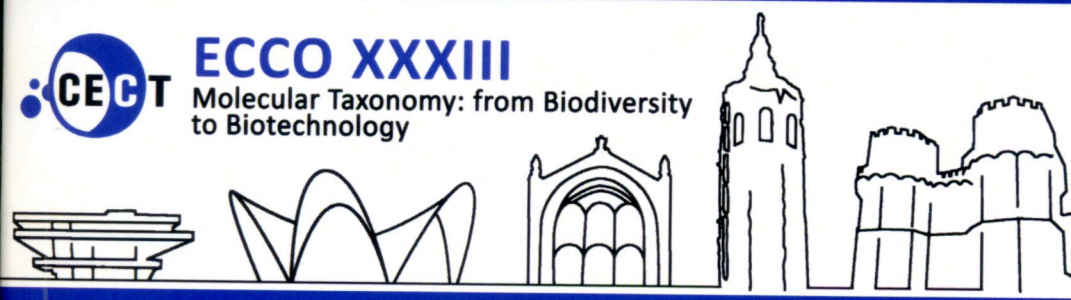

ECCO XXXIII

Molecular Taxonomy: from biodiversity to biotechnology

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ABSTRACT BOOK

Acetobacter musti sp. nov., an acetic acid bacteria isolated from Bobal grape must

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New species of acetic acid bacteria (AAB) isolated from wine have been described during the last few years. The use of new powerful molecular tools, more discriminative, high throughput, automatable, objective, and reliable, is allowing a faster and deeper knowledge of this group of microorganisms. Besides, the climate change and the modifications in the viticultural and enological practices occurred in the recent years are changing the conditions where microbes can propagate: higher pH and ethanol content, decrease in the use of SO_2, etc. Altogether, these changes explain this emergency of new species in wines.

During a study of the microbial biodiversity of spontaneous fermentation of Bobal grape must, 48 AAB isolates were collected. The isolates were investigated by an extensive polyphasic analysis using genotypic and phenotypic methods. The composite dendrogram built by comparing the results obtained from the different techniques, showed one strain (Bo7) that did not cluster with any of the reference species of the genus *Acetobacter*. Phylogenetic analysis based on 16S rRNA gene sequences allocated the Bo7 strain to the genus *Acetobacter*, and revealed *Acetobacter aceti* and *Acetobacter oeni* to be nearest neighbors. DNA–DNA hybridizations demonstrated that the isolate belongs to a single novel genospecies that could be differentiated from its phylogenetically nearest neighbors by the following phenotypic characteristics: no production of 5-keto-D-gluconic acid from D-glucose; growth on glycerol, but not on methanol and maltose, as sole carbon sources, and growth on yeast extract with 30% D-glucose. The results obtained prove that the strain should be classified as representatives of a novel *Acetobacter* species, for which the name *Acetobacter musti* sp. nov. is proposed. The type strain is strain Bo7^T (=DSMZ 2382^T =CECT 7722^T).

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RAPD typing: A useful tool for selection and implantation analysis of an *O. oeni* starter non-histamine producer in wine

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Malolactic fermentation (MLF) is considered a simple phenomenon but with a practical relevance in wine. The main value of the MLF in vinification is the biological de-acidification, which results from the transformation of L-malic acid into L-lactic acid by lactic acid bacteria (LAB), mainly by *Oenococcus oeni*. Some LAB strains can produce histamine, which can cause physiological distress in the human organism, and economical losses to winemakers. The objectives of this work were: 1) to identify the LAB strains present at the cellar, 2) to determine which autochthonous strains were able to produce histamine, 3) to monitorize the implantation of a selected safe strain in cellar vinifications and 4) to reduce the histamine content in wine by inoculating an *O. oeni* strain non-histamine producer.

To achieve the objectives 1 and 3, we used the molecular technique of Random Amplified Polymorphic DNA PCR (RAPD-PCR) and for objectives 2 and 4 we performed biogenic amines (BA) quantification by HPLC. To carry out the first aim, the population dynamics from 13 cellar vats were studied during one vintage using RAPD-PCR with the M13 primer. Eight different *O. oeni* strains were found in the cellar, three of them were able to produce histamine and they were present in almost all vats. One unique strain was present only in the 2 vats showing the lowest histamine content; this strain was not able to produce histamine. This *O. oeni* strain was selected as malolactic starter culture and was inoculated the following year in a vat of the same cellar to carry out the MLF. RAPD-PCR was applied to monitorize the dynamics of *O. oeni* natural and inoculated population. Two different *O. oeni* strains were found in the inoculated vat along the winemaking process. Isolates from the first stages of vinification showed an unique RAPD pattern that matched with that of the inoculated strain, confirming de implantation of the inoculated strain. Other strain was isolated during ageing of wine in barrels. This strain was able to produce histamine, explaining why the content of this BA increased at this vinification stage. The inoculation of the safe *O. oeni* strain allowed completing MLF and to diminish six times the histamine content. RAPD-PCR technique was a very useful tool to discriminate *O. oeni* strains and to determine the implantation of the selected safe strain.

