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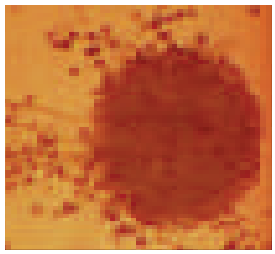

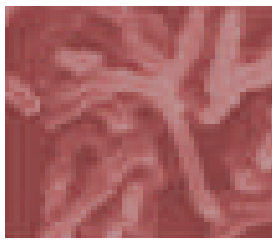

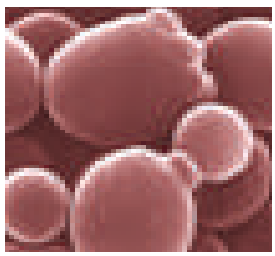

Wine Microbiology and Safety:
from the vineyard to the bottle
(Microsafetywine)

BOOK OF ABSTRACTS

Martina Franca (TA), Italy

19 - 20 November, 2009

http://www.mycotox-society.org/microsafety-2009

WINE MICROBIOLOGY AND SAFETY: FROM THE VINEYARD TO THE BOTTLE (MICROSAFETYWINE)

19-20 November, 2009
Martina Franca (Italy)

Organized by

CNR-Institute of Sciences of Food Production Lecce (Italy)

Agricultural Faculty University of Basilicata Potenza (Italy)

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Welcome to the 2nd Microsafetywine

On behalf of the Scientific Committee we are glad to welcome you in attending and taking part to the II International Symposium “MICROSAFETYWINE”, focused on “Wine Microbiology and Quality of Wine", held in Martina Franca (Taranto, Italy) on 19th and 20th November 2009. The specific title of the congress is "Wine Microbiology and Safety: from the vineyard to the bottle"

This Symposium has the principal scope to gather people interested in the enhancement of quality and safety of wine production, by emphasizing the role of micro-organisms (yeast, bacteria, fungi) on wine quality and safety.

This event, which is under the "aegis" of the International Organisation of Vine and Wine and of the International Society of Mycotoxicology, supplies a great opportunity for all the subjects involved in the wine chain since it will promote the diffusion of scientific results useful to support small and middle wine industries.

Scientific know-how will be available for technical operators with the aim to fill the existing gap between the scientific world and the industry, helping wine producers to meet the technical challenges of the twenty-first century.

Articles of selected research presentations will be published in a spread issue of the peer-reviewed scientific journal Annals of Microbiology.

We are grateful to the Sponsors who made possible the 2nd Microsafetywine in Italy, financially supporting the local committee.

We wish all participants a productive and very fun meeting, in the welcoming atmosphere of Apulia, a colourful region of Southern Italy.

The Organizing Committee

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Simona Antonella Lamorte, *OIV*

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Alessandra Ricelli, *Institute of Biomolecular Chemistry-CNR, Italy*

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Michele Solfrizzo, *Institute of Science of Food Production of CNR, Italy*

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Ana Puig Pujol, *INCAVI, Spain*

12.10 **Identification of peptides marker for the detection of caseinate used as fining agent in white wine by capLC-MS/MS method**

Linda Monaci, *Institute of Science of Food Production of CNR, Italy*

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The OIV and its role in the vitivinicultural sector in the world and with respect to EU: strategic axes and actions for microbiological quality of wine

Lamorte S. A.

Oenology-Methods of Analysis Unit of OIV, International Organisation of Vine and Wine, 18 Rue d'Aguesseau 75015 Paris

The International Organisation of Vine and Wine (OIV) is an intergovernmental organisation of a scientific and technical nature, of recognised competence for its works concerning vines, wine, wine-based beverages, table grapes, raisins and other vine-based products as defined in the Agreement of 3 April 2001 signed by the 43 member States.
Since UE Regulation 479/2008, the European Union officially referred to OIV for scientific and technical competence concerning vine and wine. The European Commission in fact when authorising oenological practices shall base itself on the oenological practices recommended and published by the OIV *(Article 30).* The methods of analysis for determining and the composition of the products covered by this Regulation and the rules whereby it may be established whether these products have undergone processes contrary to the authorised oenological practices shall be those recommended and published by the OIV *(Article 32).*
Concerning "*Rules applying to imports",* save as otherwise provided in agreements concluded pursuant to Article 300 of the EU Treaty, vitivinicultural products shall be produced in accordance with oenological practices recommended and published by the OIV *(Article 82).*
The OIV conducts its scientific activity through expert groups, sub-commissions and commissions, co-ordinated by a Scientific and Technical Committee.
The commissions and expert groups develop their outcomes according to OIV Strategic Plans, in accordance with the priorities established and Commission II "Oenology" is responsible for all matters relating to the composition and making of beverages, particularly wines, their storage conditions, packaging, transportation and consumption. Among experts groups of Commission Oenology, the MICROBIOLOGY group of experts is more specifically engaged with issues relating to microbiological quality of wine referred in Strategic Plan such as:

- K.4 Propose means for detecting and limiting contaminations and D.2 Establish guidelines for implementation of concept of sustainable development applied to product safety in the vitivinicultural sector, a clear example of these synergic axis is the Code of sound vitivinicultural practices in order to minimise levels of ochratoxin A in vine-based products adopted in 2005 (Resolution VITI-OENO 1/2005);
- E.8 Review consequences of climate change on the evolution and the activity of micro-organisms;
- G.2 Study applications of functional genomics on micro-organisms;
- H.2 Evaluate environmental impact of genetically modified organisms in vitiviniculture;
- I.4 Develop a selection guide for micro-organisms in accordance with their biodiversity and their technological interest;
- J.5 Develop methods for the identification and characterisation of micro-organisms of oenological interest (microbiology research, methods of analysis, molecular methods).

References

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OIV. Strategic Plan 2009-2012. www.oiv.int

Session I
Microbial control in the vineyard

Oral presentations

Managing ochratoxin A contamination in the vineyards

Cozzi G.[1], G. Perrone[1], P. Battilani[2], A. Logrieco[1] and A.Visconti[1]

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[2]Insititute of Entomology and Plant Pathology, Università Cattolica del Sacro Cuore, Via Emilia Parmense, 84, 29100 Piacenza, Italy

Ochratoxin A (OTA) in wine is a problem that originates principally in the vineyard. Analysis of wine samples throughout Europe have shown that there is a gradient in OTA content with a decrease in concentration from red, to rosé and white wine. In addition, some grape varieties display greater susceptibility to aspergillus rots due to intrinsic genetic characteristics and probably to the grape-bunch structure. OTA contamination is also dependent on the latitude of the production region with a positive gradient from North to South and West to East. Climatic conditions (high humidity and temperature) and geographical location (closeness to the sea) are important factors favouring OTA accumulation in grape berries. The meteorological conditions need to be monitored carefully from veraison to harvest since the severity of aspergillus bunch rot and OTA accumulation is influenced by excessive irrigation and rainfall during this time. Developing predictive models and risk maps can help to optimise preventive measures and control OTA accumulation in grapes.
"Black aspergilli" are the main source of contamination by ochratoxin A of grapes and wine. OTA producing black aspergilli include mainly *Aspergillus carbonarius*, followed by *A. niger* and possibly *A. tubingensis*. Understanding the ecology and the physiology of these fungi can provide tools for management of OTA at all stages of grape production. Black aspergilli are opportunistic fungi that, although always present in field, may develop massively on damaged berries by abiotic and/or biotic causes, from veraison to harvest, with a high incidence at ripening. Monitoring black aspergilli, and in particular *A. carbonarius* level in vineyard during this period is critical point to know the potential OTA risk and adopt the good agricultural practices in the vineyard.
Berry wounds caused by biotic and abiotic factors also provide preferential entries for black aspergilli. In particular the control of grape berry moth *Lobesia botrana,* that is a key pest in vineyards in southern Europe, may reduce significantly the OTA contamination in grape.

Work supported in part by regional project INNOWINE (POR Puglia 2000/2006, PS 008)

Reduction of ochratoxin A in grapes and wine through chemical and biological control in vineyard

Battilani P.[1], G. Cozzi [2], E. Tjamos[3] and A. Logrieco[2]

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[2]*Institute of Sciences of Food Production (ISPA), National Research Council (CNR), Via Amendola 122/O - 70126 Bari, Italy*
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Several microorganism can cause grape berries decay, but the main concern for human health is actually related to mycotoxin. Mycotoxin producing fungi are weak parasites that cause symptoms not always visible and commonly related to limited yield losses. Actions devoted to their direct control are not included among common agricultural practices as it happens for many other plant pathogens. Farmers are reluctant in using fungicides because of the limited payback perceived and industries do limited efforts to find out specific new molecules because of the supposed limited market. Nevertheless, some interesting results were recently published and they can contribute to abide the mandatory legal limits defined by European Commission.
Aspergilli belonging to section *Nigri* are responsible for ochratoxin A (OTA) contamination in grapes. They take advantage of wounds on berries in starting the infection and consequently are favoured by *Oidium tuckery – Uncinula necator*, causal agent of powdery mildew, among fungi and *Lobesia botrana* as insect pest. It was shown that a good pest and disease control in the vineyard significantly reduce OTA contamination also in high risk years and areas. A key role is played by the control of *L. botrana*; being this insect active both in spore dispersal and berries damage, a good control significantly reduces OTA content at harvest. Both chemical compounds and *Bacillus thuringensis,* as biological control agent, can give a good control. The direct control of black aspergilli, with focus on *A. carbonarius*, was also considered. Several chemical active ingredients were studied both *in vitro* and in field and the best results were reported for cyprodinil mixed to fludioxonil. The product was developed to control *Botrytis cinerea*; the same dosages and application time suggested for grey mould is also effective against black aspergilli and a significant reduction in OTA content in grapes is obtained. Very good results were also report with biological control agents, in particular with *Aureobasidium pullulans*.

Molecular tools for an early detection of ochratoxigenic fungi occurring on grapes berries in the vineyard

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Within *Aspergillus* section *Nigri*, *A. carbonarius* is the main source of OTA contamination in grapes and wines in Europe, followed by *A. tubingensis* and *A. niger*. The identification of fungal strains based on macro and micro morphological observation is time consuming and requires a high qualification and skills of the staff, especially within the black aspergilli species, as the taxonomy of this section is still unclear.
In our laboratories, an intensive search for alternative methods based on innovative DNA technologies of rapid and precise identification of ochratoxigenic fungi has been conducted in the last decade. A molecular diversity of a large number of different black *Aspergillus* strains isolated from grapes in Europe has been studied by sequencing rDNA, beta-tubulin, calmodulin, and by AFLP analysis. Then some PCR assays were set up in pure culture and grape berries: a) polymerase chain reaction (PCR) assay for discrimination of *A. niger* and *A. tubingensis* (sensitivity 10 pg DNA/reaction), b) quantitative real-time PCR assay for the detection of *A. carbonarius* in grapes (sensitivity 0.3 pg DNA/mg berries), c) DNA OLISA^{TM} microarray for identification of *A. carbonarius* (sensitivity 3.2 pg DNA/reaction) and other black Aspergilli from grapes, and d) rapid PCR-method based on single-stranded conformational polymorphism (SSCP) assay for the identification of *Aspergillus* section *Nigri* spp. A good correlation between *A. carbonarius*, quantified by qPCR, and OTA contamination was observed in the grape berries collected in 2005 from OTA risk Mediterranean area.
These methods reduce considerably the time of detection of *Aspergillus* Sect. *Nigri* and allow to monitor in field potential risk of OTA contamination in order to apply prevention strategies.

Undesirable fungal metabolites in grapes: emphasis on geosmin and ochratoxin A

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Food Mycotoxicology is the branch of mycology focused on the study of the mycotoxins (or other undesirable fungal metabolites) produced by fungi in food commodities. These are ubiquitous in nature and are considered as being natural and unavoidable. Fungi have plagued mankind before and since the beginning of organized crop production. The control of undesirable fungal metabolites is a continuous process in commodity production. They can become established and remain within the commodity anywhere throughout the production, storage, transportation and processing chain. No absolute controls over them are available, and total control is probably not economically feasible. Additionally, their control may involve either the prevention of its synthesis prior to harvest, or the prevention and decontamination after harvest.
The earthy smell is of crucial importance to grape and wine quality. The major compounds associated to this earthy smell are 2-methyl-isoborneol (MIB) and (-)-geosmin, which are produced by *Botrytis cinerea* and *Penicillium expansum* growing on grapes. Other metabolites affect the safety of grape products, being the most relevant ones patulin (mainly present in unfermented products) and ochratoxin A.
To elucidate the role of mould affected grapes in the carry-over of these metabolites to wine, 17 samples of grapes, with different pathologies, were analyzed for the presence of a mycotoxin (ochratoxin A) and nine fungal volatiles (geosmin, MIB, 1-octen-3-ol, fenchone, fenchol, 2,4,6-TCA, TeCA, TBA, PCA).
The results obtained showed that: i) Ochratoxin A was detected in one sample, at a level of 1.6 μg/kg (close to the legal limit of 2 μg/kg); ii) Geosmin, MIB, 1-octen-3-ol, fenchone, and fenchol were present in 3, 13, 17, 12 and one sample, respectively; iii) Anisoles (2,4,6-TCA, TeCA, TBA, PCA) were not present at detectable levels. Interestingly, one sample was found to contain all the compounds except fenchol and the anisoles. There was no relevant correlation between the grape disease and the presence of fungal metabolites.

Determination of the combined effects of temperature, carbon dioxide and copper on the geosmin production by *Penicillium expansum.*

Charpentier C.[3], D. Correia[1], P. Dantigny[2]and M. Bensoussan[1].

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Bordeaux mixture ($Ca(OH)_2$ + $CuSO_4$) has been applied onto vineyards since the end of the 19th century to prevent the growth of *Plasmopara viticola* (mildew). While arable land usually presents amounts of Cu between 5 and 30 $mg.kg^{-1}$, these treatments are responsible for copper accumulation in the upper layers of vineyards soils and many wine growing area exhibit Cu contents between 200 and 500 $mg.kg^{-1}$ (Deluisa et al., 1996).
Many microorganisms co-exist on the grapevine. Some have beneficial effects on the quality of grapes, others are at the origin of organoleptic defects. These last years, several earthy or musty deviations, with a strong smell of humid earth or beet, associated with the development, more or less visible, of grey rot on grapes, have been highlighted in various wine regions. Yamamoto et al. (1985) have shown that the genus *Penicillium* were predominant in soils polluted by copper.
The influences of environmental factors, as temperature and CO_2 content in the atmosphere, and copper ion, in the culture medium, on the geosmin production by *Penicillium expansum* were determined. An experimental device hermetically closed was developed for easy monitoring of the fungal growth. Experiments were carried out according a Doehlert matrix (Sautour et al., 2001): temperature ; 5 levels from 10 to 30°C; copper, 7 levels from 0 to 76.5 $ml.L^{-1}$; carbon dioxide, 3 levels from 0.03 to 3,03% in the atmosphere. In these conditions, a second order polynomial relationship between the geosmin production and the environmental factors was established with regression coefficients close to 0.96.
In these experimental conditions, temperature and CO_2 have negative effects on geosmin production, whereas copper has a positive effect :

- when the temperature increased, the geosmin production decreased
- when the copper level increased, the geosmin production increased,
- when the %CO2 increased, the geosmin production decreased

The combined effect of temperature and copper on geosmin production was also demonstrated. Both the experimental set-up and the Doehlert matrix were well suited to determine the influence of environmental factors on geosmin production.

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Session II
Starter selection and microbial control in the cellar

Oral presentations

Must treatments and wild yeast growth before and during alcoholic fermentation

Cavazza A.[1], E. Poznanski[1] and R. Guzzon[2]

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Recently, winemakers are debating about the role of wild yeast in determining the peculiar characters of wines produced in a territory. However, the information about the real activity of wild yeast in musts is still poor. In recent years, we monitored the presence of wild yeasts in grape musts collected to wineries in different ways and coming from different areas. The presence of wild yeasts in musts was checked firstly at their arrival to wineries, and along the subsequent treatments before inoculation with *Saccharomyces* starter yeast. The execution of draining, settling, filter-bed filtration, monitored in some wineries in Trentino region, revealed the presence and the activity of wild yeast during and after these operations. Differences in the microbial load of the musts draining off during pressing, were observed, depending mostly on the time elapsed between harvesting and crushing. The effectiveness in the removal of wild yeasts, together with grape solids, of cold settling was dramatically different, probably indicating that in performing the common winemaking operations, the gap between proper operation and non-conformity is very narrow. Less differences were observed in centrifuged, filtered and in floated musts.

