Determination of Effects of Genetic Diversity of *Oenococcus oeni* and Physicochemical Characteristics on Malolactic Fermentation Across Chilean Vineyards, using Multivariate Methods

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After alcoholic fermentation, *Oenococcus oeni* is the protagonist that carry out malolactic fermentation in order to improve the quality of wine. However, the effectiveness of malolactic fermentation varies according to intraspecific diversity of *O. oeni* at a genetic level and physicochemical concentrations of intrinsic factors. In this study, we assessed both components by isolating *O. oeni* from three Chilean vineyards (Maipo, Colchagua y Curicó) and measuring intrinsic components, such as biogenic amines and amino acids, during malolactic fermentation. Both data were condensed and then processed with Multivariate using the statistical method of correspondence analysis. The genetic data was clustered using the RAPD-PCR molecular method, and the physicochemical analysis was carried out using chromatography techniques. Isolation of *O. oeni* species and genetic diversity results between the vineyards revealed that *O. oeni* strains cluster according to its geographical origin, with strains similarity higher than 60% of all the samples. The wines of each valley could be characterized by the presence or absence of biogenic amines, and final correspondence analysis showed that there is a differentiation of strains between the studied Valleys, also confirming the accomplishment of the malolactic fermentation in the wines analyzed.

Keywords: Genotyping, Lactic acid bacteria, Oenococcus, Wine, Fermentation.

Wine is produced by natural alcoholic fermentation of must, which is mostly performed by *Saccharomyces* yeasts. After alcoholic fermentation, a second fermentation called malolactic fermentation is conducted to improve intensity of flavors and increase microbial stability in the wine[2]. Malolactic fermentation is carried out by lactic acid bacteria, being *Oenococcus oeni* the main species in charge of the process[3–7]. *O. oeni* is gram-positive bacteria that is able to resist low pH (lower than 3.5), high levels of SO_2 (50 mg/L) and ethanol concentration (greater than 10.5 v/v)[8]. Although *O. oeni* has good adaptation to wine environment, it has shown phenotypic and genotypic diversity at strain level[9] that may have an effect on the result of malolactic fermentation and consequently, it produces inconsistencies in the quality of the wine. This has led to the selection

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of resistant strains from different environments[6], in which intraspecific *O. oeni* biodiversity studies have been assessed comparing RAPD-PCR results and it has showed a large diversity and correlation between strains and geographical origin[10–12]. These observations give insights of the potential of identifying *O. oeni* strains from different geographical regions for the selection of resistant strains and development of fermentation starters that will improve the oenological properties of local wines.

Although malolactic fermentation enhances the wine microbial stability and flavor, the process can be risky because some biogenic amines may increase their levels generating the opposite effect: undesired aroma and flavor[13-14]. The increased of the concentration of biogenic amines has been attributed to the action of lactic acid bacteria that decarboxylates the amino acids present in the wine, which can also be influenced by intrinsic features such as amino acids concentration, sugars, ethanol concentration, etc.

The aim of this study was to evaluate the effect of genetic diversity on the consumption of amino acids and the production of biogenic amines. For this, we evaluate isolates of *O. oeni* population genetic clustering obtained of three Central Valleys of Chile: Maipo, Curicó y Colchagua and correlate them with malolactic fermentation, biogenic amines and amino acids concentrations from vintages from the same vineyards using correspondence analysis and Partial Least Square Discriminant Analysis (PLS-DA).

MATERIALS AND METHODS

Molecular characterization and O. oeni isolation from Chilean vineyards

Lactic acid bacteria were isolated from 30 Cabernet Sauvignon wine samples from three central Chilean vineyards: Maipo (n=6), Curicó (n=15) and Colchagua (n=9) taken during malolactic fermentation. In addition, seven control strains were used: *Pediococcus pentosaceus* (CR 949) from Centro de Estudios de Enologia (INTA-Argentina), *Lactobacillus plantarum* (2793) and *Lactobacillus brevis* (1366) from Laboratorio de Biotecnología y Microbiología Aplicada (LAMAP, USACH, Chile), *Pediococcus acidilactici* (B-14950) from USA Agriculture Department*,