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Genetic diversity in *Oenococcus oeni* strains isolated from musts and wines

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Oenococcus oeni has been identified as the principal organism involved in Malolactic Fermentation (MLF). This process consists in the decarboxylation of malic acid to lactic acid and carbon dioxide. As a consequence of it, decreases total acidity, increases pH, improves organoleptic characteristics and improves the microbiological stability of wine. The availability of reliable methods for strain differentiation is crucial to evaluate both the intraspecific diversity as the diversity existing in wine fermentation. One hundred and four isolates from ENOLAB culture collection obtained from 19 different cellars, located in different winemaking regions of Spain and Portugal, were genotyped in order to study the intraspecific diversity of this specie. In addition, the *O. oeni* diversity in the cellars of Utiel-Requena D.O was evaluated. To carry out this objective the Random Amplified Polymorphic DNA (RAPD) analysis with M13 primer (Zapparoli et al., 2000) and Multiple-locus Variable Number of Tandem Repeats (VNTR) analysis (Claisse and Lonvaud-Funel, 2012) were used. Individual and combined DNA fingerprinting patterns were analyzed on BioNumerics software using Unweighted Pair Group Method with Arithmetic Average (UPGMA) and the Pearson correlation coefficient. The combined analysis grouped 104 isolates in 87 clusters at a cut-off value of 97.5 % similarity; this threshold is based on reproducibility results of this technique. The discriminative power of these two combined techniques (0.995) is higher than that of each one separately: RAPD (0.98) and VNTR (0.90). The value of Diversity index ($D=1-(\Sigma n(n-1)/n(n-1))$) showed a high genomic intraspecific diversity taking into account that the max value of this diversity index is 1. Camporrobles was the cellar showing a higher diversity. RAPD and VNTR analysis are each one suitable genotyping methods to discriminate strains and to determine intraspecific diversity but the discriminative power increased when they are combined.

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Rapid detection and identification of wine microorganisms by FISH coupled to epifluorescence microscopy and flow cytometry

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Flow cytometry can be considered a useful method for microbiological quality control in wineries and for the investigation of the growth dynamics of microorganisms in wine. This technique offers a number of advantages such a high speed, high precision, simultaneous measurements of multiple cellular parameters and possibility to detect the presence of heterogeneous populations. The aim of this work was to adapt previous fluorescence in situ hybridization (FISH) protocols for wine yeasts and lactic acid bacteria to flow cytometry and epifluorescence microscopy analysis. For this, we optimized a liquid hybridization protocol with specific fluorescein-labelled oligonucleotide probes targeted to the rRNA of different wine species, such as *Saccharomyces cerevisiae* and *Lactobacillus plantarum*. The labelled probes targeted to the D1-D2 region of 26SrRNA of yeasts and the V1 region of 16SrRNA of bacteria. Hybridization protocols comprised optimized permeabilization, fixation, hybridization, and washing conditions for *S. cerevisiae* and *L. plantarum*. The application of this protocol allowed to achieve 100% hybridization of cells. The effort and time required to detect these two species could be greatly diminished by using any of the two techniques. Both methods are based on hybridization with extremely specific probes and, in addition, permit the identification of various species present in a sample while performing a single analysis.

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Analysis and direct quantification of *Brettanomyces bruxellensis* populations in red wine by plate count, FISH, flow cytometry and quantitative PCR

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The yeast *Brettanomyces bruxellensis* is a spoilage microorganism found in wine and various alcoholic beverages. This yeast is usually present on grape berries, cellar equipment and barrels, and thrives during wine ageing. *B. bruxellensis* has been characterized as the principal agent responsible for the formation of volatile phenols, namely 4-ethylphenol and 4-ethylguaiacol. Traditional microbiological methods are based on culturing on appropriate differential and selective media and incubation for 1-2 weeks. Due to the inaccuracy and long time to achieve results, more emphasis in recent years has been on using culture independent techniques such as quantitative PCR (qPCR), FISH, and flow cytometry. These methods are generally faster, more specific, sensitive and accurate. The aim of the present work was to detect and quantify *B. bruxellensis* populations in red wine by culture independent methods, such as qPCR, FISH, and flow cytometry, and compare with results obtained by plating. For this, we previously optimized the qPCR, FISH and flow cytometry methods. Calibrations were done in the range of 10^1-10^8 cells/mL. For cell quantification by flow cytometry, we optimized a liquid hybridization protocol with specific fluorescein-labelled probes targeted to the D1-D2 region of 26S rRNA of *B. bruxellensis*. To quantify cell concentration, the hybridized samples were analyzed by FISH and flow cytometry, and a calibration was performed by plotting total cell count versus number of quantified cells by flow cytometry for different cell concentrations (10^1-10^8 cells/mL). Finally, we performed a correlation in cell quantification by the methods previously described.

