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Identification of biogenic amines-producing lactic acid bacteria isolated from spontaneous malolactic fermentation of chilean red wines

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ABSTRACT

Most Chilean wineries perform malolactic fermentation by means of non-typified autochthonous lactic acid bacteria already present in grapes and oak barrels or fermenters. The objective of this research was to investigate the principal lactic acid bacteria present during spontaneous malolactic fermentation of Chilean Cabernet Sauvignon wines and to study its role in BA formation. To the best of our knowledge this is the first time that this relation has been reported for Chilean wines. Lactic acid bacteria were isolated from five wineries located in three Chilean geographical regions. Genotypic differentiation of each bacterial isolated was performed via a restriction fragment length polymorphism method using *rpoB* and 16S rRNA genes and *HinfI*, *AciI* and *MseI* enzymes. Sixty-five colonies were isolated and identified as lactic acid bacteria, identifying two species, *Lactobacillus rhamnosus* and *Oenococcus oeni*. The predominant species was *L. rhamnosus*, which, to the best of our knowledge, we are describing for the first time in the vinification process. Considering that *L. rhamnosus* was detected in wineries from different geographical viticultural regions, it could be preliminarily considered as an endemic species. Both species were biogenic amines producers, *L. rhamnosus* being mainly responsible for biogenic amines present in the Cabernet Sauvignon wines studied.

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1. Introduction

Winemaking or vinification is an ancient and traditional technological process that involves an almost perfect combination of biochemical and microbiological reactions. This process typically requires two fermentation stages: i) alcoholic fermentation (AF) performed by yeasts and ii) malolactic fermentation (MLF) conducted by lactic acid bacteria (LAB) mainly *Oenococcus oeni* species. This last step has been considered relevant and non-avoidable for red wines, because produces a decrease in total wine acidity, enhances organoleptic properties and improves microbiological stability (Capozzi et al., 2010; Lonvaud-Funel, 1995; Versari, Parpinello, & Cattaneo, 1999). LAB are naturally present in grapes, musts and wines, the predominant genera are *Leuconostoc, Pediococcus, Lactobacillus* and *Oenococcus* (Lonvaud-Funel, 1995; Versari et al., 1999). During MLF the most observed species is *Oenococcus oeni* (Garcia-Moruno & Muñoz, 2012) which is capable of proliferating in the harsh wine environment, i.e. low pH (ca. 3.5), high alcohol content (14% v/v), high concentration of SO_2 (50–80 mg L^{-1}) and low temperature (18-20 °C) (Versari et al., 1999). During MLF, besides the beneficial conversion of L-malic to L-lactic acid mainly via malate decarboxylase (malolactic enzyme), other compounds like biogenic amines (BA) are formed. These kinds of compounds, produced by free amino acids decarboxylation (Gerbaux, Villa, Monamy, & Bertrand, 1997; Lonvaud-Funel, 2001), can negatively affect the wine quality and its presence might be considered a possible health risks for some consumers (Ancin-Azpilicueta, Gonzalez-Marco, & Jimenez-Moreno, 2008; Anli & Bayram, 2009). Our research group evaluated the BA content in

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Chilean red wines finding higher concentrations than in foreign wines (Henríquez-Aedo, Vega, Prieto-Rodríguez, & Aranda, 2012; Pineda, Carrasco, Pena-Farfal, Henriquez-Aedo, & Aranda, 2012). In Chile most wineries perform MLF by means of non-typified autochthonous lactic acid bacteria already present in grapes, oak barrels or fermenters, thus, for Chilean wineries, the identification of autochthonous lactic acid bacteria is important in order to determine which kinds of bacteria are present and responsible for MLF and to establish their relation with BA formation. The objective of this research was to isolate, characterize and identify autochthonous lactic acid bacteria present during spontaneous MLF of Cabernet Sauvignon wines and to study their role in biogenic amines formation. To the best of our knowledge this is the first time that this relation has been reported for Chilean wines.

2. Materials and methods

2.1. Samples

All MLF samples were collected in 2011 from five wineries (A-E) located in three Chilean valleys: Limari (30° 34′ 29.1″S and 71° 24.45′ 45.4″W), Curico (35° 05′ 54.5″ S and 71° 18′ 37.0′ W) and Itata (36° 46′ 4.8′ S and 72° 13′ 00.5′ W). All the wineries selected carry out spontaneous MFL without commercial starter. The winemaking process was initiated with grapes harvested in March, followed by the traditional vinification practices of each winery. As a general rule, AF using freeze-dried yeast was performed in plastics bins (wineries A and B) or stainless steel tanks (wineries C, D and E) at 22-25 °C. Spontaneous MLF was carried out immediately after AF in oak barrels (wineries A, B, D and E) or stainless steel tanks (winery C) at 18-22 °C for 30–40 days. Sampling was randomly done in the cellar, 100 mL of different barrels (or tanks) were pooled to obtain 750 mL of composed sample. Chemical parameters such as pH and alcohol content were determined before microbiological assays. Samples were stored at 4 °C until be processed.

