INDIGENOUS *O. OENI* STRAIN SELECTION AS A MALOLACTIC FERMENTATION STARTER CULTURE TO AVOID THE HISTAMINE PRODUCTION IN WINE

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Poster presented at Enoforum 2013, 7-9 May, Arezzo (Italy)

Introduction

Biogenic amines (BA) are nitrogenous low molecular weight organic bases present in wine, mostly as a consequence of the decarboxylation of their respective free precursor amino acids, through the action of substrate-specific microbial decarboxylases. Histamine is produced by enzymatic decarboxylation of the amino acid histidine. It is assumed that most histamine in wine is produced by histidine decarboxylases from lactic acid bacteria during the malolactic fermentation (MLF) (Lonvaud-Funel 2001). Many authors have considered *O. oeni* as the dominant species during the MLF that produces histamine in wine (López *et al.* 2009). This BA formation seems to be strain-dependent. The objectives of this study were to identify the microorganism responsible for histamine formation in a winery from Ribera del Duero region (Spain), and the selection of a proper *O. oeni* MLF starter among the indigenous strains, unable to produce BA, able to dominate the population and decrease the histamine levels in wines.

Material and methods

Wine samples and histamine quantification
Wine samples were taken before and after the MLF from 13 vats with 20000 L of red wine from the cellar, and histamine quantification was carried out by HPLC (Peña-Gallego *et al.* 2009).

Isolation and identification of microorganisms
Total cell count with the Thoma chamber, and viable count plating decimal serial dilutions on MLO culture medium were made from all the samples before (A) and after (B) the MLF. From each sample, A and B, 10 colonies were randomly isolated from MLO plates on new culture media and the molecular technique of RAPD with M13 primer was performed to differentiate *O. oeni* strains (Zapparoli *et al.* 2000). Similarities between band profiles from the wine isolates were made employing the software BioNumerics versión 2.5 analyzed with the Pearson correlation coefficient and UPGMA clustering method.

Histaminogenic activity
The different *O. oeni* strains isolated from all samples on MLO media (Zuñiga *et al.* 1993) were grown in in liquid MLO until a final population of $2x10^9$ cfu/mL. Then $2x10^7$ cfu/mL were inoculated in MDB-mod (Landete *et al.* 2005) for 15 days. After that time the cultures were analysed by HPLC to determine if histamine had been produced.

MLF starter production and starter inoculation

The scale up of the selected strain was made through three stages procedure: 3 cultures of 50 mL (150 mL), 3 cultures of 0.5 L (1.5 L), and 3 cultures of 10 L (30 L). This 30 L inoculum was used to inoculate 20000 L. Wine in vat VI was inoculated with de selected strain ($2x10^6$ cfu/mL) and vat VU remained un-inoculated. All the samples collected under sterile conditions during the biomass production in the 10 L fermenters were also analysed microbiologically, counting the bacterial cells by Thoma counting chamber, and plating 0.1 mL of serial dilutions on MLO medium to study the growth dynamics. The plates were incubated at 28 ºC for 6 days.

Evolution of O. oeni *populations and histamine content in the inoculated (VI) and non-inoculated (VU) vats*

Just after the inoculation, samples from the two vats were taken and physicochemical and bacteriological analyses were made (VIBMLF and VUBMLF). Wine samples were taken also during the wine-making process: one week after the inoculation (1W) after the MLF (AMLF), after the SO_2 addition (SO_2), when wine was transferred to barrels (BL), and one year after the wine was transferred to barrels (BL1Y). The malic and lactic acid, histamine content and bacterial viability of both vats were analysed.

Results

Histamine determination in wine samples

The HPLC results showed that there was not histamine in wine samples from the vats before the MLF (A) was carried out (data not shown). Otherwise, histamine was found in the vat samples after the MLF (B), confirming that the LAB were the microorganisms responsible of the histamine formation. The HPLC data showed histamine contents classified as low, medium and high (Table 1).

