INFLUENCE OF INDIGENOUS NON-*SACCHAROMYCES* YEAST ON THE AROMATIC PROFILE OF RED WINES

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RESUMEN

En los últimos años numerosas bodegas están interesadas en producir vinos naturales o ecológicos, con el objetivo de ofrecer al consumidor un producto de calidad e identidad propia que les diferencie del resto. Estudios recientes han demostrado que las levaduras indígenas y especialmente las de tipo no-*Saccharomyces*, tienen un papel muy importante en el desarrollo de la fermentación y en las cualidades organolépticas de los vinos. Esto ha llevado a algunas bodegas a estudiar la posibilidad de utilizar las levaduras indígenas durante el proceso fermentativo. El objetivo de este trabajo es analizar la influencia de las levaduras indígenas previamente aisladas de las bodegas Áster y Díaz-Bayo (Ribera del Duero, España), en el perfil aromático y la complejidad de los vinos tintos. Los resultados obtenidos mediante cromatografía de gases (GC-FID) han permitido inferir asociaciones entre cepas de levaduras indígenas y presencia o ausencia de compuestos orgánicos importantes en el vino.

ABSTRACT

In recent years, many wineries are interested in producing natural or organic wines, with the aim of offering the consumer a high quality product with specific organoleptic characteristics that differentiates them from the rest. Studies carried out in the last years have shown that indigenous yeasts, especially the non-*Saccharomyces* ones, play a very important role in the development of fermentation and the organic complexity and quality of wines. This finding has motivated some wineries to study the possibility of using indigenous yeasts to carry out the fermentation process. The aim of this project is to analyze the influence of indigenous yeasts isolated from the wineries Aster and Diaz-Bayo (Ribera del Duero, Spain), in the flavor complexity of red wines. The results obtained by gas chromatography (GC-FID) allowed inferring associations between indigenous yeast strains and presence or absence of important organic compounds in wine.

INTRODUCTION

Wine identity depends largely on the particular activity of yeasts and lactic bacteria involved in the winemaking process (Hidalgo Togores, 2003; Fleet, 2003). However bouquet in wines is due mainly to organic acids, long chain alcohols, esters, and other molecules whose presence in fermented must is caused by the action of yeasts. Recently, one important trend followed by many wineries consist on the employ of standardized commercial yeasts which allows a better control on winemaking process, but causes a relevant homogenization of final product quality (Hidalgo Togores, 2003) In contrast, many wineries are really interested in the production of natural and ecologic wines, looking for offering high quality products with differentiated characteristics.

Saccharomyces cerevisiae is responsible for the fermentation of most sugars present in must (Boulton, 1998). However, other relevant yeast strains participate in the first stages of fermentation process contributing to final wine complexity (Ciani, Comitini, 2011; Carrascosa *et al.*, 2011). Yeasts from genus *Kloeckera*, *Hanseniaspora, Kluyveromyces*, *Candida*, *Pichia* and *Hansenula*, among others, are responsible for the starting of fermentation and the production of different metabolites causing particular organoleptic properties of wines (Egli *et al.*, 1998; Masneuf-Pomarede *et al.* 2007; Agnolucci *et al.*, 2007; Domizio *et al.*, 2007; Carrascosa *et al.*, 2011; Comitini *et al.*, 2011). Indeed, non-*Saccharomyces* yeasts avoid the presence of undesirable yeast strain producing metabolites that causes important defects on wines (Ciani, Comitini, 2011). According to the important role of non-*Saccharomyces* yeasts on wines, and the outstanding role they play on organoleptic complexity of wines, characterization of indigenous yeasts present in vineyards is a very useful tool to provide typical identity products.

The aim of this work is to study the influence of indigenous yeast on the aromatic profile and complexity of red wines. The yeast species included in the study are *Metschnikowia pulcherrima*, *Hanseniaespora uvarum*, *Kluyveromyces thermotolerans* and *Torulaspora delbrueckii*. These yeasts have been isolated from grape and must samples obtained from different wineries belonging to the Ribera del Duero PDO (Spain). Identification of these yeast strains have been performed using AFLP molecular markers, based on the methodology previously developed in the research team (Santos *et al.*, 2011).