Lactobacillus paracasei (R0212), *Lactobacillus hilgardii* (H013) and *O. oeni* (Lalvin 31, Lalvin, France). Serial dilutions method (10^{-6}) was used for isolation, and 100 ìL per sample were grown in basal agar-grape culture media (10 g/L yeast extract, 5 g/L Glucose, 1 mL Tween 80, 170 mL of sterile grape juice, 25 g/L agar) supplemented with cycloheximide 1%. Petri dishes were incubated for 5-7 days at 28 ºC under microaerophilic conditions. Initially, the morphology of cells was observed using Gram tinction[15] and the cells with ovoid-shaped and grouped in chains, were preliminarily identified as *O. oeni*.

O. oeni bacteria were distinguished at species level using RFLP-PCR method[16]. First, we extracted total DNA according to kit Wizard DNA purification method (Promega, USA). A fragment of 294 bp that codes for the gene *rpoB* was amplified, which PCR products were digested with *Hinf*I and *Aci*I digestion enzymes (Fermentas, USA) and separated to be detected at 4% agarose gel electrophoresis.

Samples were labeled as M[A/B/C]-X, which corresponds to molecular information from zone A: Maipo, zone B: Colchagua and zone C: Curicó, and X the isolated strain number. Then, the isolates identified as *O. oeni* a malolactic region of 1025 bp was amplified with PCR using primers On1 (5'-TAA TGT GGT TCT TGA GGA GAA AAT-3') and On2 (5'-ATC ATC GTC AAA CAA GAG GCC TT-3')[17]. PCR amplification was carried out during 30 cycles of amplification with denaturation during 45 s at 95°C, annealing for 2 min at 64°C, extension of 2 min at 72°C. PCR products were visualized in 1% agarose gel electrophoresis. Finally, *O. oeni* strains diversity was evaluated using short primer as previously described RAPD technique[10]. PCR program was repeated during 30 cycles of amplification with denaturation during 1 min at 94°C, annealing for 2 min at 40°C, extension of 2 min at 72°C, and final 10 min extension at 72°C. Products were visualized in 1.4% agarose gel. All the electrophoresis gels were staining of 5 µg ethidium bromide per L. Electropherograms were processed using the software *Quantity One version* 4.1.1 (Bio-Rad, USA) to calculate distances employing UPGMA cluster analysis, in order to build a dendrogram with the tool Free Tree versión 0.9.1.50[18] and visualized it in Figtree v1.4.2.

Biogenic amines characterization during malolactic fermentation

Wines samples collected during malolactic fermentation were chemically analyzed for: *a) Biogenic amines quantification* (histamine, tyramine, putrescine, cadaverine and phenylethylamine) from samples collected during malolactic fermentation. For this, was carried out using reversed phase HPLC method, according to previously reported conditions[14]. Biogenic amines are determined by derivatization using mainly benzoyl chloride and posterior extraction with chloroform. Final residue was resuspended in acetonitrile/water mixture for HPLC analysis. Chromatography separation was carried out at a constant temperature of 22°C using a column of 150 x 4,6 nm of dimensions and 5 μ particle size (LUNA, Phenomenex, USA). *b) Amino acids quantification* was carried out using reversed phase HPLC method[19], the samples were derivatized with phenyl isocyanate in order to form phenylthiocarbamide amino acids, which were separated and quantified through gradient chromatography at a constant temperature of 45 ºC. The mobile phase was composed of a solution (A) of sodium acetate anhydrous (0.14 M, pH 6.4) and acetonitrile (proportion 96:6). And a solution (B) composed of water and acetonitrile (proportion 60:40).

Correspondence analysis between genetic diversity and biogenic amines content

The genetic diversity of *O. oeni* and the measurements of intrinsic physicochemical parameters of the wine are two independent variables that were collected in the same vineyards. For this reason, both data were compared using the statistical method of correspondence analysis (CA), which aims to compare qualitative data into factorial analysis of both variables. CA is a multidimensional method to conforms homogenous groups from eventual similarities (or differences) of the isolates map indicating relative distance among isolates[20]. Results of CA were submitted to condensation/reduction of the dimensionality using the method of Karhunen–Loeve[21]. Following a PLS-DA were performed.