***Table 1**. Histamine content (relative) after the MLF of the 13 studied wine vats.*

Samples B	Histamine mg/L
V1	high
V2	medium
V3	medium
V4	medium
V5	high
V6	low
V7	medium
V8	medium
V9	high
V10	low
V11	medium
V12	medium
V13	medium

Bacteria isolation and identification

In all cases, typical *O. oeni* colonial morphology was observed on the plates, and cell morphology under the microscope. The cell count showed an initial bacterial population of less than 10^4 cfu/mL in all samples, and bacterial populations between 10^6 cfu/mL and $8x10^6$ cfu/mL after MLF (Table 2).

Table 2. *Total and viable count of bacteria cells of the 13 wine vats studied before the MLF (A) and after the MLF (B).*

Sample	A (cfu/mL)		B (cfu/mL)	
	Total cells (Thoma C.)	Viable cells (MLO)	Total cells (Thoma C.)	Viable cells (MLO)
V1	$<10^5$	90	$1.6x10^7$	$5.6x10^6$
V2	$<10^5$	$9x10^2$	$8x10^6$	$7.5x10^6$
V3	$<10^5$	$6.5x10^2$	$2x10^7$	$6.4x10^6$
V4	$<10^5$	$5x10^2$	$1.2x10^7$	$9x10^6$
V5	$<10^5$	$1.25x10^3$	$1.6x10^7$	$8x10^6$
V6	$<10^5$	<10	$2x10^7$	$8x10^6$
V7	$<10^5$	$4x10^2$	$8x10^6$	$1.18x10^6$
V8	$<10^5$	90	$2x10^7$	$6.83x10^6$
V9	$<10^5$	70	$1.6x10^7$	$4.65x10^6$
V10	$<10^5$	20	$1.2x10^7$	$3.27x10^6$
V11	$<10^5$	90	$2x10^7$	$4.91x10^6$
V12	$<10^5$	20	$1.2x10^7$	$1x10^6$
V13	$<10^5$	$1x10^2$	$8x10^6$	$4x10^6$

The typification by the molecular technique of RAPD with M13 primer, showed that all isolates were grouped in eight different clusters (Figure 1). In one cluster, all the isolates of the V6B and the V10B vats were grouped; evidencing that was the same *O. oeni* strain (Table 3). In both vats the histamine content were the lowest, subsequently, this strain was considered a good option to be the selected MLF starter.

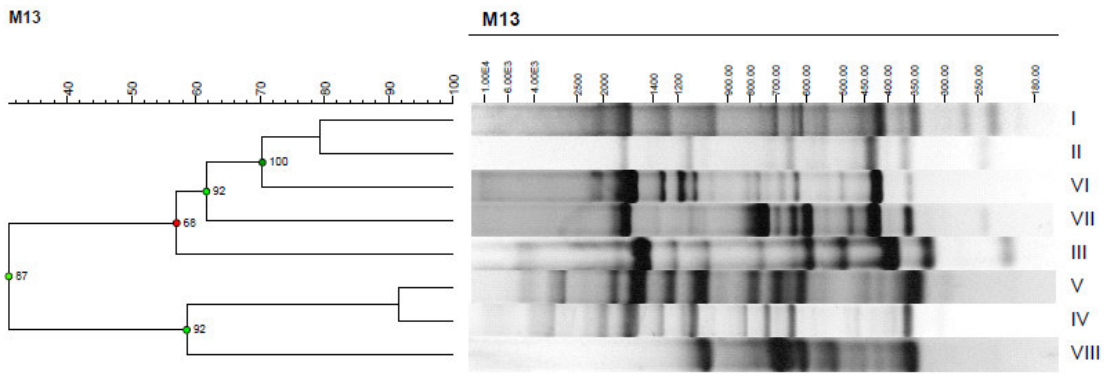


Figure 1. *Different band profiles (by RAPD with M13 primer) from O. oeni isolates, one representing isolate from each cluster (I-VIII).*

***Table 3**. Different band profiles (by RAPD with M13 primer), from O. oeni isolates, found in the wine vats before (A) and after (B) MLF.*

Sample	RAPD profiles	
	A	B
V1	IV-VI	VI
V2	IV-VI	III-VI
V3	VIII-V-VI	VI
V4	VI	VI
V5	VII-VI	VII-VI
V6	I	I
V7	III-IV-V-VI	VI
V8	VI	VI
V9	II-IV-VI	IV-VI
V10	I-II	I
V11	II-IV	VI
V12	VI	IV-V-VI
V13	IV-VI	VI

Histaminogenic activity

Three isolates, from the clusters III, VI and VIII, were able to produce histamine in MDB-mod medium (Table 4). Moreover, cluster VI, the cluster with the majority of the isolates from the wine samples, produced the higher histamine content, explaining why all the vats had histamine. The isolates belonging to cluster VI were present in all the wines containing significant amounts of histamine. The isolate V6B1 was not able to produce histamine in MDB-mod, therefore this would confirm that this *O. oeni* strain was the best option to be the MLF starter, and this strain was selected to the inoculation experiment.