MATERIAL AND METHODS

Chemicals

Dichlorometane was supplied by Sigma. Acetaldehide, ethyl acetate, 1-propanol, isobutyl acetate, ethyl butyrate, 2-methyl-propan-1-ol, isoamyle acetate, 1-butanol, ethyl hexanoate, ethyl octanoate and ethyl propionate were purchased from Dr. Ehrenstorfer GmbH. Concentrated red wine must was supplied by Mostos Españoles S.A. Glucose, peptone and yeast extract were obtained from Conda. Deionized water was used to prepare all media and stock solutions.

Isolation and identification of yeast strains

Grape and must samples obtained from wineries Áster and Díaz-Bayo, belonging to the Ribera del Duero PDO (Spain), were employed to isolate and characterize yeasts strains using typical microbiology and molecular biology techniques (Kurtzman, Robnett, 1998). Identification of theses strains was accomplished using AFLP molecular markers, based on the methodology previously developed in the research team (Santos *et al.,* 2001). Tab.1. shows the indigenous yeast strains that have been used for the present work.

Tab.1.- Isolated yeast strains from Ribera del Duero PDO wineries

Yeast	Strains
Metschnikowia pulcherrima	*Mp*U3.2 / *Mp*U3.4 / *Mp* M1.4.1 / *Mp* M1.4.5fl
Hanseniaspora uvarum	*Hu* M1.4.2fl / *Hu* M1.5.1
Kluyveromyces thermotolerans	*Kt* U3.1 / *Kt* M1.5.2
Torulaspora delbrueckii	*Td* M3.7.8 / *Td* M3.7.13
Saccharomyces cerevisiae	*Sc* U3c1 / *Sc* M1.4.2
Saccharomyces uvarum	*Sc* M1.6.2fl / *Sc* M1.6.4fl

Microfermentation assays

Small scale fermentations were carried out in laboratory both using only one strain and selected combinations of strains as shown below in Tab.2, 3 and 4. The results obtained from sensory evaluation of fermentations managed by the oenologist of Díaz Bayo Winery, were considered in order to select the proper yeast combinations. For these experiments, 50 mL of rehydrated must (15º Bx) were used and 10^7 cells·mL^{-1} was employed as total initial biomass concentration. Pre-inoculums in liquid YPD medium (20 g.L^{-1} glucose, 20 g.L^{-1} peptone and 10 g.L^{-1} yeast extract) were prepared from frozen stocks and grown over night at 22ºC. Samples were collected after 4 days of fermentation.

Gas-chromatography analysis

Gas chromatography coupled with flame ionization detector (GC-FID) was employed with samples directly collected from fermentation broth. Acetaldehyde, ethyl acetate, isoamyl acetate, ethyl butyrate, ethyl hexanoate, ethyl octanoate and ethyl propionate were determined by direct injection into a 7980A gas chromatographer (Agilent Technologies Inc., Palo Alto, CA) equipped with a flame ionization detector. Injector and detector were both set up at 280º. Samples (2 μL) were injected using the split mode (split ratio 10:1) and analyzed in a TRB-FFAP (Teknokroma, Sant Cugat del Vallès, Barcelona, Spain) fused silica column (30 m x 0.25 mm x 0.25 μm), with helium (37.3 psi) as the carrier gas and a temperature program (60°C to 200°C, 1 min initial hold, 20°C min^{-1} ramp rate) (Cedrón Fernández, 2004). Peaks were identified on the basis of sample coincidence with relative retention times of commercial standards. These analysis were performed at the Gas Chromatography Service of Biological Research Center (CIB-CSIC).

RESULTS AND DISCUSSION

As a starting point, eleven microfermentations using only one yeast strain were carried out (Tab.2). Must samples analyzed by GC-FID after 4 days of fermentation revealed some differences in the production of organic compounds important for the aromatic profile of wines (Tab.3).