PLS-DA is an asymmetric method to predict one data set separate from another while compute two sets differently. Indeed, PLS-DA is a linear regression method whereby the multivariate variables corresponding to the observations (physicochemical markers) are related to the class membership for each sample. This modeling technique establishes the relationship between two sets of predictor and response variables. It is a correlation analysis that estimates the values of one variable from a set of controllable independent variables[14].

The result was validated by full cross-validation routines, minimizing the prediction residual sum of squares function (PRESS) to avoid overfitting the models[22]. All analyses were performed using the SPSS 19 (IBM SPSS, USA, 2016) and SIMCA P+ 12 (Umetrics AB, Sweden, 2012) software.

RESULTS AND DISCUSSION

Microscopic characterization of isolates

Wine samples collected during malolactic fermentation were analyzed from three Chilean Valleys: Maipo (n=6), Curicó (n=15) and Colchagua (n=9). In total, a 35% of grown bacteria (248
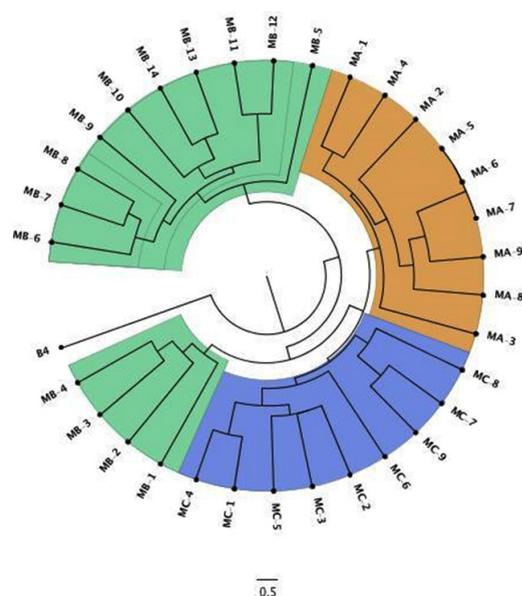