2.2. Bacterial isolation and culture media

Bacterial isolation was carried out using four different media (Table 1): Man Rogosa & Sharpe (MRS) (De Man, Rogosa, & Sharpe, 1960) from Difco (Le Pont de Claix, France); Acid Tomato Broth (ATB) using grape juice instead tomato juice (Garvie, 1967); Lactic Acid Bacteria from Wine (LABW) (Weiler & Radler, 1970) and Medium for *Leuconosctoc oenos* (MLO), supplemented with 10% v/v of tomato juice (Zúñiga, Pardo, & Ferrer, 1993). All media were prepared with ultra-pure water (18 MΩ cm), autoclaved and stored refrigerated at 4 °C. The pH (4.8–5.3) was adjusted with 1 N HCl. To avoid yeast proliferation 100 mg L^{-1} of filtered (0.22 μm) pimaricin solution (VGP Pharmachem, Vic, Spain) was added after the autoclave process (Sanchez et al., 2010). Serial dilutions were plated onto the MRS, ATB and LABW media and incubated for 24–48 h at 37 °C under microaerophilic conditions (7% CO_2). MLO plates were anaerobically incubated (BD Gaspak EZ from Sparks, Marylans, USA) for 5–7 days at 30 °C. After count (CFU mL^{-1}), colonies from different plates were randomly selected and transferred to the original broth. Each colony was transferred at least three successive times to obtain pure cultures. Each isolate was stored at −80 °C in the original medium supplemented with glycerol (20% v/v).

Table 1
Culture media composition for lactic acid bacteria isolation from MLF samples.

Component[a]	MRS	ATB	LABW	MLO
Casein trypsin digest	10.0	10.0	–	10.0
Gelatin pancreatic digest	10.0	–	5.0	–
Yeast extract	5.0	5.0	5.0	5.0
Glucose	20.0	10.0	10.0	10.0
Fructose	–	–	–	5.0
$MgSO_4.7H_2O$	0.1	0.2	0.5	0.2
$MnSO_4.4H_2O$	0.05	0.05	0.2	0.05
Grape juice	–	25[b]	–	–
Tomato juice	–	–	–	100.0[b]
$(NH_4)_2$ citrate	2.0	–	2.0	3.5
$NaCH_3COO·3H_20$	5.0	–	5.0	–
Tween 80	1.0	–	1.0	1.0
$FeSO_4·7H_20$	–	–	0.05	–
KH_2PO_4	2.0	–	5.0	–
pH	5.0	4.8	5.3	4.8

[a] g L^{-1}.
[b] mL.

2.3. Microbiological characterization of lactic acid bacteria isolated from MLF

Isolated colonies were phenotypically characterized including macroscopic and microscopic analysis, i.e. shape, color, border, surface, aspect, elevation, light and consistency. A gram test was assayed observing a positive reaction for lactic acid bacteria and a negative reaction for acetic acid bacteria. A catalase test was performed with 3% (v/v) of hydrogen peroxide (Wu, Ma, Zhang, & Chen, 2012) and a negative reaction was observed in the presence of lactic acid bacteria.

2.4. Molecular characterization of lactic acid bacteria: 16S rRNA and rpoB genes

Cells from bacterial cultures were collected by centrifugation at 15183 × *g* for 5 min. The pellet generated was subjected to DNA extraction using Power Soil DNA isolation kit (Mo Bio Laboratories Inc, Solana Beach, CA, USA) adhering to the manufacturer's protocol but using double incubation time (10 min) for DNA extraction. DNA purity and concentration were determined spectrophotometrically using a Tecan (Männedorf, Switzerland) Multimode Reader Infinite M200 Pro NanoQuant. The extracted DNA was amplified using the primers: $WLAB_1$ (5′-TCCGGATTTATTGGGCGTAAAGCGA-3′) and $WLAB_2$ (5-TCGAATTAAACCACATGCTCCA-3) (Lopez et al., 2003), that amplifies the region V4 and V5 of the 16S rRNA subunit generating a product of ca. 400 bp. For RNA polymerase β subunit (*rpoB*) the following primers were used: *rpoB*$_1$ (5′ ATTGACCACTTGGGTAACCGTCG 3′) and *rpoB2 (*5′ ACGATCACGGGTCAAACCACC 3′) obtaining an amplification product of ca. 306 bp (Claisse, Renouf, & Lonvaud-Funel, 2007; Renouf, Claisse, & Lonvaud-Funel, 2006). Each amplification reaction solution was prepared by mixing 12.5 μL of Takara kit (Bio inc, Shiga, Japan), 0.4 μM of primers and 20 ng of DNA template. The amplification was performed using a Veriti 96-wall gradient thermocycler from Applied Biosystem (Foster, CA, USA) utilizing the following program: 2 min at 95 °C for initial denaturation, 30 cycles of: 30 s at 95 °C for denaturation, 30 s at 55 °C for *rpoB* annealing and 30 s at 60 °C for WLAB annealing, and 1 min at 72 °C for extension. Afterwards a final extension step was carried out for 2 min at 72 °C. PCR products were separated by gel electrophoresis on 2% w/v agarose gel, prepared with 1X TAE (Tris acetate-EDTA, pH 8) buffer, in a MS Mini-7 horizontal chamber (Cleaver Scientific, Warwickshire, UK) applying 100V. PCR amplification patterns were visualized under a UV light using SafeView Nucleic Acid Stain (NBS Biologicals, Richmond, Canada) and 100 bp ladder (Thermo Scientific, Vilnius, Lithuania) as a DNA molecular weight marker.

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