Concerning the alcoholic fermentation, recently we monitored the kinetics and the activity of the inoculated *Saccharomyces* starter cultures in the presence of different amount of wild yeasts. The availability of DMDC, a preservative that completely degrades in some hours, allows the must sterilization without the addition of permanent preservatives like SO_2 or sorbic acid, and without heat treatments that could degrade vitamins or yeast nutrients. In order to compare the performance of starter yeast in the presence or in the absence of competing yeasts, we treated a *Pinot gris* must in a laboratory trial (as DMDC is NOT allowed in the winemaking practice). The must was divided in two parts, one was treated with 20 ml/hl DMDC and kept overnight, in order to sterilize it and to wait the complete hydrolysis of preservative, and the other was not treated and refrigerated. Two series of bottles were filled with the musts and were inoculated with 30 commercial *Saccharomyces* starter yeasts.

In the untreated must, wild yeasts were present in about 10^4 cfu/ml amount, and did compete with the inoculated starter in the first days. The alcoholic fermentation kinetics was lower in the contaminated must than in the treated one for most, if not all, the 30 commercial yeast. On the opposite, in the DMDC treated must, where all the wild yeast were killed (total yeast content <50 cfu/ml), the fermentation kinetics of the inoculated yeasts were faster.

The final wine composition was also different. The presence of apiculate yeasts during the fermentation led to wines richer in higher alcohols. Other differences were also observed, although not all of them were significant.

It is not possible to determine which role play the wild microflora in the peculiarity of wines, but it was observed that the winemaking technology has a strong effect on their growth and fermentation activity, that can depend largely on how the same operations are performed.

Control of inoculated fermentations in wine cellars by mitochondrial DNA analysis of starter yeast

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Nowadays the use of *Saccharomyces cerevisiae* starter cultures in winemaking is a common practice because it ensures a reproducible product, reduces the risk of wine spoilage and allows a more predictable control of fermentation. The use of selected *S. cerevisiae* strains in the winemaking process requires the development of techniques that can clearly differentiate the inoculated strain from wild strains present in the grape must, thus ensuring the control of the inoculated fermentation. In this way, it is possible to monitor the dominance of inoculated strain and to check if undesirable yeast species/strains were developed during the process. Among these techniques, the restriction analysis of mitochondrial DNA (RFLP-mtDNA) represents an useful tool to obtain molecular fingerprinting of yeast starter inoculated in the cellar (Querol et al., 1992).

During this research activity, a *S. cerevisiae* selected strain, isolated from Aglianico del Vulture grapes, was chosen. This strain was tested during inoculated fermentation at pilot scale in three wine cellars, producing Aglianco del Vulture wine and characterized by different typologies. In order to evaluate the capacity of the selected native strains to dominate the fermentation, the mtDNA-RFLP analysis was used. Samples were collected at different time intervals (the beginning, the middle and the end of the process) and the isolated colonies were submitted to restriction analysis of mtDNA with *Rsa*I and *Hinf*I enzymes. The dominance of inoculated strain was different in function of wine cellar. In two cellars, all the colonies isolated during fermentation showed the same restriction profile, identical to the profile of the starter. In the third cellar, on the contrary, some colonies exhibited the profile of the inoculated strain, whereas others showed mtDNA-RFLP patterns different from yeast starter profile. In this case, although the inoculated strain was found with the highest frequency, other yeasts developed, contributing to the fermentative process. These isolates might be yeasts resident in the cellar and/or in the vineyard, which reached the must by grapes.

This test was performed with the main purposes to test the effective dominance of the inoculated strain during the fermentation process. This aspect is poorly considered in winemaking, whereas several factors, such as those related to the cellar operations, grape must type, geographical characteristics, among others, can affect the implantation capacity of a wine yeast starter. Our results confirm that the capacity of a selected yeast starter to take over the fermentation represents an important parameter to be evaluated in all wine yeast selection programmes, representing an useful tool to guarantee the suitable course of the inoculated fermentation.

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Non-*Saccharomyces* wine yeasts play an important role in the biotechnological approach in winemaking

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Biotechnology applied to winemaking involves several traits of this fermentative industry such as the monitoring of microbial populations, the use of selected starters cultures, the control of spoilage yeasts. In the last decades the correct use and the control of microorganisms following a biotechnological approach get increasing importance in winemaking. The profusion of selected starter cultures has allowed the more widespread use of inoculated fermentations, with consequent improvements to the control of the fermentation process, and the use of new biotechnological processes in winemaking. Over the last few years, as a consequence of the re-evaluation of the role of non-*Saccharomyces* yeasts in winemaking, there have been several studies that have evaluated the use of controlled mixed fermentations using *Saccharomyces* and different non-*Saccharomyces* yeast species from the wine environment In this context, mixed fermentations using controlled inoculation of *Saccharomyces cerevisiae* starter cultures and non-*Saccharomyces* yeasts represent a feasible way towards improving complexity and enhancing particular and specific characteristics of wines.
Another trait of the potential use of non-*Saccharomyces* yeasts in winemaking is related to the control of spoilage microorganisms. Indeed, a more strict control of potential spoilage microorganisms during the various stages of wine production is needed. There is an increasing interest toward the use in food of natural antimicrobial agent. Killer system in yeasts could play an important role in the control of spontaneous and/or spoilage microflora. Killer toxins could be of particular interest as potential application as antimicrobial agents in partial or complete substitution of chemical ones. Indeed, natural antimicrobials would be more compatible with the requests of consumers for safe and unspoiled food products.

Influence of organic viticulture on non-*Saccharomyces* wine yeasts

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Grapes arriving to the cellar tend to have variable proportions of some yeasts with a low ethanol tolerance, such as species of *Hanseniaspora*, *Kloeckera*, *Candida*, *Pichia*, *Metschnikowia*, *Kluyveromyces*, and *Issatchenkia* and some of these non-*Saccharomyces* yeasts can proliferate in the first steps of fermentation. Numerous studies have evaluated the non-*Saccharomyces* (NS) species present in the wine ecosystem and recent research have demonstrated the impact of grape conditions on NS populations. Botrytis has been shown to alter species heterogeneity and succession (Greece) (Zott et al., 2008) and the influence of ripeness on yeast dynamics and diversity has also been investigated (Hierro et al., 2006). Information about natural must fermentation using organically produced grapes are limited. The objective of this study was to evaluate the population dynamics of *Saccharomyces* and non-*Saccharomyces* biota during spontaneous fermentation of organic musts. In addition, the wine-making was based on ancient traditional processes, such as stemming by hand and crushing by feet. Information on yeast diversity and changes at the species level during wine fermentation was obtained by combining culture dependent and culture-independent methods. By using a quantitative PCR-based method (qPCR) the counts of yeast populations were determined. A total of 543 yeast colonies were isolated, 190 from lysine medium, 254 from WL nutrient agar and 99 from YPD. To estimate yeast population dynamics during spontaneous fermentation we applied a genotypic approach comprising RFLP-PCR of 5.8-ITS, sequence determination of D1/D2 regions of the 26S rRNA gene and RAPD fingerprinting. The tools enabled us to identify the yeast isolates at the species level. Among the species identified, *S. cerevisiae*, *Hanseniaspora uvarum*, *Metschnikowia fructicola* and *Candida zemplinina* predominated, while *Issatchenkia terricola*, *Issatchenkia orientalis* and *Pichia* sp. were identified with a lower frequency. *Hanseniaspora uvarum* and *M. fructicola* species represented 43% and 31% of the total NS population isolated, respectively. A number of yeast isolates were shown to be closely related to *Hanseniaspora* spp. and *Candida spp.* on the basis of the D1/D2 sequences. We presume that different *Hanseniaspora* and *Candida* species existed in the grape musts and their complete identification will require characterization of additional molecular markers. Strain typing and differentiation was carried out by RAPD-PCR. High strain polymorphisms were observed into the different species. Some strains demonstrated appreciable properties as demonstrated by the API-ZYM test. Metabolic interactions between non-*Saccharomyces* yeasts and *S. cerevisiae* during fermentation could positively or negatively interfere with growth and fermentation behaviour of yeast species, especially with *S. cerevisiae*. Suitable strains should be selected in order to be able to design mixed starter capable of providing beneficial contributions to wine quality.

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Selection criteria for malolactic starters development: an update

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The use of malolactic starter cultures is becoming a common practice in the winemaking industry to improve fermentation process and enhance quality and safety of wine with respect to fermentation processes by native bacteria. Strains of lactic acid bacteria (LAB), mainly *Oenococcus oeni* and *Lactobacillus plantarum*, are now commercially available to winemakers for inducing malolactic fermentation in wine. A number of selection criteria has to be taken into account to design bacterial cultures specific for the different style of wine. These include: resistance to ethanol and SO_2, growth at low pH, absence of negative traits for the health of consumers (production of biogenic amines and ethyl carbamate, antibiotic-resistance), resistance to technological stress (freezing, freeze-drying, hydration and inoculation in wine), and compatibility with *Saccharomyces* selected yeasts. Further features are related to desirable enzymatic activities, such as glycosidase and tannase activities. Today, the selection of new starter cultures results from advanced technological applications rather than by the traditional screening methods and trial and error. In particular, progresses in genetics and molecular biology, as well as whole genome sequencing projects indicate a great flexibility of the genetic background of wine LAB and offer novel opportunities for a more precise characterization of these bacteria. The present contribution aims to provide an update on the new selection criteria that ought to be taken into account when selecting LAB strains suitable for winemaking. In next future, the full integration of phenotypic and genetic data will allow the development of more effective starter to be apply by winemakers in a more informed and rational manner.

The contribution of molecular methods in the understanding of the fermentation process for sweet wine production

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In the last ten years, the technological advancements in the field of molecular biology allowed the development of accurate and reliable techniques able to profile the microbial ecology of complex ecosystems. It is scientifically accepted that wine fermentations are complicated microbial consortia, in which bacteria, yeasts and, for some wines, moulds, are co-existing and interacting during the transformation process. In this scenario, the alcoholic fermentations for the production of sweet wines introduce additional factors which may affect microbial activity and fitness, resulting in sluggish or stack fermentation. Moreover, the high content of sugar may pose a risk for *Saccharomyces cerevisiae* activity, slowing down the fermentation start off and speed, and subject the yeast to metabolic stresses that can result in the production of off-flavours.

In this paper we present the results, obtained by using molecular methods, on microbial dynamics and strain biodiversity in the *Erbaluce di Calluso* sweet wine fermentation and in the first attempts to produce ice-wines in the Piedmont region in the North West part of Italy. More specifically, yeast ecology was monitored from the collection of the grapes, through the drying process and the fermentation step by isolating colonies on WLN medium, which were subsequently identified by molecular methods. Moreover, culture-independent methods were applied on the DNA and RNA extracted directly from the samples during 29 days of fermentation. A spontaneous and inoculated fermentation were followed in parallel.

The results obtained underlined a complex yeast microbiota in the fermentations considered. While in the inoculated fermentations, strains of *S. cerevisiae* immediately became predominant, in the spontaneous fermentations it showed a delay in taking over the transformation process and it was always accompanied by strains belonging to *Candida zemplinina, Hanseniaspora spp.* and *Kluyveromyces thermotollerans*. This complexity was also underlined by culture-independent analysis at DNA and RNA level. It is interesting to notice that molecular characterization of the isolated strains belonging to the starter culture highlighted that the inoculated strains not always performed well, underlining the necessity to evaluate well the use of starter cultures especially during sweet wines productions.

Exploitation of autochthonous yeast potential to enhance the quality of regional wines: the Apulian experience

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Apulia is the second Italian wine-producing region and the most important producer of red-rosé wine. One peculiar characteristic of Apulian wines is that the pedologic characteristics and the climatic conditions of this region contribute to enrich the wine of aromatic essences and to give, therefore, a characteristic and intense taste to the finished product. The Apulian wine industry is living a moment of great qualitative transformation and the challenge of the market had therefore addressed the productive row towards the use of innovative systems to guarantee and exalt the qualitative characteristics of regional wines. A heartfelt requirement is to being able to pilot and to control the productive activity to obtain wines with peculiar characteristics, with respect of the typicality, that is guaranteed by the denominations of origin. The selection and the employment of new combinations of microorganisms, obtained from the native micro flora would be a powerful instrument to improve the organoleptic and sensory characteristics of the product. Autochthonous yeasts are the micro-organisms better adapted to a specific must, which detain characteristics determined by the variety of the grapes and the terroir and, therefore they are able to exalt the peculiarities (aromas, structure and colour) of a wine.
In these last year the research activity of the Institute of Science of Food Production of C.N.R. has been directed to the exploitation of autochthonous microbiota to enhance the quality of regional wines. This research line has been granted by a number of National and Regional Projects and it has been conducted in collaboration between the ISPA and a large number of wine companies. The research activity has generated a virtuous circle, thus allowing the standardization of protocols for wine yeast enological selection and biomass production, the constitution of a Yeast Collection but, most of all, the transfer of technology to a large number of SMEs belonging of the wine production chain. Natural fermentations of Negroamaro, Primitivo and Susumaniello musts have been performed and the *Saccharomyces cerevisiae* population has been analyzed and characterized by molecular, physiological, enological and technological tests allowing the identification of four indigenous *S. cerevisiae* strains candidate as autochthonous fermentation starters. The enological properties of the above strains have been evaluated during the vintages 2006-2009, by performing 37 large scale vinification trials in 21 different industrial cellars in Apulia. The obtained results and their implications for the valorisation and the improvement of Apulian typical wines will be discussed.

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Session III
Yeast activity on wine quality

Oral presentations

Genetically modified wine yeasts and risk assessment studies covering the wine making process

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The usage of gene technology to modify the genome of wine yeasts belonging to the species *Saccharomyces cerevisiae* started in the early 90s of the last century. Starting from a pure scientifically point of view, many constructs (GMO) have been made so far covering more or less all stages of the wine making process where microorganisms or commercial enzymes play a key role, for example in aroma production (positive or negative flavours), alcohol production, pectin and protein degradation, etc. (Cebollero et al., 2007; Schuller and Casal, 2005; Pretorius, 2000).
Despite the availability of these strains, their usage is hampered by two important issues:
firstly strict legal regulations concerning the dissemination of genetically modified organisms and secondly the still existing doubts or even refuse of engineered food by the people worldwide. Many wine consumers feel threatened if a wine might be produced by using genetically modified yeasts. Only two engineered wine strains are legally on the market in the USA, Canada and Moldavia.
To demonstrate the actual behaviour of modified wine yeasts we investigated the following scenarios:

- behaviour of these strains in a vineyard located in a green house due to safety reasons and the impact of engineered strains on the natural flora,
- monitoring the persistence on the vines over several vegetation periods,
- persistence of the strains on winery equipment like oak barrels,
- and their behaviour in winery waste water and waste water treatment facilities, investigated in small scale in S1 laboratory.

It turned out that disseminated strains persist in principle but at different concentration levels, depending on the sensitivity of the monitoring system.
The presentation will display the current situation of existing modified wine yeasts, the legal situation, a market research study dealing with GMO and consumer expectations, as well as the scientific dissemination results and resulting consequences.

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Genetic stability and instability of wine yeasts

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The conversion of grape juice into wine is a complex process in which *Saccharomyces* yeasts play a central role. The *Saccharomyces* flora of the fermenting must is variable and can change in the course of fermentation so that usually several strains grow simultaneously. The properties of these strains may differ significantly and thus their contribution to the properties and quality of the wine may also vary significantly. Natural ("wild") strains are particularly prone to undergo changes during propagation and sporulation and often segregate into subpopulations. Many studies have shown that this instability is a consequence of changes in the genetic constitution (genome) of the wine yeast strains. It is believed that the changeability of the genome helps the yeast adapt to the rapidly changing environment (e.g. increasing ethanol concentration, decreasing concentrations of sugars and other nutrients, etc.) during fermentation because certain segregant subpopulations can cope better with certain changes than the parental strain. However, it also involves the risk that the whole yeast population evolves in an unpredictable and incontrollable way which then reduces the reproducibility of the wine quality. To avoid this problem, winemakers inoculate their must with starter cultures of carefully selected and characterized wine yeasts. Undoubtedly, this practice makes fermentation faster and more controllable but we should bear in mind that the commercial starters were developed from naturally occurring wine strains and may have retained some ability to segregate. This "residual" instability may account for the phenomenon that a commercial starter, even when used to ferment the same juice under identical conditions, can yield different wines in terms of sensory characteristics. Thus, improving genetic stability of commercial starters and obtaining stable variants of newly selected natural yeast strains are of great importance for further development of modern winemaking technologies. Numerous laboratories worldwide search into the mechanisms of alterations of the yeast genome during fermentation with the purpose of finding way(s) to make these changes controllable.

Comparative genomics of *Saccharomyces cerevisiae* wine strains

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The array-CGH (microarray based comparative genome hybridization) technique is a powerful method to investigate the genetic features of yeast strains and to understand which differences in genome organization might contribute to specific traits of the strain. On the basis of these considerations we have performed the array-CGH analysis on 10 autochthonous wine yeast strains selected for their fermentative performances. The results obtained allowed us to identify a common set of genome variations in all the autochthonous strains analyzed as respect to the laboratory strain S288C and commercial wine strains. These variations could be related to specific enological traits shown by the autochthonous strains.

