicity has led to the general agreement that they should not be allowed to accumulate in food (Ladero et al., 2010a). Some authors have reported that some *Enterococcus* isolates from cheese (Linares et al., 2009) and wine (Marcobal et al., 2004; Capozzi et al., 2011; Coton et al., 2010) are involved in BA production, mainly tyramine. For this reason, it is also interesting to assess the potential of isolated

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strains to produce these compounds and their contribution to the BA concentrations in wine, since BA levels of up to the legal threshold could prevent their exportation.

To the best of our knowledge, a study to characterise from a safety point of view, indigenous *Enterococcus* strains isolated from MLF of wine has not been reported. In this study, 129 *Enterococcus* isolates obtained from wine samples taken during MLF, from different wineries of the Castilla-La Mancha region were genetically characterised and assayed for antibiotic resistance, presence of virulence genes and aminobiogenic capacity, both in decarboxylase medium and wine.

## 2. Materials and methods

### 2.1. Bacterial isolates and culture media

A total of 129 isolates from our collection obtained from wine samples of different grape varieties (Tempranillo, Merlot, Syrah, Cabernet Sauvignon and Garnacha) isolated during malolactic fermentation at different wineries (designated using letters A–E) in the Castilla-La Mancha region (Spain) during the 2009 vintage, were analysed. These had been isolated from MRS plates (De Man et al., 1960) supplemented with 50 mg/L sodium azide and 100 mg/L cycloheximide (Sigma, USA), purified by sub-culturing on plates of the same medium and maintained at −80 °C in MRS broth supplemented with 20% glycerol as a cryoprotector. These isolates were presumptively assigned to the *Enterococcus* genus on the basis of cell morphology, Gram staining, catalase and $CO_2$ production and growth in the presence of 6.5% NaCl.

### 2.2. Genotyping of isolates by randomly amplified polymorphic DNA (RAPD) analysis

DNA extraction was carried out as described by Rodas et al. (2003). RAPD-PCR using the M13 primer (5′-GAGGGTGGCGGTTCT-3′) (Bonsai Technologies Group, Madrid, Spain) and the reproducibility study were performed in accordance with the procedure described by Ruiz et al. (2008). This study was carried out on four isolates and four iterations of the entire procedure of RAPD-PCR to determine the minimum percent similarity (*r*) necessary for strain discrimination. Comparison of the obtained RAPD-PCR patterns were made by Pearson product–moment correlation coefficient and cluster analysis by unweighted pair group method with arithmetic average (UPGMA).

### 2.3. Identification of isolates

Representative isolates from genotypes obtained in the RAPD-PCR analysis were identified at the genus level by amplification and restriction analysis of the 16S-rRNA gene (16S-ARDRA) using the restriction enzyme *MseI* (New England BioLabs, UK) as described by Rodas et al. (2003). The strains *E. faecium* $410^T$ and *Enterococcus faecalis* $481^T$ from the Spanish Type Culture Collection (CECT) were used as references.

In order to identify the isolates at the species level, species-specific PCR reactions were carried out. Primers Efm1 (5′-TKCAG-CAATTGAGAAATAC-3′)/Efm2 (5′-CTTCTTTTATTTCTCCTGTA-3′), Efs1 (5′-CTGTAGAAGACCTAATTTCA-3′)/Efs2 (5′-CAGCTGTTTTGAAAG-CAG-3′) and Eh1 (5′-AAACAATCGAAGAACTACTT-3′)/Eh2 (5′-TAAATTCTTCCTTAAATGTTG-3′) from *E. faecium*, *E. faecalis* and *Enterococcus hirae*, respectively, and conditions reported by Bensalah et al. (2006) were used. The expected bands of the amplicons were 190, 209 and 263 bp, respectively.

In order to confirm the identity obtained with the species-specific PCR analysis, a number of the representative isolates were also identified by sequencing. The PCR product of the 16SrRNA gene (1500 bp) obtained for these isolates from previous 16S-ARDRA analysis, was sequenced (Macrogen Inc., The Netherlands), and the sequences compared with those available from GenBank (www.ncbi.nlm.nih.gov/blast/blast.cgi).

Representative isolates from genotypes obtained were selected for subsequent analysis.

### 2.4. Safety evaluation

#### 2.4.1. Antibiotic resistance

ATB STREP EU (08) strip (BioMérieux, France) was used to determine the susceptibility of the strains to 15 antibiotics. A suspension of each isolate in saline solution was transferred into the medium supplied by the manufacturer and inoculated into the strip following the manufacturer's instructions. Results were recorded after incubation for 24 h at 37 °C according to standards published by the Clinical and Laboratory Standards Institute (CLSI, 2008).

#### 2.4.2. Determination of virulence genes

Multiplex PCR reactions, using primers and conditions described by Martín-Platero et al. (2009), were carried out to detect the presence of genes involved in the expression of gelatinase (*gelE*), hyaluronidase (*hyl*), the aggregation substance (*asa1*), enterococcal surface protein (*esp*), cytolysin (*cylA*), the collagen adhesion (*ace*), endocarditis antigen (*efaA*) and vancomycin resistance (*vanA* and *vanB*).

#### 2.4.3. Biogenic amine production (BA)

The potential to produce the biogenic amines tyramine, histamine, putrescine (via ornithine decarboxylase), and cadaverine was assessed by using both liquid and solid decarboxylase medium (DM) (Bover-Cid and Holzapfel, 1999). One percent (w/v) of each precursor amino acid (L-histidine monohydrochloride, L-ornithine monohydrochloride, tyrosine disodium salt, and L-lysine monohydrochloride) purchased from Sigma (St. Louis, MO), and 0.005% (w/v) of pyridoxal-5-phosphate, a cofactor for the decarboxylation reaction, were added to the decarboxylase medium. Each strain was cultured both in 1.5 mL test tubes containing 1 mL of DM and on plates with and without amino acid (controls). The test tube and plate cultures were incubated for 48 h at 37 °C under aerobic and anaerobic conditions (Gas Pack System, Oxoid, Ltd., Basingstoke, Hampshire, UK). All assays were performed in duplicate. Positive reactions were recorded when a purple colour appeared in DM broth or on the plates, except for tyrosine plates where a positive reaction was considered when tyrosine precipitates disappeared around the colonies (Bover-Cid and Holzapfel, 1999).

#### 2.4.4. Detection of the tyrosine decarboxylase gene

Genomic DNA was extracted as described by Rodas et al. (2003). The presence of the tyrosine decarboxylase (*tdc*) gene was assayed by specific PCR reactions following the procedure described by Capozzi et al. (2011). Primers used were TDC/1 (5′-AACTATCGTATGGA-TATCAACG-3′) and TDC/2 (5′-TAGTCAACCATATTGAAATCTGG-3′) (Fernández et al., 2004), which produce an amplicon of 720 bp.

#### 2.4.5. Determination of BA production in wine by microvinification assays

Tempranillo wine after the completion of alcoholic fermentation was used. It was provided by a winery of the Castilla-La Mancha region and filtered through a 0.2-μm pore size module (Millipore, Billerica, MA) for sterilisation. The chemical composition of the wine was as follows: 12.76% (v/v) alcohol content, 6.31 g/L total acidity, 0.18 g/L volatile acidity, pH 3.43, 2.18 g/L malic acid, 0.09 g/L citric acid and 0.15 g/L lactic acid. BA concentrations, determined by