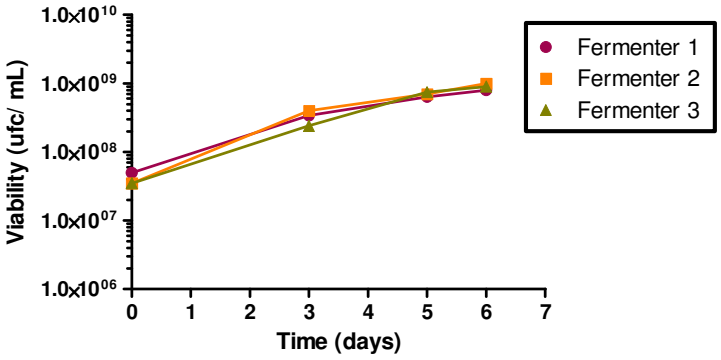
***Table 4.** Histamine content (mg/L) produced from the O. oeni isolates (one per each cluster formed) grown in MDB-mod for 15 days at 28 ºC.*

Cluster	Isolates	Histamine (mg/L)
I	V6B1	0
II	V9A1	0
III	V7A1	0.41
IV	V13A1	0
V	V12B7	0
VI	V5B2	17.37
VII	V5B10	0
VIII	V3A7	6.86

MLF starter production and starter inoculation

The final bacteria concentration of V6B1 in the three 10 L fermenters of starter production medium was $2x10^9$ cfu/mL after 6 days of incubation at room temperature (Figure 2).

***Figure 2**. Kinetics of viable V6B1 O. oeni cells (cfu/mL) from the three 10 L fermenters during 6 days.*



In the cellar, the vat VI was inoculated with the MLF starter previously prepared at a 1/1000 proportion to obtain a final concentration of $2x10^6$ cfu/mL bacteria. The VU vat was kept as non-inoculated control.

Before the inoculation experiment, samples from the two vats were taken and physicochemical and bacteriological analyses were made (VIBMLF and DUBMLF) (Table 5).

***Table 1.** HPLC analysis of vats VI and DU before the inoculation.*

Sample	Glucose (g/L)	Fructose (g/L)	Malic Ac. (g/L)	Lactic Ac. (g/L)	Ethanol % (v:v)	pH
VIBMLF	0	0.48	2.08	0.39	16.58	3.73
VUBMLF	0	0.47	2.12	0.34	16.58	3.73

Evolution of O. oeni *populations and histamine content in the inoculated (VI) and non-inoculated (VU) vats*

MLF took place in both vats but in VI began sooner. In both cases *O. oeni* population increased to beyond $1x10^7$ cfu/mL at the end of MLF (Figure 3).

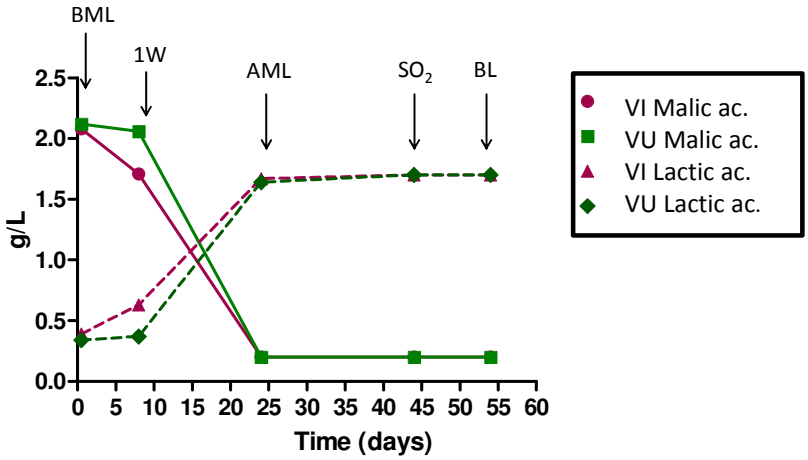


Figure 3. *Malic acid consumption and lactic acid formation from the different samples taken from the inoculated vat (VI) and the non-inoculated vat (VU), before the starter culture inoculation (BMLF), after 1 week from the inoculation (1W), after the MLF (AMLF), after the SO_2 addition (SO_2) and when wine was transferred to barrels (BL).*