Yeast influence on wine flavour as tool to select indigenous starter cultures

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The aroma and flavour of wine are one of the main characteristics that define the differences among the vast assortment of wines produced throughout the world. The chemical composition of wine is determined by many factors and yeasts have a prominent role. Yeast impact upon wine flavour is largely determined by the array of volatile substances produced by the metabolism of grape juice components. Acetic acid, acetaldehyde, ethyl acetate, higher alcohols account for more than half of these volatiles, the other half being distributed among 600–800 minor volatile compounds present in very low amounts. The diversity and the composition of the yeast population significantly contribute to the sensory characteristics of wine, The growth of each wine yeast species/strain is characterized by a specific metabolic activity, which determines concentrations of flavour compounds in the final wine. The concentration levels of these metabolites depend on the activity during alcoholic fermentation of the predominant different yeasts, that can contribute to wine flavour by producing compounds formed 1) with a minimum level of variation in each species or 2) with a wider variability within the species. The first case regards by-products considerable as differentiating characteristics among the various yeast species, allowing the individuation of the metabolic profiles typical for each species. An example is acetoin and 2,3-butanediol that are produced with an inverse correlation in function of yeast species. Predominant non-*Saccharomyces* yeasts of the early fermentation phase exhibit the characteristics to be high acetoin and low 2,3-butanediol producers, whereas the main actor of alcoholic fermentation, the species *S. cerevisiae*, exhibits the characteristics to be low acetoin and high 2,3-butanediol producer. The second case regards by-products considerable as differentiating characteristics among strains of the same species. It means that within each species a significant, sometimes also considerable, strain variability can be find. Thus, different strains of *S. cerevisiae* determine different levels of fermentation by-products and the organoleptic quality of the final wine varies considerably as a function of the strains which performed and/or dominated the fermentation process. In this context, the wide use of starter cultures can ensure a balanced wine flavour, but it may also cause a loss of characteristic aroma and flavour determinants, which are typical for each vine cultivar. Another key parameter to optimize wine flavor is to take into account strain relationship with grape must composition. The beneficial contribution from the strain increases when starter cultures for winemaking are also able to complement and optimise grape quality and its individual characteristics. Within the total production chain, the alcoholic fermentation of grape juice by yeasts is a key process where winemakers can creatively engineer wine character and value through better yeast management and, thereby, strategically adapt wines to a changing market.

This review considers the importance of yeast species/strain biodiversity and yeast metabolic reactions in determining wine flavour, and then discusses new directions for exploiting yeasts in wine fermentation. It covers criteria for selecting and developing new commercial strains.

Microbiological approach to improve quality of Grappa, an Italian distillate from fermented marc

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Grape marc is the raw material that is processed to produce Grappa, a traditional Italian beverage. It is mainly composed of grape skins, the most aromatic part of grapes, seeds, and in some cases, stalks. After separation from must, grape pomace is usually pressed and then delivered to the distillery. In the case of marc from white varieties that were normally separated from must before fermentation, alcohol has to be obtained during an appropriate period of storage. Due to favourable conditions, such as pH value relatively higher than that of must, presence of sugars and other nutritional compounds, increasing temperature determined by the ongoing fermentation, a copious development of indigenous microorganisms rapidly takes place during this period. Sugar degradation and alcohol production are the main transformation processes, carried out mainly by yeasts, but a number of other compounds, that can affect the quality of the future distillate, can also develop. Modern distilleries have developed technological supports to avoid spoiling of the product due to unsuitable storing conditions, such as acidification treatment, control of temperature, preservation of an anaerobic environment and finally, only at experimental level, the management of microbial populations by means of selected yeast introduction. Since very few data are available on composition, biodiversity, evolution and activity of yeasts and bacteria during marc storage, the dynamics of natural microbial populations, both at species and strain level, that evolve during the fermentation of natural and acidified marcs were investigated by molecular methods. At the beginning of storage, species also found in the very first stages of fermentation of musts and common on grape berries, such as *Hanseniaspora*, *M. pulcherrima* and *T. delbrueckii* are abundant. Then, a succession of yeast species takes place leading to a dominance of *S. cerevisiae*. Among this species an extremely high level of strain variability, by means of mitDNA profiles, was found, comparing with must natural fermentation. Concerning bacterial population the dominant species belong to *L. plantarum* group, whereas after acidification treatment *O. oeni* was the most present. Finally in order to detect the effect of inoculum of yeasts (collected from grape pomace during previous experimentations) on the quality of the final distillate, a laboratory-scale fermentation of grape marcs has been performed. As the inoculated yeasts showed to be good colonizers of the grape pomace, they were considered the principal agents of sugar consumption, that is completely ended after five days of fermentation. A time course analysis of fermentation products by mean of gas-chromatography was performed to determine the impact of the inoculated strains on the main aromatic compounds, that are concentrated in the product after distillation.

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Influence of *Saccharomyces cerevisiae* wine strains on total antioxidant capacity

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Phytochemicals with strong antioxidant activities are ubiquitously distributed as throughout the plant kingdom and exhibit a wide variety of biological effects in mammalian organisms. Polyphenols are found in considerable quantities in fruits, vegetables, tea and wine and consequently they are common components of the human diet. In addition to the antioxidant action, polyphenols also display anti-carcinogenic, anti-atherogenic, anti-inflammatory, antibacterial and antiviral properties. The aromatic fraction of wines is composed by a wide variety of compounds with different aromatic properties and antioxidant capacity. Some of these compounds are already present in the musts, others are modified during the vinification process, and finally, others are produced during the fermentative process by yeast activity. In particular, phenolic compounds contained within the skin, seeds, and flesh of grapes are extracted into red wines during the process of vinification. Vineyard factors affect the phenolic compounds that accumulate in grapes, but also starter cultures performing the fermentation process can play a significant role on the total antioxidant capacity (Brandolini et al., 2007). Taking into account the increasing interest towards wine quality, our interdisciplinary research group is working actively on studies regarding yeast strain parameters related to the human health, such as a high total antioxidant capacity. Here we report results on the evaluation of the total antioxidant capacity in commercial wines and in experimental wines.
The total antioxidant capacity, calculated as ascorbic acid equivalents was measured by Photochem apparatus (Analytik Jena AG), which is based on the photochemiluminescence method suggested by Popov and Lewin (2000). This methods employed for the determination of antioxidant capacity allows to evaluate the activity of original food against the free radicals generated by Photochem. The main characteristics of this apparatus are the high sensitivity, short time of analysis (max 3 minutes), small volumes and high reproducibility.
Laboratory fermentations were carried out in grape must inoculated with different selected autochthonous strains of *Saccharomyces cerevisiae* and at the end of the fermentations the total antioxidant capacity was measured.
The results, statistically analyzed, revealed a significant influence of the strain on the total antioxidant capacity in the final wine. The majority of experimental wines showed an increased total antioxidant capacity, whereas only a few samples had a decreased capacity. In view of the importance of total wine quality, the different strain influence on antioxidant capacity assumes a technological significance, determining the final quality of the wine by influencing also parameters related to the human health.

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Yeasts and wine off-flavours: a technological perspective

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In wine production, yeasts have both beneficial and detrimental activities. *Saccharomyces cerevisiae* is the main responsible for turning grape juice into wine but this species and several others may also show undesirable effects in wines. Among these, technologists are particularly concerned with the production of off-flavours that may occur during all stages of winemaking. Typical spoiling activities include the production of ethyl acetate by apiculate yeasts before fermentation, of hydrogen sulphide by *S. cerevisiae* during fermentation phases, of acetaldehyde by film-forming yeasts during bulk storage, and of volatile phenols by *Dekkera bruxellensis* during storage or after bottling. The occurrence of these hazards depends on the technological operations designed to obtain a given type of wine and most of them may be avoided by current preventive or curative measures. On the contrary, good manufacturing practices must be strengthened to deal with the problem of volatile phenol production in red wines. Appropriate monitoring of *D. bruxellensis* populations and quantification of 4-ethylphenol is advised during storage, particularly when oak barrels are used, and absence of viable cells must be guaranteed in bottled wines. This work is based on our experience at winery level, aiming to provide adequate technological strategies to deal with the problem of off-flavours produced by yeasts.

Microbial formation of key sulfur aroma compounds in wine

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The identification of fermentation-derived aroma compounds to better describe wine quality and style, and development of yeast strains to optimize wine aroma have been of significant importance to both researchers and wineries for many years. A current focus of our research is to limit yeast derived sulfur off flavors such as H_2S, CS_2 and methylmercaptan, while at the same time maximising formation of varietal thiols such as 3-mercaptohexanol during fermentation. This requires a detailed understanding of the interplay between different yeast strains and grape juice composition, flavor precursors and nutrients. With the ever-growing importance of screwcaps as alternatives to cork closures, additional focus is on minimizing the risk to develop "reduced characters" during bottle storage, and to better understand the role of yeast nutrients and yeast sulfur metabolism in controlling sulfur compounds.
This paper explores how analysis of volatile sulfur compounds can be utilized for yeast strain development; we summarize studies targeting fermentation-derived sulfur off flavors, and highlight recent developments and remaining challenges with the analysis of varietal thiols and their precursors in wine.

Session IV
Methods for detection of micro-organisms affecting wine safety and quality

Oral presentations

PCR methods for the detection of biogenic amine-producing bacteria on wine

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The presence of biogenic amines in wine is of considerable public concern for the wine industry and the regulatory agencies, since given the potential health hazard, there is a growing demand from consumers and control authorities to reduce the allowable limits of biogenic amines in wine. Several toxicological problems resulting from the ingestion of wine containing biogenic amines have been described. Biogenic amines are mainly produced by the decarboxylation of certain amino acids by microbial action. Since the ability of microorganisms to decarboxylate amino acid is highly variable, being in most cases strain-specific, the detection of bacteria possessing amino acid decarboxylase activity is important to estimate the risk of biogenic amine content and to prevent biogenic amine accumulation in wine. Rapid and simple methods are needed for the analysis of the ability to form biogenic amines by bacteria in order to evaluate the potential risk of bacterial occurring in wine. Analytical chromatographic methods used for routine biogenic analysis of food substrates have been applied to bacterial cultures. Specific differential culture media for the presumptive identification of biogenic amine-producer bacteria have been developed. However, the detection of biogenic amine-producer bacteria by conventional culture techniques is often tedious and unreliable, exhibiting disadvantages such as lack of speed, appearance of false positive/negative results, low sensibility, requirements for costly and sophisticated equipment, as HPLC, or that only one biogenic amine is detected. Molecular methods for detection and identification of food-borne bacteria are becoming widely accepted as an alternative to traditional culture methods. PCR and DNA hybridization have become important methods and offer the advantages of speed, sensitive, simplicity and specific detection of targeted genes. Genetic procedures accelerate getting results and allow the introduction of early control measures to avoid the development of these bacteria. Since molecular methods are fast, reliable and culture-independent, they are an interesting alternative to solve the shortcomings of traditional methods. Moreover, PCR methods detect potential biogenic amine risk formation in food before the amine is produced. During the last decade several molecular methods have been described for the unambiguous detection of bacteria capable to produce one or several biogenic amines. Several oligonucleotide primers have been described to detect amino acid decarboxylase encoding genes by PCR. The designed oligonucleotide primers are based on amino acid regions conserved in specific decarboxylases, therefore primers targeting a specific amino acid decarboxylase are useful for the detection of all the bacteria producing the specific biogenic amine. PCR assays provide methods that can be successfully used for the routine detection of bacterial strains potentially producers of histamine, tyramine, putrescine, and cadaverine on wine.

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Managing Biogenic Amines in Australian Wines

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Biogenic amines are organic nitrogenous low molecular weight bases which are found in a range of fermented foods and beverages, including wine. Absorption of these compounds in elevated concentrations may induce headaches, gastro-intestinal and respiratory distress akin to an adverse allergic reaction. The main biogenic amines found in wine are histamine, tyramine, cadaverine and putrescine. Even though concentrations of histamine in wine are generally 10-fold lower than found in some fresh and other fermented foods, their presence may contribute to an adverse reaction when consumed in combination with other histamine containing foods. In addition, wines containing higher concentrations of biogenic amines, particularly histamine, risk exclusion from certain export markets.

It is well established that the main contribution of biogenic amines in wines is from lactic acid bacteria metabolism, especially during or after malolactic fermentation (MLF). Management of an efficient MLF though inoculation with selected *Oenococcus oeni* starter cultures began in the mid 1990's. Today, it is a well established protocol in Australian winemaking.

A survey of Australian red and white wines produced during 1988-1992 for biogenic amine content demonstrated a wide range of concentrations. A second survey of biogenic amine content in red and white wines produced during 2007-2009 has been undertaken. Comparisons and possible implications between the two wine biogenic amine surveys will be explored from an Australian winemaking perspective.

Chemical and biological methods to control of biogenic amines production in wine: application to the study of commercial yeast and bacteria starters

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Wine, like other fermented foods, may contain biogenic amines (BAs). The most dangerous amines in wine are histamine, putrescine and tyramine. Several research groups support the view that BAs are formed in winemaking mainly during malolactic fermentation due to the decarboxylation of free amino acids. According to several authors, the formation of BAs may be due to spoilage bacteria before, during or after food processing and it is associated with food hygiene and technology.
It is known that commercial starter preparations contain lactic acid bacteria contaminants but no previous studies have taken into account if these bacteria are able to produce BAs.
The aim of this work was to investigate if contaminating microorganisms, eventually present in bacteria and yeast preparations used as commercial starters in winemaking, have the ability to produce the biogenic amines histamine, putrescine and tyramine.
For the screening of 30 starters (14 yeasts *Saccharomyces cerevisiae* and 16 bacteria *Oenococcus oeni*) a TLC method (Garcia-Moruno et al., 2005) was carried out; it constitutes a simple solution to the previous reports describing false-positive reactions in routine screening procedures which generally employ differential medium containing a pH indicator.
To detect decarboxylase genes in the bacterial cultures, a simple and fast method of PCR-multiplex was used (Marcobal et al., 2005; Costantini et al.,, 2006).
Fermentation tests with yeast preparations containing bacteria contaminants able to produce BAs were performed. BAs formation in wine was determined by HPLC using a method described by Costantini et al. (2006).
The results obtained showed that one of the possible origin of BAs in wine can be due to the presence of contaminating bacteria able to produce biogenic amines in commercial starters, routinely employed in winemaking practice.

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Polyphasic approach based on culture dependent and independent methods as useful tools for the detection, *in vitro* and *in vivo*, of biogenic amine producing strains in regional wines

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The improvement of fast and reliable culture dependent or independent molecular tools, usually based on qualitative or quantitative polymerase chain reaction (PCR or qPCR) approaches, has recently allowed for rapid and accurate detection of biogenic amine (BA) producing bacteria in fermented beverages. Oligonucleotides based on genes coding for amino acid decarboxylase (*hdc*, *odc*, *tyrdc*) or deiminase (*agdi*) enzymes involved in BA production pathways have been developed. These tools that target several genes individually or simultaneously are able to detect all potential BA producing lactic acid bacteria (LAB) in a given sample. Although these PCR based tools can provide novel data on potential BA-producers, it should be noted that they may also be limited. For instance, some false positive may arise due to genes unrelated to BA production. In addition, even if the identified gene is highly homologous to known BA coding genes, the BA production pathway may be incomplete. As a result, the strain is usually unable to produce BAs. Therefore, after detection of a BA pathway coding gene, the potential for the LAB strain to actually produce BAs must be further analysed. For example, thin layer chromatography (TLC) has been observed to be a robust and effective analytical technique and is best suited for high-throughput semi-quantitative analysis. Combination of PCR and TLC analysis is surely a very robust approach in order to detect BA-producing strains. It can also provide new information on BA-producing species. Moreover, detection of BA pathway genes is usually performed after bacterial isolation and therefore will only be able to detect bacteria that can grow on defined media. For this reason, use of molecular tools that detect potential BA-producing LAB directly from wine samples is preferred. Development of appropriate nucleic acid preparation techniques allows for viable but non-cultivable (VNC) BA-producing bacteria to be detected directly from wine samples.

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Effect of nitrogen addition during alcoholic fermentation on final biogenic amine content in wines.

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An aspiration of the wine industry is to control critical technological factors to produce wines with low levels of biogenic amines. Histamine, tyramine and putrescine are the biogenic amines present due to the decarboxylase activity of lactic acid bacteria during malolactic fermentation (Landete et al., 2007). Not only does the production of biogenic amines in wine depend on the presence of biogenic amine producing bacteria, but also it depends on other parameters of wine such as amino acid precursors content, pH or MLF duration (Martin-Alvarez et al., 2006). Among these factors, amino acids and ammonium ions are essential growth factors for development of yeasts and lactic acid bacteria during alcoholic and malolactic fermentations. Nitrogen is often a limiting nutrient for Saccharomyces cerevisiae during batch alcoholic fermentation. It is recommended to supplement such depleted musts with nitrogen to ensure a good alcoholic fermentation (Bely et al., 1990). But this action can be contradictory with the aim of controlling biogenic amine content. To rationalized nitrogen addition, fermentation experiments at the pilot scale (100 L) were conducted with grapes (Syrah and Grenache) coming from the Rhône Valley. Amino acid and biogenic amine contents were determined simultaneously using the method described by Gómez-Alonso et al. (2007). Populations of bacteria producing histamine present in wine during malolactic fermentation were analyzed using PCR as developed by Lucas et al. (2008). The purpose of this work was to assess the effect of nitrogen addition on amino acid and final biogenic amine concentration during wine-making conditions.