Tab.2.- Fermentation experiments carried out with one yeast strain

Fermentation No.		Strain
1	*Metschnikowia pulcherrima*	U3.2
2		M1.4.1
3		U3c3
4		M1.4.5fl
5	*Hanseniaspora uvarum*	M1.4.2fl
6		M1.5.1
7	*Kluyveromyces thermotolerans*	U3.1
8		M1.5.2
9	*Torulaspora delbrueckii*	M3.7.13
10	*Saccharomyces cerevisiae*	U3c1
11	*Saccharomyces uvarum*	M1.6.4fl

Tab.3.- Volatile compounds and their associated flavour

Compound	Retention time	Flavour/s
	min.	
Acetaldehyde[(a)]	1.267	Apple
Ethyl acetate[(a)]	1.607	Varnish, acetone
2-Methyl-propan-1-ol[(b)]	1.668	Alcoholic sensation
Isobutyl acetate[(b)]	2.725	Fruity, banana, strawberry
Ethyl butyrate[(b)]	3.112	Fruity, apple, papaya
Isoamyl acetate [(a)]	4.658	Banana
Ethyl hexanoate[(a)]	6.721	Banana, apple
Ethyl octanoate [(a)]	9.218	Pineapple, pear

[(a)] (Catania, Avagnina, 2007); [(b)] (Cedrón Fernández, 2004)

Fig.1 shows the relative production of six of the previously mentioned compounds important for wine aroma in the single fermentation experiments. Relative production is calculated as the ratio between the chromatographic signal of a particular compound and the maximum signal obtained. This way allows comparing the production of specific molecules among the different tested yeasts. According to this, different flavour profiles associated to each yeast strain used in this single strain fermentation can be differentiated. Hence, fermentation carried out by *M. pulcherrima* M1.4.1 (Ferm. 2) and M1.4.5fl (Ferm. 4) strains achieves the highest production of acetaldehyde. *M. pulcherrima* M1.4.1 is also responsible for an important accumulation of ethyl hexanoate. Fermentation carried out using only *T. delbrueckii* M3.7.13 (Ferm. 9) has led the most important levels of ethyl acetate, 1-propanol, ethyl octanoate and ethyl propionate. Fermentation carried out by *M.*

pulcherrima U3.2 (Ferm. 1) also produced a balanced combination of volatile compounds due to the big production of acetaldehyde, 1-propanol, ethyl hexanoate, ethyl octanoate and ethyl propionate. Both fermentation carried out by *Saccharomyces* strains (Ferm. 10 and 11) resulted in complex volatile profiles according to the quantities of esters produced during these fermentations.

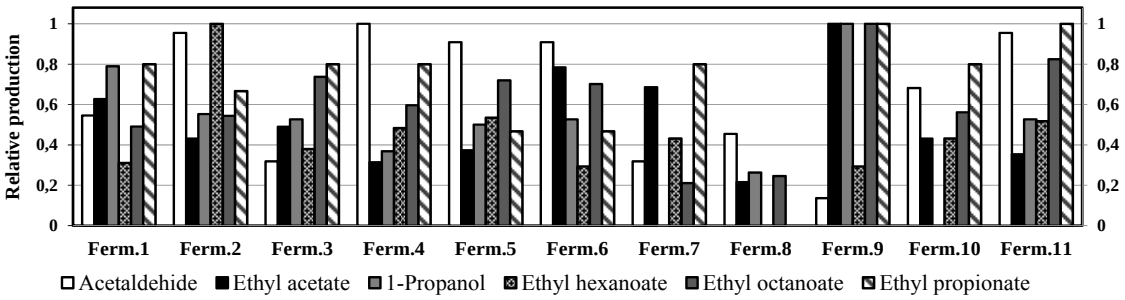

Fig.1.- Volatile compound profiles obtained using one yeast strain.

After these results, new experiments were designed combining *Saccharomyces cerevisiae* U3c1 and the previously mentioned non-*Saccharomyces* strains (Tab.4.)