Fig. 1. Dendrogram calculated from UPGMA. It corresponds to *O. oeni* strains molecular information of isolates from three Chilean vineyards. Samples were labeled as M[A/B/C]-X, which corresponds to molecular information from zone A: Maipo, zone B: Colchagua and zone C: Curicó, and then the isolated strain number. B4 is *B. bruxellensis* outgroup.
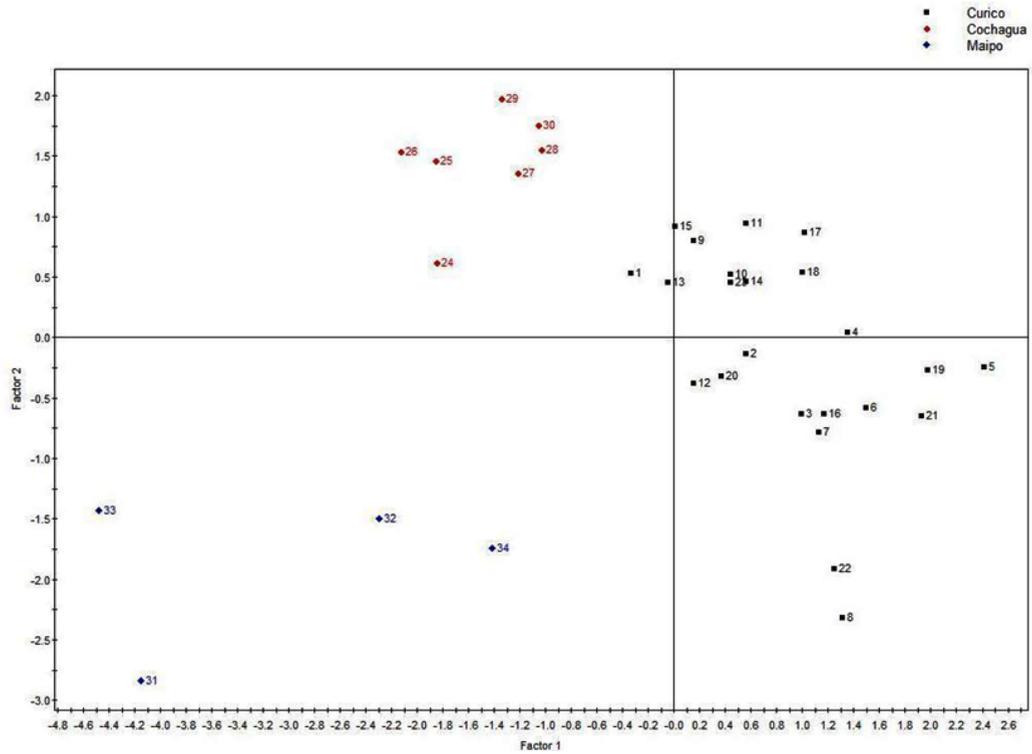
Fig. 2. Partial least squares discriminant analysis (PLS-DA) from HPLC biogenic amines quantification. Samples corresponds to three Chilean Valleys: Curicó (black: samples 1-23), Colchagua (red: samples 24-30) and Maipo (blue: samples 31-34)
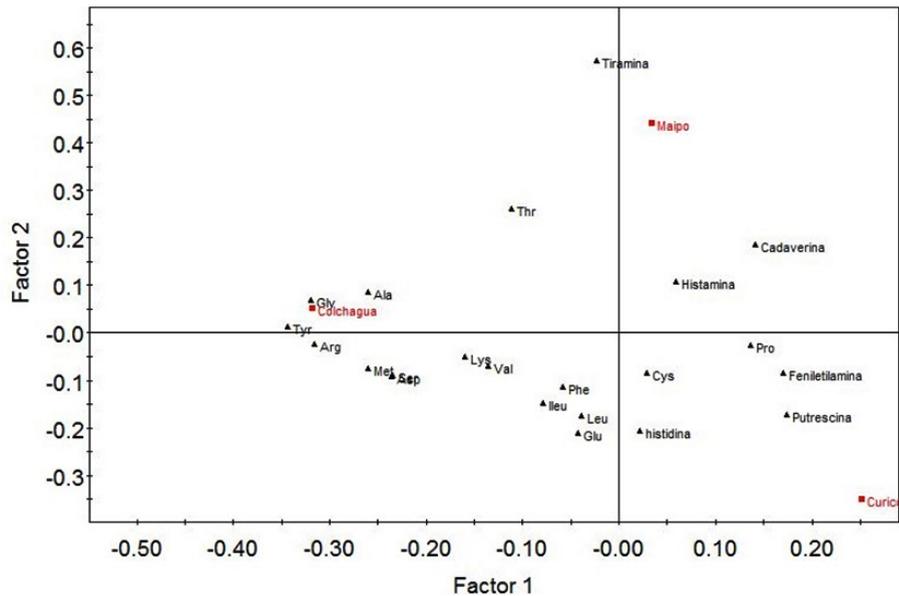

Fig. 3. Partial least squares discriminant analysis (PLS-DA) of molecular information and biogenic amines quantification. The first independent variable corresponds to data from RAPD-PCR results of the genetic variations of *O. oeni* across three Chilean vineyards: Maipo, Colchagua and Curicó. The second variable corresponds to HPLC quantification of biogenic amines (histamine, tyramine, putrescine, cadaverine and phenylethylamine)

isolates) were randomly selected from petri dishes cultures, which correspond to 63 samples from Maipo Valley, 117 samples from Curicó Valley and 68 samples from Colchagua Valley. Microscopic characterization of isolates revealed that all isolates showed morphologic characteristics associated with *O. oeni* [8].

Molecular characterization of O. oeni.

After DNA yield and purity was confirmed, results from PCR amplification of *O. oeni* specie-specific fragments of malolactic enzyme were analyzed. In total, 239 samples showed presence of the 1025 bp fragment (96%) and those with absence (9 samples) were discarded (data not shown). The amplification of the 1025 bp fragment amplifies species-specific primers targeted to the gene encoding the malolactic enzyme of *O. oeni*[17] Therefore, in all the wines analyzed the population of this species was the predominant one. In contrast, Henrriquez-Aedo et al.[23] reported that also found *Lactobacillus rhamnosus* in the samples of Chilean wines analyzed by them. The same authors described that the latter species were endemic to some Chilean winegrowing areas (Limari, Curicó and Itata), which is a species that was not identified in the present study (a case of the Curicó area).