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Development and application of a duplex PCR for the detection of *Aspergillus carbonarius* occurring in grapes

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Ochratoxin A (OTA) is a mycotoxin naturally produced by certain fungal species of the genera *Penicillium* and *Aspergillus*. Many studies confirmed that this mycotoxin is nephrotoxic, hepatotoxic and immunotoxic in experimental animals, and due to its carcinogenic properties, the International Agency for Research on Cancer evaluated OTA as a possible carcinogen (2B group) in humans in relation to the exposure (IARC, 1993).
OTA was found in several commodities of animal and plant origin. Contamination in viticultural products is well documented and it is considered one of the most important contributor to human exposure to OTA (Zimmerli and Dick, 1996). Therefore, it is necessary to reduce OTA contamination and bring it below the threshold limit fixed by the EU.
A possible way to control this contamination and protect consumer health is to detect the presence of OTA-producing fungi rather than their contaminant metabolite, since it was found that grapes, although infected with moulds, did not have a detectable concentration of OTA (Zimmerli and Dick, 1996). Anyway, this detection should be sensitive, reliable and rapid to better manage grape and wine production. DNA-based technologies seem to fulfil these requirements, since they have been successfully applied for the detection of GMO, food allergens, food-borne pathogens, etc.
In this study, the development of a duplex PCR for the specific detection of *Aspergillus carbonarius*, a potent OTA producer (Sage et al., 2004), is presented. This method resulted very accurate and sensitive for the detection of low amounts of the mycotoxigenic mould occurring in grapes. In addition, an optimized protocol for the isolation of genomic DNA from a polyphenol-rich fruit like grape is described.

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Early detection of ochratoxin A and ochratoxigenic fungi in wine

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Mycotoxins are metabolites hazardous for animal and human health produced by different fungal species mainly belonging to the *Aspergillus and Penicillium* fungal genera. In particular on wine grapes mycotoxins are represented by ochratoxin A (OTA) which is produced mainly by *A. niger* and *A. carbonarius*. This toxin is suspected to promote kidney cancer and for this reason it is regulated by EU legislation. Up to date the control of OTA biosynthesis has not been fully achieved and a rapid, reliable and cheap quantification of this mycotoxin and of the toxigenic fungi could help in the control of its contamination. Molecular biology methods, based on suitable extraction procedures and SYBR green Real Time PCR amplifications with the use of specie-specific primers, were set to early detect minimal quantity (1-5 conidia) of *A. carbonarius* in grapes. Moreover OTA was detected by a novel device based on the use of an amorphous silica photosensor. This technique using very simplified and rapid extraction procedures has allowed to detect OTA from red wine contaminated at 2ppb. The use of both the considered approaches could improve wine safety and quality.

Vinification and repassage as effective processes to remove ochratoxin A from contaminated grape

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Ochratoxin A (OTA) contamination of red wine grape might be quite severe in certain high-risk regions and vintages thus requiring corrective measures to fulfill acceptable standard for human consumption. Knowledge about the fate of OTA during vinification and its distribution in wine and winery by-products is important to manage OTA risk in red wine grape.
The distribution of OTA in red wine and winery byproducts during vinification (at laboratory and winery scale) of naturally contaminated Negroamaro and Primitivo was thoroughly monitored. Musts and wines were analysed by the EN 14133 standard method while a new HPLC method was developed for the analysis of grape pomaces, seeds and lees. Experimental results showed that during the 5-6 day fermentation and maceration of crushed grape berries the amount of toxin dissolved in juice/must and adsorbed on pomace reached a dynamic equilibrium with only 4% OTA dissolved in the wine, 95% retained in the pressed grape pomaces and 1% in the lees. OTA was homogeneously distributed within pomaces during fermentation/maceration of crushed grape berries (5-7% variability in triplicate analyses from the same tank). After fermentation/maceration OTA concentration remained unchanged in all liquid fractions collected during vinification (free run wine, press wine and wine after first and second decantation) as well as in wine after one year aging. Similar results were obtained from experiments conducted at winery scale for two consecutive vintages. Although most of the toxin is retained by pomaces during vinification process, OTA concentrations higher then 2 μg/kg (EU limit) can be found in must/wine produced in certain high-risk regions and vintages which requires additional decontamination measure.
The high affinity of grape pomaces for OTA suggested that repassage of contaminated must/wine over pomaces with no or low OTA levels could be used to remove the toxin. Time course experiments showed that OTA adsorption by pomaces is a rapid process, reaching the equilibrium in less than 10 h, and is not affected by the tested toxin concentrations. Specific experiments carried out at laboratory and winery scale showed that up to 65% of toxin was removed when contaminated red must spiked with 2-10 μg/kg OTA was repassed over clean red pomaces. Grape pomaces maintained a good efficacy in removing OTA after four time re-usage. Unlike other oenological fining agents, the use of grape pomaces to remove OTA from red wines of the same grape variety (Primitivo) did not affect relevant wine quality parameters, including color intensity and health promoting phenolics content (*trans*-resveratrol, quercetin, total polyphenols). The proposed repassage process can be applied in a modern winery to decontaminate must/wines that still contain OTA at concentrations higher then 2 μg/kg at the end of vinification process.

Ethyl carbamate content in wines with malolactic fermentation induced at different moments of vinification process

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Ethyl carbamate (EC), also known as urethane, is a carcinogen compound found in fermented food and beverages, as wine. Content of EC is greater in products with high alcoholic degree and aging. The major precursor involved in EC production in wine is urea produced by metabolism of arginine by yeast, but there is also evidence that EC levels could increase after malolactic fermentation (MLF). Some lactic acid bacteria (LAB) can degrade arginine present in must and wine via the arginine deiminase pathway and produce citrulline and carbamyl phosphate. Both compounds can react with ethanol in acid conditions and produce EC.
Our research group is studying the influence of MLF induced at different moments of wine-making process (at the beginning of alcoholic fermentation (AF), at 10 g/l of sugars to the end of AF and when AF is finished) in the quality of wine. Among other parameters, it has been evaluated the content of toxic compounds as EC. Until now the results we have obtained show that EC levels at the end of MLF were quite low (less than 3 μg/l) in all cases. In almost all wines, after 8 months of storage EC concentration increased as it has been described by other authors. In those wines that MLF was carried out by selected LAB the increase of EC concentration was lower.

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Brettanomyces bruxellensis prevalence in wines produced or marketed in Spain

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Brettanomyces bruxellensis is a yeast responsible for the presence of 4-ethylphenol and 4-ethylguaiacol in wine. The non controlled accumulation of such molecules in wines leads to sensory defects which compromise the wine quality and consequently it can cause serious financial losses. The presence of this spoilage microorganism is increasingly common in the cellars. Its presence in wines is hard to detect using conventional culture methods, which are long and, sometimes, non-specific. Fast, specific and early detection of the yeast during the wine-making process enables the oenologist and producer to take preventive measures before the phenolic aspect appears. Real Time PCR (RT-PCR) method fits with all these criteria, with high speeds, sensitivity and specificity.

The aim of this work has been to validate a new commercial kit to detect *Brettanomyces* yeast based on RT-PCR, comparing the results with those obtained with traditional microbial counting in selective medium and another molecular method based on the amplification of the internal transcribed spacers (ITS1 and ITS2) of the rRNA 5.8S. We have analyzed 86 red wines produced or marketed in Spain (commercial wines or just before being bottled) with or without aging to study the prevalence of this spoilage yeast. Sixteen wines (18.6%) were positive for *B. bruxellensis* presence by microbiological plate counting after 5-7 days of incubation and twelve of them (14 %) were also positive with RT-PCR analysis, obtaining the results only in 7 hours. RT-PCR could thus be applied as a quick and reliable detection and enumeration method of *Brettanomyces* during early steps of wine-making and also just before bottling.

Identification of peptides marker for the detection of caseinate used as fining agent in white wine by capLC-MS/MS method

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Proteins are typically present in wines at low levels, most of them having a remarked technological and economical relevance. As proteins can affect wine stability and clarity, a variety of procedures have been developed for the specific removal of proteins from wines (Ferreira et al., 2002). Among fining agents used in wine processing to reduce off-flavour ingredients, special milk proteins, with particular regard to caseins, can be employed for binding phenolic compounds that may affect wine taste and colour. Although it is assumed that fining agents are likely to be removed during the manufacturing process to date there is no evidence for the wine. Besides, the lack of good manufacturing practice can arise some risk for allergic consumers. In this regard, the analysis of fining agents like caseins that can be present as residues in wines is of paramount importance to safeguard the health of allergic individuals and to comply with the recent legislation issued in the field of food allergens. Last Directive 2007/68/EC regards the need of labeling for food allergens. In particular, light is cast on all fining agents containing allergenic proteins, used for the manufacture of alcoholic and non alcoholic beverages and that is mandatory to declare in the relative label.
To date the only method available for detection of milk proteins used as fining agents in wines is based on ELISA (Rolland et al., 2008) while MS based methods have not been investigated yet. The potentials and features offered by mass spectrometry make this approach suitable for the identification of allergenic proteins by MS/MS approach (Monaci et al., 2009; Monaci et al., 2008). In this presentation a method is described using capillary LC separation combined with Q TOF mass spectrometry for the separation and MS detection of casein markers in fortified white wines. Specific peptide markers were identified in the chromatograms upon careful analysis of the acquired spectra and the MS/MS fragments found confirmed the goodness of the attribution. Peptides arising from α casein and β casein were highlighted in spiked white wines and their identity was confirmed by database search and *de novo* sequencing. The method appears to be very useful for screening purposes as well as a confirmative method to corroborate positive results obtained by ELISA.

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Session I
Microbial control in the vineyard

Poster presentations

Novel integrated strategies for ochratoxin reduction in grape-wine chain foreseen in MYCORED EU FP7 large collaborative project

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In order to comply with the needs of EU and address global strategies for mycotoxin reduction, a four years large collaborative project, MYCORED as acronym, has been recently approved within the European FP7-"Food, Agriculture and Biotechnologies" Work Programmes.
MYCORED aims at developing strategic solutions to reduce contamination by mycotoxins of major concern in economically important food and feed chains. The following toxins and commodities are especially considered in the project: aflatoxins, trichothecenes, zearalenone, fumonisins in wheat/maize food and feed chains; ochratoxin A in grape-wine and wheat chains; and aflatoxins in dried fruit chain. Novel methodologies, efficient handling procedures and information, dissemination and educational strategies are considered in a context of multidisciplinary integration of know-how and technology to reduce mycotoxins exposure worldwide.
In particular the following strategies in grape-wine chain are carried out: a) use of biocontrol agents to reduce the Aspergillus black rot disease and ochratoxin accumulation in grapevine berries in field trials located in a potential high OTA risk area in Argentina (WP 2); b) development both of predictive model for OTA producing fungi in vineyard and of decision support system for pre- and post-harvest management of grape to minimize OTA (WP 3); c) study the biodiversity of black Aspergilli at global level, development both of mycotoxicological risk map and more rapid, sensitive and inexpensive methods for the detection of toxigenic fungi (WP 6).
One additional objective of the project is to support, stimulate and facilitate education and cooperation with countries having major mycotoxin concerns related to (international) trade and human health (WP8). The direct involvement of ICPC countries (Argentina, Egypt, Russia, South Africa) and international organizations (CIMMYT, IITA) together with strong scientific alliances with International Experts will strengthen the project through sharing experiences and resources from several past/ongoing mycotoxin projects in a global context.

Effect of heat treatment and of enzymatic preparations on the phenolic profile of must for vinification

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Maceration displays a dualistic effect on the vinification process, if on one hand it increases the extraction of phenolic compounds; on another it also increases enzymatic oxidation rates. Therefore, the search for procedures that minimize the damaging effects is an important research area in wine making. This study evaluated the effect of a pre-treatment, thermal and enzymatic, on Bordô grapes regarding the phenolic and color profile for the elaboration of artisanal wine in the state of São Paulo, Brazil. 50 g of fruit were bleached under saturated vapor and softly squeezed, receiving then the addition of 5 mL of enzymatic preparations. The enzymatic preparation N31, produced by the fungus *Thermomucor indicae-seudaticae*, was diluted to a total protein content of 0.6 mg/mL while the enzymatic preparation Vinozym Vintage FCE (Novozyme) was diluted according the manufacture's specifications (4 g /100 Kg of fruit). Samples were incubated at 37 ºC under agitation (100 rpm), at must's natural pH (3.3), for different times (0, 5, 1, 2, 3 and 4 hours) and, afterwards, pressed to obtain the must. Two controls were carried out, one sample in nature and one bleached, both with only addition of 5 mL of water. The experiments were carried out in duplicate. Samples were evaluated regarding color parameters (color index, T coloration, composition of red color intensity), content of anthocyanins, total phenolic compounds and methanol. Samples thermally treated presented content of monomeric anthocyanins (75.65 ± 0.81 mg/L) and phenolic compounds (1509.43 ± 33.34 GAE) significantly higher than the ones found in must obtained from the in natural fruit (10.24 ± 0.47 mg/L and 569.73 ± 60.45 GAE, respectively). These results are in agreement with the color indices found in the samples, showing increase of red color after the heat treatment. Therefore, the use of this procedure, besides minimizing enzymatic browning resulted in tissue softening and, consequently loss of cell cohesion and cell wall's mechanical resistance, which facilitated the transfer of pigments from the peel to the must. The application of enzymatic preparation N31 on the grapes assured improvement on the liberation of anthocyanins from the must and on visual characteristics. The musts treated with enzymatic preparation Vinozym Vintage FCE presented maximum anthocyanin content (113.25 ± 12.20 mg/L) and total phenolic compounds (1697.55 ± 196.98 GAE) after 4 hours of treatment while the musts treated with enzymatic preparation N31 presented maximum content of anthocyanin (94.35 ± 18.25 mg/L) and phenolic compounds (1677.25 ± 62.36) with 1 hour of treatment. It can be noted that the results obtained with the use of enzymatic preparation from the fungus *Thermomucor indicae-seudaticae* N31 are very close to the ones obtained with the commercial product, however in a shorter period of time, which encourages the continuation of the studies with this enzymatic preparation.

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Influence of environmental parameters related to global warming on the growth of fungal species responsible for alteration of vine

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During the past 10 years, off-flavours like earthy-musty tastes have been described in wines from several regions of France. The two main molecules that have been associated to that organoleptic defect are 2-methylisoborneol and geosmin, respectively produced by *Botrytis cinerea* and several *Penicillium* species. As end products of the mevalonate pathway, these molecules are secondary metabolites whitch productions are environmental factor-dependent. Molds from vineyards of Burgundy (Beaune, Corton, Givry and Pommard) were counted, isolated and identified. Overall, the number of fungal isolates from uncontaminated grapes is fairly uniform and ranged between 1 and 4.10^2 UFC.g^{-1}. Nevertheless, whatever the sampling point, and despite a more heterogeneous distribution of the contamination, contaminated berries coming from the same vineyard exhibit a fungal load greater than 10^3 UFC.g^{-1}. From a qualitative point of view, the identification of fungal contaminants revealed, by decreasing frequency, the presence of the genera *Botrytis, Penicillium, Aspergillus, Fusarium*, *Alternaria, Cladosporium* and *Trichothecium*. The experimental approach involved is that of predictive mycology (Dantigny *et al*., (2005). On Potato Dextrose Agar (PDA) medium the effects of temperature and water activity on the growth rates of *Botrytis cinerea* and *Penicillium expansum* were modeled using the protocols described by Rosso *et al.* (1993) and Sautour *et al.* (2001). The cardinal values (optimum growth rate: μopt ; temperatures minimum, optimum and maximum and water activity minimum, optimum and maximum, respectively T_{min}, T_{opt} and T_{max}, $a_{w\text{-}min}$, $a_{w\text{-}opt}$ and $a_{w\text{-}max}$, for growth,) were calculated. In comparison with the cardinal values obtained on PDA medium, growth rates of *Botrytis cinerea* and *Penicillium expansum* were validated on synthetic and natural grape juice media using « *bias and accuracy factors* » (Ross, 1996). Two factorial designs were used to study the combined effects of *i*) two factors (temperature and carbon dioxide) and *ii)* three factors (temperature, copper ions and nitrate) on *Penicillium expansum* growth and sporogenesis. If carbon dioxide increase (0,03 to 3%) induced better growth than temperature increase (15 to 25°C), the best effect of copper increase on sporogenesis was observed at low temperature level. The combined effects of environmental factors associated with global warming on the growth of some fungi have been evaluated. The measurements of these effects on the development of these moulds will contribute to a better use of pesticides to prevent organoleptic deviations of biological origin.