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Evaluation of the behaviour of two organic yeasts in organic bobal must vinifications: Implantation and fermentation abilities

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The demand for organic wines has changed the vine culture and vinification techniques in order to adapt them to the legislation controlling their production. Although the use of selected yeast or bacteria cultures is allowed for organic wine product ion, producers of these kinds of wines usually prefer not adding them or, if they do, use "organic" microorganisms. Organic microorganism must be isolated from organic grapes or organic vinifications. There is an interesting market opportunity for this type of microorganisms because its use allows to maintain the organic character of wine and to control fermentation.

In a previous work, 132 yeast isolates were obtained from organic vinifications of Tempranillo, Bobal and Cabernet-Sauvignon organically cultured grapes. They were identified, by restriction analysis of Intergenic Transcribed Spacers (ITS) [1] and then characterized at the strain level by restriction analysis of mitochondrial DNA [2]. Their metabolic, enzymatic, and technological characteristics were also studied in order to select the best strains to conduct alcoholic fermentation. Twenty four different strains of *Saccharomyces cerevisiae* were identified and two strains were retained after the selection program: 55A and 110A.

The aim of this work was to study the behaviors of these two yeasts during vinification and to compare them with that of the commercial yeast "Excellence SP" (Lamothe-Abiet). To perform this objective, yeasts were inoculated at two different concentrations in 50 L of Bobal must. Implantation of selected and commercial yeasts was estimated by plating samples corresponding to middle and final stages of fermentation. Finally, wines were physico-chemically characterized. Results obtained were compared with each other and the findings of the experiment were derived.

The sugar concentration was below 0.45 g/L whatever yeast and inoculum level used, although strain 55A consumed 8,5% more sugar. There were not significant differences in ethanol content among wines. Glycerol content was higher when a lower concentration was used for inoculation: a 4,6% and a 7,2% higher for 55A and 110A, respectively. In sensory analysis, the wines fermented with strains 110A and 55A achieved a higher score than those fermented with the commercial strain. Strain 55A showed the highest percentage of implantation whereas the other two were unable to overtake the native population. Due to this fact, results obtained for wines inoculated with 110A strains cannot be attributed to this strain.

Keywords : wine , organic yeast , implantation , yeast selection , Bobal, identification, typing, alcoholic fermentation.

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A PMA-qPCR assay for a rapid detection and quantification of *Saccharomyces cerevisiae* viable cells from wine

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Saccharomyces cerevisiae plays a beneficial and essential role in winemaking, in which it is the predominant microbial species. Nevertheless, *S. cerevisiae* is able to spoil wine after the alcoholic fermentation if the yeasts are not properly eliminated or controlled and residual sugars remain, causing refermentations. The spoilage can be very important because yeasts resist high ethanol concentrations. In wineries, culture-dependent methods are the most commonly used, but it often cause confusion since they are heavily dependent on the microorganisms' physiological status and growing capacity. Quantitative PCR (qPCR) techniques have already been developed, but usually these methods are incapable of discriminating between viable and dead cells in a sample. Recently, some dyes, as propidium monoazide (PMA), have been used in conjunction with qPCR to selectively detect live cells. PMA selectively penetrates the membrane of dead cells and forms stable DNA monoadducts upon photolysis, resulting in DNA that cannot be amplified by PCR. Furthermore, the PCR reaction is frequently inhibited by substances present in wine, typically but not only phenolic compounds. In the present study we have developed a fast and reliable qPCR method in conjunction with propidium monoazide (PMA) – PMA-qPCR. Altogether, the optimized method involves the removal of the wine inhibitors and the ability to distinguish between live and dead yeast cells by their different responses to the PMA-qPCR reaction. Numerous standard curves were built, growing *S. cerevisiae* in must or wine. The small standard errors with these replicas proved that the assay is reproducible and highly robust. Conventional (cfu) counting were also performed. The PCR assay confirmed to be specific for *S. cerevisiae* and correlated to the conventional plating method. PMA-qPCR provides a useful tool for the rapid and direct detection and quantification of viable *S. cerevisiae* in wine, enabling winemakers to take wine processing decisions more quickly, in order to reduce the threat of spoilage by *S. cerevisiae*.

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