Metadata of those 9 samples revealed that they were taken from wines at the end of alcoholic fermentation in two different valleys, which is in agree with previously reported observations[3–8,10]

O. oeni diversity in Cabernet Sauvignon

Out of total 239 *O. oeni* samples, 32 (13%) were selected from zone A: Maipo, zone B: Colchagua and zone C: Curicó. According to Zapparoli et al.[10] method RAPD-PCR clustering analysis showed high diversity and 29 unique samples in four different cluster were identified (Figure 1). Cophenetic correlation of 0.84 indicates good relation between the dendrogram structure and *DICE* coefficient, but all the sample showed similarity higher than 65%, which has been also reported in Australian wine[24]. The observed heterogeneity in the samples with respect to the native populations of *O. oeni* results has been important to study both from the ecological point of view and by possible technological projections. Bon *et al.* [25] showed a possible association between the presence of oenological sequences and characteristics of interest. So, the study at the molecular level is interesting not only from one point the taxonomic view, but also from ecological and technological aspects.

Correspondence Analysis (CA)

To identify a relationship between the geographical origin and physicochemical components produced by the strains, we used the CA method linked to PLS-DA. The correspondence analysis revealed that the strains isolated from Colchagua Valley (Figure 2) are grouped, as observed in the RAPD-PCR clustering analysis, supporting the idea of phylogenetic and phenotypic similarity of these strains. It was also possible to appreciate that the Colchagua Valley was characterized by higher levels of lactic acid in its wines, suggesting that malolactic fermentation was performed. Curicó valley was characterized by higher levels of malic acid, which could indicate that the malolactic fermentation was not fully accomplished (Figure 3).

The Maipo Valley, unlike the Curicó and Colchagua valleys, was characterized by the presence of histamine, tyramine and cadaverine. It is important to notice that histamine and tyramine have been suspected to cause toxicological problems[26]. In our case the levels of this amines were much lower than the physiologically acceptable thresholds[27]. These biogenic amines are related to microbial activity and their formation occurs mainly in alcoholic and malolactic fermentation[28,29]. It is thus that this valley (Maipo) showed a low presence of precursor amino acids of the mentioned biogenic amines (Figure 3). On the other hand, the Curicó valley is characterized by the presence of putrescine related with vines suffer stressful conditions[30] and phenylethylamine. The presence of the different biogenic amines is related to the amino acid content present in the samples. In this way, the Colchagua valley presents higher levels of amino acids and absence of biogenic amines, which suggests that the bacteria that metabolize malic acid do not metabolized the precursor amino acids associated with biogenic amines. This valley is characterized by the presence of Glycine, Alanine, Tyrosine, Threonine, Arginine, Methionine, Lysine, Valine, among others.

Guerrini et al. [31] determined the biogenic amine-producing capability of *O. oeni* strains, isolated from different Italian wines, and this capability was quali-quantitatively variable among the strains. Out of the 44 strains investigated,

more than 60% of the strains were able to produce histamine, in a range of concentrations between 1.0 to 33 mg/L, and about 16% showed the additional capability to form both putrescine and cadaverine.

Also, Landete et al. [32] found that although *O. oeni* was the most frequent histamine producer, this species also produced the lowest concentrations and that this production is strain dependent[33]. Coton et al. [34] reported in a study of 113 wine-isolated *O. oeni* strains, than 12 strains were positive for the biogenic amine-encoding genes histidine decarboxylase (10 strains) and ornithine decarboxylase (two strains). The ability of one or more strains to predominate in their natural habitat will allow not only to control the production of biogenic amines in wine, but also to deliver properties of oenological interest such as SO_2 resistance and sensory characteristics[35]. Our results showed that biogenic amine-production phenotype is a characteristic strain dependent.

In summary, most of isolated strains from Cabernet Sauvignon were identified as *O. oeni*. Strain showed similarity higher than 60% in all the samples, but they were clustered according to its geographical origin, indicating adaptation of *O. oeni* in each Valley and the condition of each vineyard.

ACKNOWLEDGEMENTS

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