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Specific PCR primers for the detection of OTA-producing *Aspergillus carbonarius* in vineyard

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A polyketide synthase (*pks*) gene of *A. carbonarius* strain AC06 isolated in Piedmont (Northern Italy) was isolated, cloned and sequenced. The nucleotide sequence showed high homology to the *pks* domain of other *Aspergillus carbonarius* strains. The sequence data were used to design a new set of primers in order to identify strains of *A. carbonarius* able to produce ochratoxin A (OTA). The specificity of the primers was checked against different species of *Aspergillus* and *Penicillium*. The PCR results showed that the primers specifically detected *A. carbonarius* strains, and did not amplify other species of *Aspergillus*, such as *A. ellipticus*, *A. tubingensis*, *A. niger*, *A. aculeatus*, *A. japonicus*, *A. brasiliensis*, and *A. ochraceus*, or other ochratoxigenic species of *Penicillium*, including *P. nordicum* and *P. verrucosum*. Further, reverse transcriptase (RT)-PCR was done to assess the involvement of the *pks* gene during OTA biosynthesis. The *pks* gene was differentially transcribed when the fungus was grown on yeast extract sucrose (YES) or on potato dextrose broth (PDB) medium, and incubated at 15°C and 30°C. In particular a higher level of transcription was observed when the fungus was grown on YES than on PDB, and at 30°C than at 15°C. No transcription was observed when the fungus was grown on YES and PDB medium and incubated at 10°C. Similarly, the level of OTA produced by the strain was higher on YES than on PDB, and higher at 30°C than at 15°C. We demonstrated that the primers developed in the current study specifically amplify a polyketide synthase gene produced by *A. carbonarius* and involved in the biosynthesis of ochratoxin A, and that the transcription level of the *pks* gene is closely related to the release of OTA in the medium.

Integrated technology platform for the quality and safety of Nebbiolo grapes and wines.

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Grapevine is one of the most widespread and highly valuable fruit crop. Severe genetic and sanitary protocols are carried out to improve grapevine performances and to propagate healthy plant material. The detrimental effect of viral infections on vine vegetative behaviour and yield is well known, whereas the infection influence on quality and safety of final products, as well as its interaction with the environment, are still unclear.
The project "Tech4wine" involves three Institutes of the Agro-food Department of CNR and four private companies and it is funded by Regione Piemonte to support the production of Piedmont typical wines based on 'Nebbiolo' grapes through an integrated technology platform. The project is aimed to investigate the relationships among virus infection, growing environments, soil arbuscular mycorrhizal fungi and grape and wine quality.
Healthy and virus-infected progenies of three registered 'Nebbiolo' clones were compared in three different environmental conditions. These locations differ mainly in terms of soil texture and pH, slope and site climate. The diseased clonal progenies are infected by Grapevine fanleaf virus (GFLV), and by mixed infection of Grapevine leafroll virus 1 (GLRaV-1) or Grapevine leafroll virus 3 (GLRaV-3) with Grapevine virus A (GVA), respectively.
The sanitary status of each plant was checked by ELISA. New primers and TaqMan probes were designed on the RNA-dependent RNA polymerase gene of each virus in order to further evaluate the influence of viral infections, and specific RT-Real time PCR protocols were developed for the quantification of the four grapevine viruses above mentioned.
Preliminary results indicate that the same clone performed differently in terms of vegetative growth, yield, juice and wine quality due to the virological status, and in most cases the healthy progenies gave better results. The healthy clonal lines also showed a different productive and qualitative response depending on the growing environments.
Soil samples and roots from experimental vineyards were analyzed using specific primers to partially amplify the small subunit (SSU) of the ribosomal DNA genes for arbuscular mycorrhizal fungi (AMF). Phylogenetic analyses highlighted an high rate of species richness, specially related to the vineyard with sandy and acid soil. Glomeraceae family resulted the most represented phylogenetic group in both compartments (soil and roots) suggesting a correlation between intra and extra radical communities. Results suggested a difference in AMF populations between the experimental sites considered, probably due to the different soil features.
As a final step, 2D electrophoresis was used to compare the protein fingerprint of the grape berries under analysis, in order to isolate the most interesting proteins involved in the infection and in adaptation to the environment. The same approach is being used for the characterization of the protein profile of the wine obtained from vines under analysis, in order to assess whether biotic and abiotic stresses have any influence on the risk of allergies to wine.

Fumonisin B_2 occurrence in grape affected by *Aspergillus* bunch rot

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Aspergillus niger recently has been reported to produce fumonisins, and in particular fumonisin B_2 (FB_2). *A. niger* also belongs to a complex of *Aspergillus* Section *Nigri* species, including *A. carbonarius, A. tubingensis* and *A. uvarum* involved in *Aspergillus* bunch rot that is a grape disease. In order to evaluate the natural occurrence and the toxicological risk in terms of levels of fumonisin B_2 contamination in grape affected by *Aspergillus* bunch rot, 49 wine grape samples (*Vitis vinifera*) of two varieties ("Primitivo" and "Negroamaro") were collected during 2008 grape-harvest. The samples, characterized by only berries with evident symptoms of black aspergilli colonization were picked from 18 vineyards in Salentum (Apulia), a grape–growing area of Southern Italy and were assessed for FB_2 contamination. Natural occurrence of FB_2 was found in 12% of the slurry samples and the levels of toxin ranged from 10 to 1,940 ng/g of fresh berries although all contaminated samples showed similar black *Aspergillus* contamination (CFU around 10^6/g of grape slurry). When grown on Czapek Yeast Autolysate with 20% sucrose (CY20S) agar, 30% of black aspergilli strains (14/47) isolated from the contaminated samples produced FB_2, ranging from 90 to 8,765 ng/g. One of the FB_2 contaminated samples resulted also contaminated by ochratoxin A. This study point out that the *Aspergillus* bunch rot may be significantly contaminated by high levels of FB_2 produced by some black *Aspergillus* toxigenic strains and that this plant disease represents a potential mycotoxin risk source for grape-wine chain.

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Influence of *Lobesia botrana* field control on black aspergilli rot and ochratoxin A contamination in grapes

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The grape berry moth *Lobesia botrana* is a key pest in vineyards in southern Europe. Damage caused by *L. botrana* larvae may encourage growth of black aspergilli, leading to ochratoxin A (OTA) accumulation in grapes. Field trials were conducted during three grape growing seasons (2005 through 2007) in Apulia, Italy, to evaluate an insecticide control strategy *for L. botrana* in the vineyard as an indirect method of reducing OTA contamination by reducing black aspergilli on the grapes. In the 2005 field trials, the insecticide treatment controlled attacks by *L. botrana* larvae and reduced OTA concentrations by up to 66% in the must samples of Negroamaro and Primitivo grape varieties. Significant differences ($P \leq 0.05$) also were observed in the incidence of black aspergilli. Environmental conditions in 2006 and 2007 resulted in a natural low level of infestation by *L. botrana*, low levels of OTA in both treated and untreated samples, and no significant differences between treated and no treated samples. The results of our field study confirm previous reports that *L. botrana* is an important risk factor for OTA accumulation and are consistent with the hypothesis that controlling *L. botrana* in vineyards reduces OTA concentrations in grapes.

This research was partially supported by the Strategic Regional Project “INNOWINE”, and by Bayer CropScience (Milan, Italy).

Different susceptibility of clones of "Primitivo" grape variety to *A. carbonarius* infection and ochratoxin A accumulation in relation to resveratrol content.

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The natural occurrence of ochratoxin A (OTA) in red wines has been widely reported by several authors, as well as a group of stilbenes including *cis* and *trans* resveratrols and related glucosylated forms. In this respect stilbenes content, and in particular resveratrols, have been recently correlated to ochratoxin A presence in grapes as a defence response of the plant to ochratoxigenic fungi infection (Bavaresco et al., 2003; 2008); in addition a survey conducted by Perrone et al. (2007) on samples of retail red wines from Southern Italy showed a positive correlation between levels of ochratoxin A and total stilbenes. In particular, the highest levels of ochratoxin A were recorded in Negroamaro and Primitivo based wine samples, showing also the highest content of stilbenes. On this respect, an *in vitro* study was conducted to investigate the susceptibility to *A. carbonarius* infection of 13 different clones of "Primitivo" grape variety, by inoculation of grape with different berry status (intact and damaged) using a mixture of five ochratoxigenic strains of *A. carbonarius*. The assay, carried out with grape berries at ripening was aimed to assess the different clonal response in relation to OTA accumulation, disease severity and resveratrol content in berries after infection. The incidence of infected berries was about 73% higher in damaged berries than in the intact ones. The differences in OTA contents were less remarkable, being 46% higher for damaged berries than for intact ones. Resveratrol content in the infected (intact and damaged) berries was more than twice respect to non inoculated berries, while among the infected berries it was 27% higher in damaged in comparison with the intact ones. Moreover a negative correlation was observed between soluble solids (Brix) and OTA content. In general, no statistical difference was observed among the clones in terms of incidence of contaminated berries (susceptibility to the disease); while OTA contamination and resveratrol varied significatively. These results confirmed the correlation between OTA levels and *cis* and *trans* resveratrols content as a response to the infection by ochratoxigenic fungi, and suggest the possible use of these two parameters as tool for the selection of grape clones less susceptible to infection by ochratoxigenic fungi.

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Antimycotic activity of *Bacillus amyloliquefaciens* against fungi of vineyards soil origin

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Preliminary investigations on grapevines (cv Catarratto) with symptoms of "esca" allowed the isolation of different bacterial colonies (Alfonzo et al, 2009). A Gram-positive, spore forming isolate, able to inhibit fungal growth, was subjected to identification. On the basis of the whole 16S rRNA gene sequence, it showed a similarity of 99% with *Bacillus amyloliquefaciens*. There are numerous reports on the antagonistic activity of this species towards several phytopathogenic microorganisms. For this reason, the potential of the bacterial strain against the fungi commonly associated to the soil of vineyards (*Alternaria alternata*, *Aspergillus ochraceus, Aspergillus carbonarius, Cladosporium cladosporoides, Fusarium oxysporum, Penicillium brevicompactum* and *Verticillium dahliae*) was studied. The antagonistic activity was investigated *in vitro* by two different protein extracts (CME = Crude Metabolites Extract and CEP = Crude Extract Protein) obtained by different methodologies (McKeen et al., 1986; Wichitra et al., 2008). Antagonistic activity was assayed by the agar-well diffusion and critical dilution assay. One active unit AU ml^{-1} was defined as the reciprocal of the highest dilution that gave growth inhibition of the fungi species (Villani et al, 1995). The results showed that both CME and CEP have a powerful antagonistic effect towards all fungal organisms tested. In particular, CEP showed greater efficacy than CME. Fungi were less sensitive to the activity of the CEP accordingly to the following order: *A. ochraceus* (10240 AU ml^{-1}), *A. alternata* (1920 AU ml^{-1}), *A. carbonarius* (1280 AU ml^{-1}), *V. dahliae* (960 AU ml^{-1}), *F. oxysporum* (800 AU ml^{-1}), *P. brevicompactum* (600 AU ml^{-1}) and *C. cladosporoides* (160 AU ml^{-1}). Diversity has been the antibiotic activity of CME: *A. alternata* (1920 AU ml^{-1}), *A. carbonarius* (960 AU ml^{-1}), *V. dahliae* (800 AU ml^{-1}), *P. brevicompactum* (240 AU ml^{-1}), *A. ochraceus* (160 AU ml^{-1}) *F. oxysporum* and *C. cladosporoides* (120 AU ml^{-1}). Further studies are needed to determine the chemical nature of the active metabolites and to evaluate their potential in biological control programmes.

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Treatment efficacy by insecticide/fungicide on *Aspergillus* section *Nigri* contamination and OTA content in grape

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From 2005 to 2008 several trials were carried in order to evaluate the effect of crop protection strategies and insecticide treatments against grape moth, *Aspergillus Nigri* contamination and OTA content in grapes. Over these four years, trials were carried out in many places of Apulia focusing on the following grape wine cultivars: Negroamaro, Primitivo, Montepulciano, Sangiovese, Bombino Nero.
In the 2005 field trials, the insecticide application (PRODIGY®) showed a leading role in containing the development of Black Aspergillus; it is important also to apply the insecticide during the first generation.
In the must samples of Negroamaro and Primitivo cultivars, the insecticide treatment controlled attacks of *L. botrana* larvae and reduced OTA concentrations by up to 66%. Significant differences ($P \leq 0.05$) were also observed in the incidence of Black Aspergillus. The "Primitivo" and "Negroamaro" varieties were selected because of their wide distribution in Apulia and their susceptibility to *A. carbonarius* and OTA contamination.
In the 2006 field trials the previous year activity was considered in order to evaluate the impact of different crop protection programs against *L. botrana*, OTA content and *Aspergillus Nigri* contamination in wine grapes.
This trial was carried out on a Sangiovese cultivar, comparing 4 theses: thesis 1 (Bayer CropScience spray program), thesis 2 (farmer program), thesis 3 (Bayer CropScience spray program without insecticide treatment), thesis 4 (Bayer CropScience spray program without treatment against botrytis).
Even if they are not statistically different, thesis 1 showed a lower OTA level compared to thesis 2.
A good protection against botrytis and powdery mildew may reduce OTA level.

Session II
Starter selection and microbial control in the cellar

Poster presentations

Effect of oleic acid and ergosterol supplementation on oxidative stress resistance in wine strains of *Saccharomyces cerevisiae*

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The lipid composition of cell membranes strongly influences their physiological functions through effects on the activities of many membrane-associated enzymes and transporters (Vigh et al., 1998). During must fermentation, a process that generally occurs under hypoxic/anaerobic conditions, the biosynthesis of unsaturated fatty acids and sterols is impaired. In these conditions, wine yeasts can assimilate exogenous lipids (Redón et al., 2009) and unsaturated fatty acids, and incorporate them into their cell membranes to promote cell growth and fermentative activity (Belviso et al., 2004). However, not all lipids have the same effects on cell membranes. According to Redón et al., (2009), C16:1 supplementation has a positive effect on wine-yeast fitness, as it reduces the fermentation length and increases yeast viability. On the contrary, the outcome of ergosterol feeding is rather controversial, as it can result in a dramatic reduction in cell viability and an increase in fermentation rate (Redón et al., 2009). As the plasma membrane is an important sensor of stress conditions (Vigh et al., 1988; Redòn et al., 2009) and a target for intracellular ROS (Landolfo et al., 2008), the aim of this study was to determine whether lipid nutrition can serve as a means to mitigate oxidative stress during fermentation of a high-sugar-containing must. To do this, oleic acid and ergosterol were added to synthetic must, and the oxidative damage to the yeast-cell structures and the antioxidant response of *S. cerevisiae* wine strains were analyzed and compared to the absence of these lipid nutrients.

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FYL - Fermenting Yeasts Laboratory: isolation, characterization and genetic stability for selection of starter yeasts for winemaking

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The biochemical transformation or influence of all grape juice constituents by yeasts has emerged, in recent years, as an important, additional mechanism whereby yeasts substantially impact on the total quality of wine. It is now accepted that wine fermentations, whether natural or inoculated, are ecologically complex, and not only involve the growth of a succession of non *Saccharomyces* and *Saccharomyces* species, but also involve the successional development of strains within each species. Nowadays, studies on yeast ecology of the fermentation have demonstrated that the dominance of the inoculated strain of *S. cerevisiae* and its metabolic impact on wine character is much more complex than previously thought. With this greater knowledge and understanding, the role of yeast starter is now seen as a key process which can provide new challenges for innovation in wine fermentation. The strategy is to develop wine character through better yeast management and to take part at the international competition within the wine market for newer styles of wines in function of consumer demands. As yeasts are part of the natural microflora of grapes, reasonably, grapes are always considered a potential source of new wine yeasts. Moreover, there is an attraction that unique strains of yeasts will be associated with particular grape varieties in specific geographical locations and, through this association, they could introduce significant diversity and regional character or " terroir" into the winemaking process. Thus, in the interests of preserving biodiversity and regional influence on wine character, grapes of the region would represent an important source of yeasts for starter culture development.

Here we describe the phases of wine strain selection program pointed out and applied in our laboratory in the Basilicata University: Fermenting Yeasts Laboratory (FYL). The selection program is finalized to characterize wild yeasts for parameters of technological interest, for strain genetic polymorphism and stability, for metabolic performance and influence on health wine quality. Each characterization program is designed on the specific request of the winemaker, by selecting "universal" yeast starter cultures, suitable for a wide array of grape varieties and producing a balanced flavour or selecting specific strains for specific grape fermentations, such as "local" or "varietal" yeast selected cultures, as a function of the vine variety characteristics in order to take the major advantage from the combination grape must/*S. cerevisiae* strain.