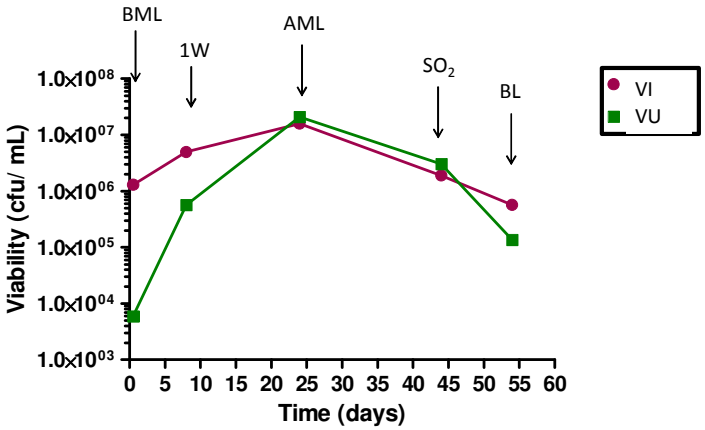


Figure 4. *Viability of the O. oeni population (cfu/mL) from the different samples taken from the inoculated vat (VI) and the non-inoculated vat (VU), before the starter culture inoculation (BMLF), after 1 week from the inoculation (1W), after the MLF (AMLF), after the SO_2 addition (SO_2) and when wine was transferred to barrels (BL).*

The HPLC results showed that histamine was not found before the MLF in any vat but when the MLF was finished there was 5 times more histamine in vat VU than in vat VI. This amount was increased after the addition of SO_2 and when wine was transferred to barrels. The vat VU increased 10 times more the histamine content that the vat VI at this moment. And after a year of aging in barrels, the histamine concentrations were 3 times higher in vat VU than in vat VI, proving that the inoculation of the MLF starter decreased significantly the levels of final histamine in commercial wines (Figure 4).

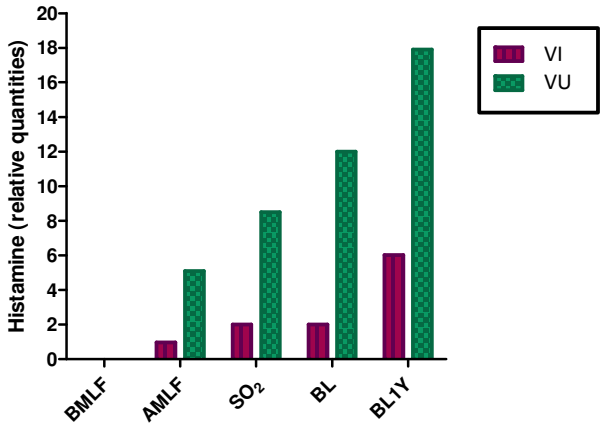


Figure 5. Evolution of the histamine content (relative quantities) from the different samples taken from VI and VU, before the starter culture inoculation (BMLF), after 1 week from the inoculation (1W), after the MLF (AMLF), after the SO_2 addition (SO_2), when wine was transferred to barrels (BL) and after1 year in barrels (BL1Y).

Conclusions

The results show that in the cellar studied there were both autochthonous *O. oeni* histamine producer and non-producer strains. From those non-producers *O. oeni*, a selection program was performed, and a strain chosen as a good candidate to become a starter. This starter was produced in semi-industrial levels to inoculate 20000 L of wine. Wines inoculated with the selected strain showed less histamine content than non-inoculated wines, after one year of ageing in barrels. This work shows the success of a selection program of an autochthonous *O. oeni* strain in a cellar to reduce dramatically the levels of histamine in red wines.

Bibiography

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