Tab.4.- Fermentation experiments carried out with two yeast strains

Fermentation No.							
12	13	14	15	16	17	18	19
M. p. U3.2	*M. p.* M1.4.1	*M .p* M1.4.5fl	*H. u* M1.4.2fl	*H. u.* M1.5.1	*K. t.* U3.1	*K. t* .M1.5.2	*T. d.* M3.7.8
S. cerevisiae U3c1							

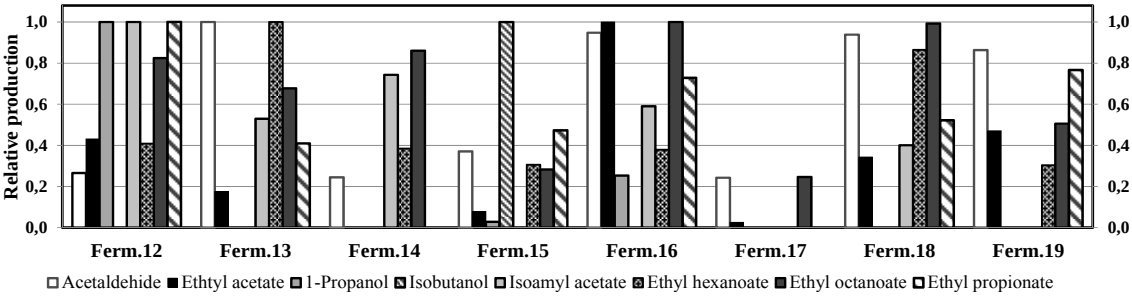

Fig.2.- Volatile compound profiles obtained from fermentation carried out with two yeast strains.

The results in Fig.2 show that combinations of *S. cerevisiae* U3c1 with *M. pulcherrima* U3.2 (Ferm. 12) and *H. uvarum* M1.5.1 (Ferm. 16) achieve the most complex volatile profile, causing no accumulation of isobutanol. *M. pulcherrima* 1.4.1 (Ferm. 13) and *K. thermotolerans* M1.5.2 (Ferm. 18) also offered an interesting profile, taking into account both the sensory evaluation carried out and the demonstrated outstanding presence of isoamyl acetate and ethyl esters of hexanoic, octanoic and propionic acid. Consistently

with results obtained in the single strain fermentations, *M. pulcherrima* U3.2 and 1.4.1 in combination with *S.cerevisie* produce a balanced mixture of aldehydes and esters. In the case of *H. uvarum* M1.5.1 and *K. thermotolerans* M1.5.2, the combination with *S. cerevisiae* U3c1 show synergic effects enhancing their joint fermentation action. Hence, although fermentations carried out using these strains separately did not get a high production of organic compounds, the combined fermentation with *S. cerevisiae* U3c1 yielded high build up of acetaldehyde, 1-propanol, isoamyl acetate, ethyl hexanoate and ethyl octanoate.

After these single and binary experiment sets, following the oenologist advice, five complex fermentations were carried out in which a common base of yeasts was inoculated in all cases and supplemented with the strains indicated in Tab.5. The yeast set consisted on a common combination of *M. pulcherrima* U3.2, U3.4, M1.4.1 and M1.4.5fl, and *H. uvarum* M1.4.2fl and M1.5.1.

Tab.5.- Fermentations carried out with complex combinations of yeast strains.

Fermentation No	Strains
20	*S. c.* M1.4.2[(c)]
21	*K. t.* U3.1, *H. u.* M1.5.2, *S. c.* M1.4.2[(c)]
22	*K. t.* U3.1, *H. u.* M1.5.2, *T. d.* M3.7.8, *S. c.* U3c1, *S. u.* M1.6.2fl [(c)]
23	*T. d.* M3.7.13, *S. u.* M1.6.4fl [(c)]
24	*H. u.*U3.1, *H. u.* M1.5.2, *T. d.* M3.7.8, *T. d.* M3.7.13, *S. c.* U3c1, *S. u.* M1.6.2fl, *S. u.* M1.6.4fl[(c)]

[(c)]Besides the previously mentioned set of yeasts.