Disinfectant susceptibilities of wine spoilage yeasts and bacteria in planktonic state and in biofilms

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Microbial spoilage of wine can occur at multiple stages of the vinification process. The common spoilage effects are film formation in stored wines, cloudiness or haziness, sediments and gas production in bottled wines, and off-odours and off-tastes at all stages of wine production. Spoilage microorganisms include lactic and acetic acid bacteria and yeasts of the genera *Brettanomyces*, *Candida*, *Hanseniaspora*, *Pichia*, *Metschnikowia*, *Saccharomycodes*, *Saccharomyces, Schizosaccharomyces* and *Zygosaccharomyces*. The *Calgary biofilm device* (CBD) has been used to evaluate the biofilm forming ability of microorganisms, particularly bacteria, as well as to determine the biocide susceptibilities of microbial biofilms (Ceri et al., 1999). The aim of this work was to verify the ability of six yeast and two bacterial strains to form biofilms in mono- or co-culture using the *CBD* and to assess the efficacy of various types of commonly used disinfectants against these spoilage microorganisms both in suspension and in biofilms.
The results obtained showed that tested *B. bruxellensis*, *S. cerevisiae, S. ludwigii, S. pombe* and *Acetobacter aceti* strains formed biofilm both in wine and in synthetic medium, meanwhile the *Z. bailii* strain formed biofilm only in wine and the strains of *P. guillermondii* and *Lactobacillus hilgardii* formed biofilm just in synthetic medium. Furthermore, when acetic bacteria was grown together with lactic bacteria in wine, an increase of 3 log was observed in biofilm formation when compared with monoculture. According to both suspension and biofilm tests, the alkaline chlorine-based disinfectant was the most effective in decontaminating yeast. Furthermore, results showed that sodium hydroxide-based detergents and peracetic-based disinfectant were efficient against suspended cells but at least 10-fold more concentrated solutions were needed to remove biofilms.
In our knowledge, this is the first study in which the CBD is used to test the ability of yeast and bacteria contaminating the wine to form biofilm and to asses their susceptibilities to disinfectant agents.

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Oenococcus oeni biodiversity during malolactic fermentation for production of Barolo

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Conduction of the malolactic fermentation (MLF) is indispensable for production of some types of wine. During vinification for production of Barolo, a prestigious wine produced solely in the Piedmont region in the Northwest part of Italy, MLF is favoured since it not only leads to significant deacidification but also adds sensorial complexity to the final product. Winemakers have the choice of conducting MLF with the autochthonous lactic acid bacteria (LAB) flora or through inoculation of starter cultures. However, LAB starters used in wine production, and in particular *Oenococcus oeni*, the LAB species ideally adapted for MLF, are 'universal', in other words, they are used for production of different wines around the world. A possible drawback of such application is the 'standardization' of the final products. For this reason, we sought to characterize the LAB autochthonous flora, responsible for MLF, during vinification of Nebbiolo grapes for production of Barolo wine. Three fermentations were followed, with grapes originating from three different vineyards in the same geographical region. Conditions of alcoholic and malolactic fermentations were identical for the three productions. During MLF, samples were taken for both microbiological and chemical analysis. The LAB isolates were first identified and then characterized, by molecular methods. Although the only species isolated from all three fermentations was *O. oeni*, significant differences, in terms of time for completion of the MLF, were observed. The genetic characterization of the isolates suggests that during MLF, there is a succession of different populations of *O. oeni*. Physiological characterization of the isolates is underway in order to identify the ideal candidate to be used as starter for the MLF in Barolo wine.

Oak barrel spoilage yeasts: effect of physical sanitization and influence on the evolution of alcoholic fermentation

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Oak barrels are tools of primary importance for winemakers: they are commonly used to ferment white wines or for the ageing of red wines. High porosity and physical inertness characterize the structure of wood; these properties favour the proliferation of spoilage microorganisms, such as many species of non-*Saccharomyces* yeasts (Swaffield et al., 1997). Their eradication from the barrel is very difficult because wood can protect their cells against chemical and physical agents. Several treatments have been proposed to obtain an effective sanitization of barrels (Oelofse et al., 2008), based either on chemical or on physical agents (SO_2, O_3, aqueous steam, UV radiation, microwaves and ultrasounds), but the definition of an effective protocol is still an actual request of winemakers. The use of chemicals is restricted to very mild treatments, to avoid depleting the wood character (Marko et al., 2005), so the use of physical treatments appears the most suitable way to obtain an effective control on the barrel spoilage microflora.
In this work we compared the efficiency of two physical sanitization treatments: UV irradiation of the internal barrel surface, and the treatment with aqueous steam.
For each treatment, different times have been tested, and the effectiveness of sanitization was evaluated by measuring the cellular density into the barrel after the treatment.
The research of yeast species in the oak barrel before and after the sanitization treatments was carried out with PCR-DGGE and their identification by 26S rRNA gene sequencing.
Considering an approximate barrel volume of 250 litres, the mean total yeast density before the treatments was $1.0\pm0.5\times10^3$ CFU/ml; apart from *Saccharomyces cerevisiae*, that was found in the majority of samples, four different non-*Saccharomyces* yeast species were found. A prolonged treatment with steam was the most effective sanitization method, able to eliminate the 90% of the original yeast microflora. No differences in terms of species present into the barrel were found before and after the treatments.
The interaction between isolated non-*Saccharomyces* yeasts and five different *Saccharomyces cerevisiae* commercial strains was studied in laboratory microvinification. Fermentations were monitored by measuring the weight loss due to sugar consumption; results are discussed in terms of fermentation kinetics, and final chemical composition of obtained wines.

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Zymocidial activity of two killer yeasts to keep under control the development of *Brettanomyces*/*Dekkera* in winemaking

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Dekkera/Brettanomyces bruxellensis is recognized as a common contaminant in wine. Actually, it can produce strong off-flavors. Some key periods are important to "managing brett". At the beginning of the fermentation for the indigenous *Brettanomyces* but also during other key /risky moment in the wine process (end of fermentation, maturating, bottling step) the risks for "brett" note deviation are real.
Considering that the synthetic fungicide sulphur dioxide is an important consumer issue and reduce or avoid the maturation of wine , that wine filtration could affect the body and viscosity of red wines, that steam treatments for barrels could damage wooden barrels, a microbiological alternative to help curtailing the growth of *Brettanomyces* would be well accepted by winemakers.
In this work we reported the results of an investigation on two yeast killer toxins active against a large spectrum of spoilage yeasts belonging to the genus *Dekkera/Brettanomyces.* Those yeasts *Pichia anomala* (DBVPG 3003) and *Kluyveromyces wickerhamii* (DBVPG 6077), produce killer toxins, respectively named Pikt and Kwkt with different molecular weight, complementary biochemical properties and mode of action (Comitini et al., 2004). Interestingly, the fungicidal effect exerted by Pikt and Kwkt against *Dekkera bruxellensis* is stable under winemaking condition. Results obtained in wine or must samples, inoculated at different stage of the wine process with *Dekkera/Brettanomyces* sensitive strains, showed an efficient anti-spoilage effect. The two killer toxins really control both growth and metabolic activity of sensitive spoilage yeasts. Contents of molecules considered to be the most involved in "brett" default in wines i.e. ethyl phenols, are reduced.
Thus, the potential application for these two toxins as antimicrobial agents active on *Dekkera/Brettanomyces* during fermentation, wine ageing and storage can be a biological strategy as alternative of sulphites.

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Diversity in a single *Oenococcus oeni* population during spontaneous malolactic fermentation of a typical grape cultivar of Salento region

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Aim of this study was to investigate on indigenous population of *O. oeni* responsible of spontaneous malolactic fermentation (MLF) of "*Malvasia nera"* wine, an economically important red wine of the Salento Region (Apulia, Italy). Strains of this specie, identified by species-specific PCR and 16S rRNA sequence analysis, were molecularly characterized by the Amplified Fragment Length Polymorphism (AFLP) technique. Three main groups resulted by clustering analysis and showed intraspecific homology higher than 78%. Moreover, this technique provide a total of 8 subgroups, representative of the three groups considered AFLP clusters. Enzymatic activities, such as esterase, β-glucosidase, protease, and consumption rate of L-malic acid, citric acid, acetaldehyde and arginine was assessed in the representative strains of each eight subgroup selected. The results displayed different enzymatic activities and consumption rates of tested metabolites among the strains. No correlation between molecular and biochemical data was observed. The evidence of genetic and phenotypic variability observed among *Malvasia nera* strains demonstrated that several strains can contributed to the spontaneous fermentation according to their ability to dominate the natural environment. The high degree of heterogeneity existing within natural *O. oeni* populations represents an interesting ecological source that can be useful for technological purposes.

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P-2-8

Fruit flies (*Drosophila* sp.) are essential to the development of sour rot in grapes

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Sour rot is a disease characterized by grape pulp browning, disaggregation of the internal tissues, detachment of the rotten berry from the pedicel, grape dropping and strong smell to vinegar and ethyl acetate. *Drosophila* flies are systematically found near the affected bunches. However, we are not aware of research demonstrating their role in the spread of yeast species associated with sour rot grapes. Therefore, the purpose of this work was to study the influence of *Drosophila* sp. on the spread of sour rot disease and on the dissemination of yeasts.
During the 2008 vintage, grapes were collected after veraison (beginning of ripening) and at time of harvest. The grapes were maintained free or protected inside a plastic structure covered by a nylon cloth. Six types of samples were analysed: i) free sound bunches; ii) protected sound bunches; iii) free sound bunch with artificial wounds; iv) protected sound bunch with wounds; v) free bunches with sour rot and vi) protected bunches with sour rot symptoms. The yeast flora was recovered by general purpose (GYP) and selective/differential culture media (DBDM and ZDM) and identified by PCR-RFLP of 5.8S-ITS rDNA and sequencing of domains D1/D2 of the 26 rRNA gene. *In vitro* grapes were inoculated with different yeast species and with or without contact with fruit flies.
The observation of sour rot symptoms was only observed when *Drosophila* sp. was in contact with the grapes. Even the artificially wounded berries did not show sour rot when kept free of insects. Accordingly, the yeast flora of wounded grapes without insect contact was similar to that of sound grapes, dominated by basidiomycetous species and *C. zemplinina*, *H. guilliermondii*, *C. amapae* and *C. diversa*. Grapes with sour rot, besides these ascomycetous species, showed a higher species diversity including *H. uvarum*, *Issatchenkia* spp., *Lachancea* spp., *Z. hellenicus*, *Z. bisporus*, which are characteristic of damaged grapes. The species *H. uvarum*, *I. terricola* and *Z. hellenicus* were recovered after enrichment cultures of insect bodies. *In vitro* assays showed that sour rot only developed in the presence of fruit flies, irrespective of the yeast strain present.
This work demonstrated the importance of *Drosophila* sp. in the development of sour rot disease and on the dissemination of wine contamination yeast species.

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Isolation and molecular identification of wine yeasts from a Brazilian vineyard.

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Traditional winemaking is carried out by spontaneous yeasts from the vineyard that continue throughout the alcoholic fermentation. This study examined the diversity of yeast species isolated from grape skin and musts of three varieties of *Vitis labrusca* from a vineyard in the southeast region of Brazil (Jales, São Paulo), which produces artesanal wines. Molecular identification was achieved by combination of PCR-RFLP/sequencing of the internal transcribed spacers (ITS) and sequencing of D1/D2 domain of ribosomal DNA (Fernandez-Gonzalez et al., 2001; Clemente-Jimenez et al, 2004). Eighty yeast samples were isolated from grapes and musts and seven different species were identified. The diversity of species varied according to the grape variety. The most frequent species were *Hanseniaspora uvarum* with 28 isolates, followed by *Issatchenkia occientalis* with 19 isolates and *Issatchenkia orientalis* with 16 isolates. Other species with a lower number of isolates were: *Issatchenkia terricola*; *Saccharomyces cerevisiae*; *Aureobasidium pullulans* and *Sporidiobolus pararoseus*. Our results showed that molecular identification is a very powerful identification method in which natural isolates of ascomycetous or basidiomycetous yeasts can be rapidly and reliably identified with reproducibility and higher throughput over conventional phenotypic methods. This is the first report of the diversity of indigenous yeast species from a vineyard from Brazil.

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Safe malolactic fermentation: the use of silica entrapped *Oenococcus oeni* cell under lysozyme protection

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Different species of lactic acid bacteria are implicated in spontaneous malolactic fermentation. Their evolution in wine represents a risk for the wine quality and safety, and their impact on the wine aroma is frequently negative. Moreover, an increase of biogenic ammine concentration, due to the degradation of aminoacids may be observed in spontaneous malolactic. Therefore, a strictly control of spoilage of lactic acid bacteria in wine, during the winemaking, is mandatory to obtain safe and high-quality wines. Two methods are the most widely used to avoid the evolution of lactic acid microflora: the addition of sulphur dioxide at the grape crushing, and after the alcoholic fermentation, or the addition of lysozyme to the must. The use of sulphur dioxide may be carefully evaluated for its toxicity versus the microorganisms involved in the wine fermentation and for the human. Instead, the Lysozyme is small single peptide with a muramidase activity, harmless versus the eukaryotic organisms. It can be added to the must to inhibit the growth of lactic acid bacteria. Lysozyme minimizes the spoilage of wild lactic acid bacteria, but unfortunately, its use definitely suppresses the evolution of successive malolactic fermentation, inoculated by selected bacteria. Therefore, a new approach is required to maintain a strictly control of wild lactic acid bacteria, during the winemaking. The cell immobilization represent a solution: *Oenococcus oeni* cells have been immobilized in Calcium alginate microbeads, covered with a sol-gel made silica membrane. The silica layer was obtained by two subsequent treatments: the first one by dipping of alginate microbeads in a silica sol suspension, the second one by direct reaction between silica precursors in gas phase and already coated silica/alginate microbeads. The silicon alkoxide precursors utilized, and the peculiar synthetic route allowed the creation of a continuous membrane with a defined porosity with a cut-off about 30 KdA. The physic/chemical properties of silica membrane have been characterized by Scanning Electron Microscopy and solid state NMR, demonstrating that a silica lattice was completely formed around the micro-spheres with 14g of silica deposed for m^2 of bead surface. The ^{29}Si NMR analysis showed that the silica membrane was composed by 19.6% of $Si(OSi)_4$, 12.5% of $HO\text{-}Si(OSi)_3$, 33% $MeSi(OSi)_2OH$ and 34.9% $MeSi(OSi)_3$.

The cell density internal to the microbeads was adjusted to obtain the higher fermentative activity; the cell viability was evaluated during and after the immobilization process by plate count. The duration of entrapped cell and the cell leaking experiments were carried out in synthetic medium and in wine. The sterical protection of silica membrane due to its defined cut-off allows the protection of entrapped cells from the action of Lysozyme. Two set of experiments were carried out in laboratory (1 L of must) and micro-winemaking (15 L of must) conditions. Free or immobilized bacteria were inoculated in the presence of two levels of lysozyme, simultaneously to the yeast in must. In the tests performed by free bacteria the lysozyme totally inhibit the evolution of malolactic fermentation: only the alcoholic fermentation occurs regularly. In the Tests inoculated by the immobilized bacteria the malolactic fermentations occurs regularly and it was concluded together the alcoholic fermentation. The immobilization allows safe and reliable malolactic fermentation in must or wine and, at the same time, the complete control of the spoilage of wild lactic acid bacteria.

Molecular biodiversity and variability for urea production in *Saccharomyces cerevisiae* wine strains of different origin

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The yeast *Saccharomyces cerevisiae* represents the principal agent of winemaking and universally known as "the wine yeast". Numerous studies have revealed the existence of high variability both for technologic and genetic characters among wild wine *S. cerevisiae* strains (Romano et al., 2008). Thus, the increasing interest to evaluate the natural biodiversity of each ecosystem has stimulated the development of molecular approaches, in order to individuate a significant level of genetic variability that seems to determine also differences in the expression of oenological parameters. The present research reports results on the technological and molecular characterization of indigenous *S. cerevisiae* strains, isolated from spontaneously fermented grapes collected from different Centre-Southern Italian regions and belonging to the collection of the Fermenting Yeasts Laboratory of the Basilicata University. These strains were submitted to RAPD-PCR analysis with different primers, as M13 and P80 and, in the same time, to amplification by PCR of AGA1, SED1 and DAN4 genes, encoding cell wall proteins. These genes are characterized by length polymorphisms and may be constitute molecular markers for the characterization of different individuals within the species (Marinangeli et al., 2004). The studied strains exhibited a significant level of genetic polymorphism, correlated, in some cases, with grape origin. In the second phase of the work, the strains were submitted to technological tests, such as laboratory fermentations to evaluate strain fermentative performance. The experimental wines obtained were analyzed for urea, arginine and ammonium content by enzymatic kit to determine yeast strain ability to affect nitrogen composition of wine. In particular, the attention was focused on urea production, which is involved in the formation of ethyl carbamate, a carcinogenic compound, in presence of ethanol produced during fermentative process. The results confirmed that urea was produced in significant different amounts. In this way, it could be possible select wine strains, useful as starters, able to produce wines characterized by reproducible quality in the safeguard of consumers health.

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Wine yeast immobilization on "food-like" matrix for inoculated fermentation: preliminary results on spray-drying treatment of yeast cells

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An upsurge of interest in cell immobilization for alcoholic beverages has taken place recently. This is mainly due to the numerous advantages that cell immobilization offers including enhanced fermentation productivity, feasibility of continuous processing, cell stability and lower costs of recovery and recycling. Numerous biotechnological processes are advantaged by immobilization techniques and therefore several such techniques and support materials have been proposed. For applications in winemaking, immobilization supports should satisfy prerequisites such as natural products, cost effectiveness, availability and should not influence the sensorial wine composition. The most commonly used polysaccharides for cell immobilization in winemaking, are alginates, cellulose, carrageenan, agar, pectic acid and chitosan. Sodium, calcium and barium salts of alginates have been extensively used for cell entrapment but calcium alginate gels are considered more suitable for alcoholic fermentation. In order to satisfy the demand for natural products and combine it with consumer acceptance, some researchers have proposed the use of fruit pieces as cell immobilisation carriers for wine or beer production and reported products with fine taste and aroma and a distinct fruity character. Apple and quince (Kourkoutas et al., 2002, 2003) pieces were considered cheap, abundant supports of food grade purity of immobilization and led to a product with improved sensory characteristics. Other authors (Tsakiris et al., 2004) proposed the use of grape products, such as residual grape skins, as a support for the immobilization in wine-making. We are developed a research project with the aim to set-up a food-like support for cell immobilization of *Saccharomyces cerevisiae*, the first actor of grape must fermentation. The final goal will be to transfer this system at cellar level, promoting the use of immobilized starter cultures. One main objective is to immobilize on natural matrix yeast cells submitted to drying treatment. The ability of dried yeasts to be stored for long periods without loss of cell viability or fermentation ability makes this biocatalyst very attractive for industrial use. The first phase of this study was focused on the setting up of spray-drying conditions of chosen strains of *S. cerevisiae* in order to assure the preservation of the viability and the metabolic characteristics of the tested strains The test was carried out by applying different physical conditions of spray drying and different protective substances to cells of two strains grown for different times. The first results on cell viability after spray-dried treatment demonstrated that cells of 72h exhibited the best viability in skim milk as protective.

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Isolation and clonal selection of enological *Saccharomyces* from Susumaniello natural fermentations

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Spontaneous grape must fermentation, induced by the indigenous micro flora, is believed to be associated with a specific vineyard and to give a distinctive style and quality to that wine. The alcohol-tolerant *Saccharomyces cerevisiae* strains invariably dominate the latter stage of natural wine fermentation. The *S. cerevisiae* population and other specific yeasts present in the vineyard niche habitats are considered autochthonous and their involvement in natural fermentation allows the production of wines with particular features in each microclimatic area. The present study was aimed to the individuation of autochthonous yeast strains useful in the improvement of oenological production of Salento, which is a very important wine-producing area of Southern Italy. Grapes were sampled from the most representative area of Salento region (Brindisi) for "Susumaniello" production and separately subjected to natural fermentation in an experimental scale. The identification of micro biota present during the last step of wine fermentation (>1 °Bé) of Susumaniello grapes, was carried out to select autochthonous yeast strains for industrial wine production. Aliquots of must samples of several dilutions were isolated after spreading on YPD agar medium supplemented. As preliminary screening, averages of 1500 colonies for each of the four fermentations were assayed for H_2S production on BIGGY agar. The colonies appearing white or light brown (about the 10% of initial 1500 individual clones) were selected and recognized as *Saccharomyces cerevisiae* by a microbiological screening based on the implementation of the taxonomical keys for identification of yeasts belonging to *Saccharomyces* genus. All the yeasts analysed were identified as *S. cerevisiae*.
The criterion for *S. cerevisiae* strain differentiation was provided by the PCR analysis of polymorphism of amplified δ interspersed element sequences, which has been demonstrated to share a similar discriminative power of the mtDNA restriction patterns. The identified *S. cerevisiae* strains were assayed by micro fermentation studies. The results of chemical and technological analyses of the above micro vinification tests and their implications for the selection of autochthonous industrial starters will be discussed.

P-2-14

Amplified fragment length polymorphism: a novel tool to investigate the biodiversity of *Oenococcus oeni* in wine.

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Molecular tools were widely used to study microbial diversity of grapes and wine and they have proved to be particularly useful in identifying strains within species that are homogeneous at the genome level, such as for *Oenococcus oeni* species. Genotyping diversity among strains of this species was performed using DNA fingerprinting based on restriction analysis of genomic DNA, restriction endonuclease gel electrophoresis (REA-PFGE), polymerase chain reaction-based methods, random amplified polymorphic DNA, and 16S–23S rDNA intergenic spacer region analysis-PCR. Results obtained by applying the above methods made it possible to group *O. oeni* strains according to their level of similarity. By the numerical analysis of DNA fingerprinting, REA-PFGE is recognized to be highly reliable in the differentiation of this closely bacteria strains.
Successively an alternative to this tool was proposed that applied differential display PCR to discriminate strains of this wine bacterium. By this technique, it has been demonstrated that genotyping, besides having a taxonomic value, has practice relevance for the differentiation of strains of interest for biotechnological processes. Several strains of *O. oeni* were identified by distinct genomic finger printing according to the geographical origin.
Recently we applied the AFLP analysis on these bacteria strains in order to distinguish between bacteria genotypes and to compare the genetic diversity of indigenous bacteria selected during MLF. To our knowledge this is the first time that AFLP analysis is used for *O. oeni* and our results indicate that this technique is reliable in order to genotype and discriminate strains of this species.
In particular a total of 87 out of 220 lactic acid bacteria, isolates from "*Primitivo*" wine (Apulia, Italy) undergoing MLF, identified as *O. oeni* by species-specific PCR and 16S rRNA sequence analysis, were studied by AFLP analysis. Four main clusters were distinguished and three of them showed intraspecific homology higher than 60%. Moreover a indigenous population of *O. oeni,* (82 from 215 isolates) responsible of spontaneous malolactic fermentation (MLF) of '*Malvasia nera*' wine, an economically important red wine of the Salento Region (Apulia, Italy), was investigate. The results obtained, by clustering analysis, evidenced three main groups resulted and showed intraspecific homology higher than 78%; this technique provide a total of 8 subgroups, representative of the three groups considered AFLP clusters.
In conclusion, this study indicated a valid approach to individuate *O. oeni* strains destined for a selection program for malolactic starter cultures. Apart from its applicative this study, also represents a contribution in understanding the population dynamics of these bacteria strains, during wine malolactic fermentation.

Dissemination of oenological yeasts through bird migration in Sicily

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The composition of yeast communities on shown to be dependent on several factors, including geographical location of the vineyard, type of soil, age of the vineyard, grapevine variety and harvesting technique. Moreover, insects and birds were in part responsible for the dissemination of fermentative yeasts during their feeding (Francesca et al., 2009). In order to evaluate the dissemination of fermenting yeasts, migrating and vineyard inhabiting birds were caught and ringed in four experimental sites: "Riserva Naturale Integrale Lago Preola e Ghorghi Tondi" (Mazara del Vallo, TP) and the Sicilian islands of Lampedusa, Ustica and Linosa. The last sites represent important stop-over for migrating birds. A total of 344 birds, belonging to different species, were captured and their mouths and cloacae were plugged with sterile cotton swabs and streaked onto malt extract agar for yeast isolation. A total of 125 yeast isolates were clustered into five groups based on colony appearance onto Wallerstein Laboratory (WL) nutrient agar (Pallman et al, 2001), while seven groups were recognized by optical microscopic observation. Yeast identification was preliminary carried out by amplification of ITS-5.8S rRNA region (Esteve-Zarzoso et al., 1999) and, subsequently, by D1/D2 region of the 26S rRNA gene sequencing. *Metschnikovia pulcherrima, Candida stellata*, *Pichia guilliermondii, Hanseniaspora uvarum, Torulaspora globosa*, *Torulaspora delbrueckii, Zygosaccharomyces bailii* and *Saccharomyces cerevisiae* were found. Furthermore, the study was focused on the nine strains of *S. cerevisiae* isolated. They were characterized for technological traits with interest in wine production such as hydrogen sulphide production, ethanol and potassium metabisulphide resistance. Strains showing the best performances were used to carry out sterile must micro-fermentations to select yeast starter cultures.
To our knowledge, this research showed a new finding regarding the ecology of vineyards: for the first time it has been proved that *Saccharomyces cerevisiae* strains can be disseminated by sedentary and migrating birds. Among them some strains with oenological potential were found.

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Ecology and technological capability of lactic acid bacteria associated with Grillo grapevine used as base wine for Marsala production

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Lactic acid bacteria (LAB) have a defining role in winemaking process since their activities determine an important contribute to wine quality. Besides sulphur dioxide, lysozyme is becoming a common supplement in wine for bacterial growth inhibition (Sonni et al, 2009). It is a natural enzyme with muramidase activity working against a wide range of LAB, including *Oenococcus* spp., *Pediococcus* spp., *Lactococcus* spp. and *Lactobacillus* spp. (Cunningham et al., 1991).
To obtain a first mapping of LAB inhabiting Marsala wine production area, grapes of "Grillo" variety were harvested from five vineyards different for climatic and agronomic parameters. A Marsala base wine large-scale process was followed and samples were collected from must to bottling. The influence of lysozyme and SO_2 on LAB was also evaluated through two experimental micro-vinification processes. Microbial communities and conventional chemical parameters were periodically analysed. Total microflora on grapes was barely around 10 CFU mL^{-1}; while must from large-scale vinification hosted a higher concentration, around 10^3 CFU mL^{-1}, that decreased during wine process. No difference in terms of microbial load was detected between the two experimental micro-vinifications (both containing around 10 CFU mL^{-1}) and the chemical parameters were those commonly reported in literature. A total of 146 bacterial isolates were analyzed: only 35 cultures were presumptively identified as LAB (Gram positive, catalase and oxidase negative). On the basis of isolation source and cell morphology, 16 isolates were genetically identified. 16S rRNA gene sequencing revealed the presence of *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Enterococcus lactis*, *Leuconostoc fallax* and *Sporalactobacillus nakayamae* subsp. *nakayamae*. Subsequently, strains were characterized for lysozyme and SO_2 resistance. *Lactococcus lactis* subsp. *lactis* strains, most frequently isolated during winemaking, showed the highest resistance to SO_2 and to lysozyme: up to 1600 mg L^{-1}, namely a concentration higher than the one usually employed in commercial vinification processes. *Sporalactobacillus nakayamae* subsp. *nakayamae* strains, isolated during the first stage of large-scale wine aging, were inhibited by 100 mg L^{-1} lysozyme, but were resistant to 800 mg L^{-1} SO_2. The other species, collected from different steps of winemaking, showed a medium-high resistance to the tested inhibitory concentrations.
In conclusion, this study, in agreement with previous works (Delfini et al., 2004), underlines the lack of antimicrobial activity of lysozyme and SO_2 against *Lactococcus lactis* subsp. *lactis* strains. Furthermore, the presence of *Sporalactobacillus nakayamae* subsp. *nakayamae* in wine has been reported for the first time. However, its presence might be associated to the LAB contamination of commercial yeast starter culture used in winemaking.

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Effect of propolis extract on wine microorganims

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Propolis, that is a mixture of vegetable resins, wax substances, pollen and salivary secretion, is produced by bees and it is known for its antiseptic, antimycotic and antioxidant activities. Therefore, in agriculture and in food processing industry, it can be used as an antimicrobial natural product. In this work, the effect of the hydroalcoholic propolis extract on wine microorganisms occurring on grapes such as yeasts, lactic acid bacteria, acetic bacteria and the mould *Botrytis cinerea* (an important pathogenic agent of grapevine) was evaluated. Experimental assays were carried out using the following microorganims: twenty-five yeast strains belonging to the most frequently yeast species occurring in winemaking (*Saccharomyces cerevisiae*, *Metschnikowia pulcherrima, Kloeckera apiculata* and *Candida zemplinina*), nine strains of wine lactic acid bacteria (three *Oenococcus oeni* strains, five *Pediococuus* spp strains and one *Lactobacillus plantarum* strain), eight strains belonging to *Acetobacter* spp. and one culture of *Botrytis cinerea*. Propolis, that is usually used in agriculture as hydroalcoholic extract at the recommended concentration of 1μL/mL, was used in the experimental assays also at the following higher concentrations: 5-10 and 100 μL/mL. The extract was added to culture media inside Petri plates and microoorganisms were inoculated by pour plating and spread plating methods. Because of the high content of ethanol in the plates with concentrations of propolis of 10 and 100 μL/mL, control plates containing equivalent ethanol concentrations were performed in order to rule out any possible interferences on results. After appropriate incubation the occurrence or the absence of microbial growth were recorded. At the concentration of propolis of 1 μL/mL the microbial growth was not affected, whereas it was completely suppressed by the highest assayed concentration (100 μL/mL). When the content of propolis was of 5 and 10 μL/mL neither lactic acid bacteria belonging to *O. oeni*, *Pediococcus* spp. and *L. plantarum* species nor acid acetic bacteria were inhibited. On the contrary, yeasts showed different behaviour according to the species and the strain examined. Indeed, at the concentration of 5μL/mL one *S. cerevisiae* strain and three *M. pulcherrima* strains resulted inhibited, while in the presence of propolis at 10 μL/mL other two *S. cerevisiae* strains and one *K. apiculata* strain were not able to grow. The possible interference in the results for the presence of ethanol in the plates containing 10 μL/mL of propolis was excluded as no growth inhibition occurred at such alcoholic concentration. All the strains of *C. zemplinina* were not affected by the different content of propolis. In conclusion, *C. zemplinina* and *K. apiculata* resulted more resistant towards the effect of propolis than *S. cerevisiae* and *M. pulcherrima* species. The growth of *Botrytis cinerea* was inhibited in the plates containing 10 μL/mL of propolis. The findings allow to suppose that propolis at the concentration of 10 μL/mL has an anti-*Botritys* action without notable alterations for other microorganisms of oenological interest.

Fish (fluorescence *in situ* hybridization): a useful tool for wine microbial control

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The microbiological control in cellars needs from increasingly rapid and trustworthy systems to give response to the needs of the winemaking industry. The fluorescence microscopy systems, although used since many years ago, have evolved largely in the last years, allowing the counting and identification of individual cells, or even the estimation of their metabolic state directly from wine and very rapidly.
As equipment, few elements of low or moderate cost are needed, as a fluorescence microscope, a bench centrifuge, a filtration system and a water bath.
FISH's technology is used for the identification of microorganisms through the use of specific probes, and results very useful in fields as enology (Blasco et al., 2003). The must or wine to be analyzed can be filtered directly without any previous treatment, adjusting the filtered volume to the concentration of microorganisms present in the sample. After fixation and permeabilization of the cells, the sample hybridizes with the desired probe (or the series of specific probes). The hybridization is based on the specific union of every fluorescent probe with the ribosomal sequences present in every cell. As there exist a high number of copies of these molecules, the signal level is high enough to be observed with a fluorescence microscope. Also, general probes can be used that will allow us to detect and to count all the present yeasts and bacteria. After a washing and drying step, it proceeds the visualization in the microscope of fluorescence. As the sample volume is known, the result is quantitative and completely accurate. With the modern computer aided image analysis software (some free), it is even more easy and accurate to count cells directly from grape must or wine samples.
As various specific probes can be employed in the same sample, we can identify and count at the same time several microorganisms, according to the specificity of each probe: for example, a general bacterial probe will count total bacteria, a probe of the *Leuconostoc* genus will identify the cells belonging to this group, and a probe specific for *Leuconostoc mesenteroides* will give us the count and the identity of those cells belonging to this species.
The whole process may last a few hours: in most cases, in less than 3 hours starting from the sample to the count and complete identification. The cost per sample (only consumables) can vary from 0.45 to 3.00 €. The training of the personnel is simple and rapid.
The main applications of FISH in wine microbial control are the identification in a little time of desirable and spoiling microorganisms, to estimate the efficiency of the inoculation with commercial starters, to make possible the control of frauds and protection of patented strains, limitation of the quantities of sulphites added in the vinification process (as a result of a better follow-up and control of the microbial populations), etc. This technique can be used easily by cellars, bottling lines, service companies, and administration centres.