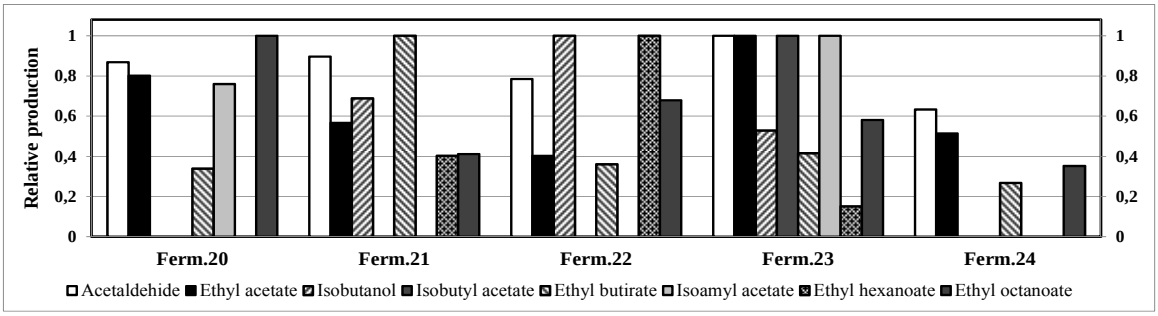

Fig.3.- Volatile compound profiles obtained after 4 days of fermentation using complex yeast combinations.

Considering the differences between the five profiles revealed by GC-FID (Fig.3), since the complexity in yeast strain combination is not directly linked with complexity in the volatile compound profiles obtained, a further analysis need to be done. For instance, fermentation No. 24, carried out by a large number of yeast strains resulted in a relative poor aromatic profile consisting on acetaldehyde, and low levels of ethyl butyrate an octanoate. In contrast, simpler combinations (Ferm.20 and 23) seemed to produces bigger quantities of acetaldehyde and esters. To separate crossed effects, statistical analysis of the obtained results was performed in order to study the influence of these strains on the production of volatile compounds, showing significant differences on the role of these

yeasts on aroma profile of red wines. As a result of this study, two groups of yeasts have shown an outstanding role on the production and build up of organic compounds involved in aroma. On one hand, the non-*Saccharomyces M. pulcherrima* U3.1, *K. thermotolerans* M1.5.2 and *T. delbrueckii* M3.7.8 and M3.7.13, showed different responses related to production of ethyl acetate, isoamyl acetate, ethyl butyrate and ethyl octanoate. On the other hand, *S. cerevisiae* U3c1 and M1.4.2 and *S. uvarum* M1.6.2fl and M1.6.4fl which are responsible for acetaldehyde, isobutanol and isobutyl acetate production in different levels. These effects were studied and separated as explained below.

CONCLUSIONS

Single and complex fermentation experiments carried out with indigenous yeasts revealed the role of some of them in the aromatic profile of red wines and showed differences between indigenous yeast strains of the same species. For instance, it has been shown that *T. delbrueckii* M3.7.8 contributes to the accumulation of ethyl octanoate and the reduction in the levels of ethyl butyrate. However, *T. delbrueckii* M3.7.13 seems to cause the most important build up of ethyl acetate. Combined action of *K. thermotolerans* U3.1 and M1.5.2 causes an intense production of ethyl butyrate and the consumption of ethyl acetate, isoamyle acetate and ethyl octanoate. Among the results related to *Saccharomyces* yeasts, *S. uvarum* M1.6.4fl is responsible for the highest accumulation of acetaldehyde, while *S. uvarum* M1.6.2fl assists its elimination.

In conclusion, it can be inferred that combined action of *T. delbrueckii* M3.7.13 and *S. uvarum* M1.6.4fl is responsible for a big build up of isobutyl acetate. However, it seems that simultaneous absence of strains *S. uvarum* M1.6.4fl, *S. cerevisie* M1.4.2 and *T. delbrueckii* M 7.13 induce the highest production of isobutanol.

Moreover, combined GC-FID technique and statistical analysis, have been proved to be a good tool for the elucidation of the role played by different indigenous yeast strains in fermented musts.

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