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Session III
Yeast activity on wine quality

Poster presentations

The effect of pyraclostrobin and fenamidone over natural wine yeast population

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Natural fermentations produce wine characterized by peculiar aromas and flavour due to the complexity of the natural micro flora, mainly composed by non-*Saccharomyces* yeasts. At the beginning of natural fermentations *S. cerevisiae* is present at low concentration. In the following stages, a succession of different ethanol-tolerant *Saccharomyces* strains are established and they complete the synthesis of volatile fermentative compounds (Clemente-Jimenez et al., 2005; Fleet, 1990). In the last years this complexity has been revaluated to produce new types and styles of wines (Pretorius, 2000). In this work we evaluated the effects of two fungicides over the natural microflora of wine yeasts during natural fermentation of must by Nieddera and Vernaccia, two Sardinian wine grape varieties. The fungicides were used at the maximum residue level (MRL) and ½ the MRL. The results obtained at the beginning of fermentation showed significant differences concerning the concentration of *Kloeckera apiculata* during the shifting from MRL to ½ MRL of pyraclostrobin. On the contrary fenamidone showed an activity over *S. cerevisiae* particularly at middle fermentation in red grape must and at the end of fermentation in white grape must. In the latter case significant differences were observed in the ethanol production.

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Characterization of potential spoilage wine yeasts and their possible use in winemaking process

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The yeasts belonging to the genera *Pichia, Zygosaccharomyces* and *Saccharomycodes* are generally believed spoilage wine yeasts because they are often isolated from stuck or sluggish fermentations, or from wines with anomalous analytical and sensorial profiles.
However, while these yeast genera in pure culture fermentation often lead to the production of wines with principal negative features, their presence in a mixed fermentation with *Saccharomyces* may provide the final product with particular sensorial characteristics. Indeed, in the last few years, other authors have shown that in natural fermentations *Saccharomyces* and non-*Saccharomyces* yeasts do not passively coexist but they seem to interact; in these conditions some oenological traits of the non-*Saccharomyces* yeasts are not expressed, or they can be modulated by the *Saccharomyces* yeast cultures (Bely et al., 2008; Anfang et al., 2009).
Other studies have also pointed out the abilities of most of the yeasts belonging to these genera to produce high amounts of different metabolites and enzymes able to release aroma from precursors present in grapes (Fernandez et al., 2000) therefore influencing the perceivable characteristics of the final product (Romano et al., 2003).
Based on these observations we evaluated their possible use in fermentation processes in mixed culture with *S. cerevisiae*, to increase the complexity of the final wine. Preliminarily we evaluated the fermentative performances of pure cultures of yeasts belonging to *Pichia, Zygosaccharomyces* and *Saccharomycodes* genera. In a second step we selected two cultures for each genera and utilized them in mixed fermentation with a commercial *Saccharomyces cerevisiae* yeast strain at different inoculum ratio.
This study represents a part of the research project coordinated and financed by Consorzio Tuscania, Firenze.

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INNOWINE: INNOvative biotechnological approaches to improve quality and safety of typical Apulian WINE

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Apulia region is one of the most important grape and wine producer of Italy, second only to Sicily, and it is the biggest producer of red-pink wines. In the past, Apulian wines were mostly employed to adjust the alcoholic graduation and flavour of wines produced in other regions. However, recently they are becoming appreciated due to a general trend towards the improvement of final quality instead of product quantity. Wine production in Apulia region is strongly affected by the typical environmental conditions in which the cultivation occurs, that confers characteristic and deep flavour the end products. The Apulian wine making sector is going through a process of technology and quality improvement. Therefore, in the traditionally important wine areas it is becoming urgent for wine producers to learn the parameters that mainly affects the safety and quality of niche and typical products. In order to comply with these needs a four years Apulia regional project, INNOWINE as acronym, has been recently approved.

According to the above concepts, this research project is composed of the following activities:

- Oenological microbiology: production, validation and transfer of new and efficient protocols for autochthonous microorganisms (yeasts and lactic bacteria) selection as specific fermentation starter. The selection procedure will take in account the capability of microorganisms to give high quality and peculiarity to regional wines and to reduce undesired compounds (ochratoxin, amines etc.) in wine.
- Molecular, biochemical and health traceability of regional wines: development of innovative systems for molecular traceability of the most relevant Apulian grape cultivars, identification and quantification of the antioxidant compounds typically associated to produced wines and their health fingerprint.
- Sensor-assisted analysis of regional wines: development of a novel application system of microsensors for regional wines qualitative and comparative analysis and identification of volatile compounds to be used as quality markers during the different stages of wine production chain.
- Toxigenic fungi detection and OTA decontamination: optimization of innovative molecular diagnosis systems for the early detection and quantification of ochratoxigenic fungi in grape, developments in applying novel methods for microbiological and biotechnological OTA detoxification of Apulian wines.
- Collection and maintenance of autochthonous microorganisms: yeast and bacteria strains will be deposited in the ITEM collection (http://www.ispa.cnr.it/Collection/); preparation of a database by collecting and arranging the molecular and biochemical characteristics of the deposited strains.

Does *Saccharomyces cerevisiae* glutathione pathway affect the production of H_2S?

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Aging wine in the presence of active yeast lees, termed biological aging, has been shown to have positive effects on wine quality. However, one problem associated with this practice is the development of sulfur-containing volatile compounds that have offensive odours, like hydrogen sulphide imparting a rotten egg-like aroma in wine. Origin of S-compounds during aging could be due to the degradation activity of yeasts on sulfur containing amino acids, methionine and cysteine, which could occur during yeast autolysis. Alternatively, biochemical degradation of tripeptide glutathione (GSH), which contains cysteine, could also be a possible source of sulfur taints. The aim of this work was to create *Saccharomyces cerevisiae* mutants, defective in GSH degradation, to be used in synthetic juice fermentation, and to evaluate if the production of H_2S was affected by GSH accumulation.
S. cerevisiae mutants were created from the commercial strain *S. cerevisiae* 950 deleted in the gene ECM38, encoding for the enzyme γ-glutamyl transpeptidase (γ-GT), from γ-glutamyl pathway, and in the gene DUG1, encoding a protein belonging to the metallohydrolase family, from a novel GSH pathway. In addition, *S. cerevisiae* mutants, defective of both genes ECM38 and DUG1, were also obtained. Fermentation dynamics of *S. cerevisiae* mutants were tested in synthetic juice, at 25°C, for seven days, in triplicate, and H_2S production was followed by using H_2S detecting tubes. Deletion in DUG1 and ECM38 genes did not affect fermentation efficiency of mutants which acted like *S. cerevisiae* wild type commercial strain. Concerning H_2S production, ecm38Δ *S. cerevisiae* mutants showed a lower production compared to the wild type. On the contrary, dug1Δ and dug1Δecm38Δ *S. cerevisiae* mutants showed a higher H_2S production.

Physiological study of *Brettanomyces* strains in wines from the Rhône Valley

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After two runs of ecology study in Rhône Valley, *Brettanomyces* has been confirmed as one of the main risks of alteration. The aim of this study, based on an experimental design using statistical tools, was to better understand the physiology of *Brettanomyces*. This experimental design was performed in order to study, with a minimum of analyses, the effect of a large number of wine parameters on the growth of *Brettanomyces* (Conterno et al., 2006). These parameters were chosen based on practical and technical observations recognized as influencing the development of yeasts. A wine with modified analytical parameters (alcoholic content, pH, temperature, sugars, tannins, free sulphur dioxide) was inoculated with two strains (Du Toit et al., 2005; Uscanga et al., 2000).
Results can be separated into two distinct parts: the impact of enological parameters on the growth of *Brettanomyces* and the impact of these same parameters on the production of volatile phenols (*i.e.* impact on the microorganisms metabolism).
Growth and volatile phenols production were influenced by different parameters. Although growth was strongly influenced by the temperature and the alcoholic content, the production of volatile phenols was more impacted by the level of free sulphur dioxide (Barata et al., 2008) combined with a high temperature. In spite of the limited number of strains, we also showed that they had a different potential of volatile phenols production.
This study allowed us to create a model to predict *Brettanomyces* behavior in wines. This template can be used by enologists to manage the potential risks in wines.

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Using molecular biology and proteomic approaches to discover the enzymes responsible for production of ethyl-phenols in *Brettanomyces bruxellensis*

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Yeasts of the genera *Brettanomyces* and *Dekkera* (its sexual state) have long been recognized as contaminants of industrial alcoholic fermentations, during production and storage of fermented beverages. Particularly *Brettanomyces bruxellensis* is considered to be the main cause of wine spoilage, especially of premium red wines matured in oak casks, often leading to serious economic losses.
The principal spoiler compounds associated with *Brettanomyces* spp. are reported to be two volatile phenols, 4-ethylphenol and 4-ethylguaiacol, isovaleric acid (3-methylbutyric), certain tetrahydropyridines and acetic acid.
Volatile phenols, associated with the activity of the *Brettanomyces* yeast genus, are produced from the transformation of the hydroxycinnamic acids, p-coumaric and ferulic acids, naturally present in grapes and must. The mechanism of conversion involves a sequence of two enzymatic reactions. In the first, the enzyme phenolic acids decarboxylase (PAD) decarboxylates the hydroxycinnamic acids into the corresponding vinyl derivative (4-vinylphenol from p-coumaric acid or 4-vinylguaiacol from ferulic acid) and in the second reaction, a vinyl phenol reductase (VPR) converts the vinyl phenols into the corresponding ethyl compound (Edlin et al., 1995). The nucleotide sequences of the genes encoding these two enzymes are still unknown.
In this work we have applied a molecular biology approach, as well as a proteomic approach, in order to discover the nucleotide and the aminoacid sequences of the enzymes. By multi alignment of the nucleotide sequences present in Gene bank from other bacteria and yeasts species degenerate primers for the PAD and VPR genes were designed. PCR with the degenerate primers led to the amplification of DNA fragments corresponding to the genes most likely encoding the two enzymes. Furthermore, with the proteomic approach we have used two different conditions of growth (with and without the precursor molecules) in order to identify possible differences in the production of the two key enzymes.

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Intron splice site PCR analysis to differentiate *Dekkera bruxellensis* at strains level

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D. bruxellensis yeast can develop off-flavours in wine. In particular volatile phenols are often produced in amounts higher than the perception threshold with a loss in product quality. Recent observations suggest that "brett spoilage" is strictly strain-dependent and, therefore, a rapid and reliable identification at strain level of *D. bruxellensis* becomes a key point for an efficient prevention (Vigentini et al., 2008). Among the techniques used to analyse DNA regions with high rate of sequence evolution, introns splice site amplification (ISS-PCR) has allowed to detect polymorphisms in commercial yeast strains of *S. cerevisiae* (De Barros et al-, 1996). Recently, the genome of a wine strain of *D. bruxellensis* has been sequenced and the results have shown that about 2% of the *D. bruxellensis* genes contain introns, a value similar to that found in other hemiascomycetes (1% in *D. hansenii*; 4% in *S. cerevisiae*). Moreover, the *D. bruxellensis* introns have 5', 3', and branch site motifs that are very similar to the consensus motif in *S. cerevisiae* (Woolfit et al., 2007). It was reported that the use of 5' intron-exon spice site as target for ISS-PCR in *D. bruxellensis* did not allow the discrimination at strain level (Oelofse et al., 2009), but an optimization of primers that are complementary to the ISSs of this yeast could permit the development of a consistent tool for the typing of the species.
In the present study, 17 *D. bruxellensis* strains belonging to the international CBS collection has been investigated for the ISSs employing specific oligonucleotides. *D. bruxellensis* contains two 5' consensus sequences: GTATGT (like *S. cerevisiae*) and GTAAGT (De Barros et al., 1996; Woolfit et al., 2007). Preliminary results have shown that most yeast collection was discriminated at strain level by the use of different combinations of primers (up to 80-90%). Therefore, to simplify the approach a multiplex PCR that generated stable genetic profiles was set up.

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Exploring *Aspergillus carbonarius* genome: mycotoxin biosynthetic gene clusters and *pks* genes.

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Recently, a draft genome sequence for *Aspergillus carbonarius* strain ITEM 5010 was generated in collaboration with the US Department of Energy Joint Genome Institute (JGI) and the assembly is now open to the public (http://genome.jgi-psf.org/Aspca1), while the automatic annotation is in progress. *Aspergillus carbonarius*, the main agent of ochratoxin A (OTA) contamination of wine, is closely related to *Aspergillus niger*, an important model organism as well as a fermentation organism used for the production of industrial enzymes and organic acids, and lately sequenced (Baker, 2006). A preliminary analysis based on similarity to the putative OTA cluster of *A. niger* strain CBS513.88 has led to the identification of a putative OTA biosynthetic cluster also in *A. carbonarius* genome. Differently, the putative biosynthetic cluster for fumonisin found in *A. niger*, recently shown to produce fumonisin B_2 (Frisvad et al., 2007), is lacking in *A. carbonarius*. Moreover, on the basis of similarity to the most highly conserved ketosynthase (KS) domain, about 25 *pks* genes have been identified by using the BLAST tool available at *A. carbonarius* genome portal. Fungal polyketide synthases (PKS) are key enzymes required for the biosynthesis of several mycotoxins and other secondary metabolites. We have initiated a phylogenetic analysis of the *pks* genes of *A. carbonarius* in comparison to the *pks* genes identified in *A. niger* and other fungal *pks* involved in the production of important mycotoxins. First results confirm the high diversity of PKS enzymes due to the high diversity of roles they may have in the fungal metabolism.

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Gene expression in *Oenococcus oeni* under stress conditions

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Oenococcus (O.) oeni is the lactic acid bacteria (LAB) species most resistant to the hostile environment of wine in which various physical-chemical factors affect the LAB growth responsible for the malolactic fermentation (MLF). Although several studies (Carreté et al., 2002; da Silveira et al., 2003) analyzed few mechanisms that enable *O. oeni* to withstand stress conditions, more information about the mechanisms involved in the adaptation of *O. oeni* to stress conditions is required, particularly under winemaking conditions. A better knowledge of stress physiology may be useful to optimize survival of starter cultures of *O. oeni*. Recently, the Fluorescent Differential Display (FDD) technique was carried out to observe the differential expression of genome in *O. oeni* strains, previously isolated from Aglianico wines (Basilicata region, Southern Italy), investigated for molecular identification and characterized on the basis of technological features in our laboratory (Sico et al., 2009).
In this study the FDD products were selected, isolated and then sequenced. In particular, in S12 *O. oeni* strain grown in presence of ethanol 12%, DNA sequence information allowed to identify a transcript, 279 bp long, with significant similarity to the geranylgeranyl pyrophosphate synthase (GGPPs) (NCBI no. ABJ56986) which was originally found to be expressed in *O. oeni* PSU-1 complete genome (NCBI no. CP000411). GGPPs is an enzyme that belongs to the family of E-prenyl diphosphate (prenylPP) synthases and catalyses the condensation of isopentenyl diphosphate (IPP) with its allylic isomer, dimethylallyl diphosphate (DMAPP), to produce geranylgeranyl pyrophosphate (GGPP), an essential isoprenoid involved in several biosynthetic pathways such as the biosynthesis of terpenoids and carotenoids, but also the synthesis of quinones and chlorophylls and the prenylation of proteins (Velayos et al., 2003). Eubacteria, like plants, have the type II synthase: in particular, in *O. oeni* this enzyme is implicated in the biosynthesis of secondary metabolites, mainly in terpenoids and steroids biosynthesis. The presence of GGPPs in secondary metabolism processes of *O. oeni* was confirmed by specific primers design and the following PCR amplification, revealing the presence of GGPPs gene in S12 *O. oeni* strain under both stress and optimal conditions. However, the expression level of gene was studied and probed by quantitative analysis.

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Characterization of the volatile fraction of Aglianico and Fiano wines obtained with an autochthonous non-*Saccharomyces* yeast

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Wine fermentation is a complex microbiological reaction involving the sequential development of various yeast strains that contribute to the flavour of wines. For many years, wines have been produced by natural fermentation carried out by yeasts that originate from both the grapes and the cellar. Several oxidative and apiculate yeasts (non-*Saccharomyces*) are predominant on the surface of grapes, but after 3–4 days of fermentation, *Saccharomyces cerevisiae* "sensu stricto" predominates, playing the major role in alcoholic fermentation (Jackson, 1994; Fleet, 2003).
Nevertheless the non-*Saccharomyces* yeasts have a prominent role in the fermentation dynamics, compositions and flavour of wine. The aromatic compounds responsible for varietal aroma in wine are mainly terpenes (others are norisoprenoids, C6 alcohols, aromatic compounds), which are metabolites derived from mevalonic acid and are characterized by multiples of branched, five-carbon units resembling isoprene. The most important group, from an oenological point of view, are the monoterpenes (10-carbons compounds) because of their volatility and odour if present in a free form. The glycosidically-bound forms can be converted into the free odours compounds by hydrolysis with glycosidases produced by yeasts (Palmieri et al., 2007).
From a screening of non-*Saccharomyces* yeasts in grapes and must typical of South Italy (Irpinia), in particular *Aglianico* and *Fiano* cultivars, we isolated a yeast strain (identified as a *Rhodotorula spp.*) showing an high extra-cellular glycosidase activity. The isolated yeast has been utilized for an experimental winemaking process to produce *Aglianico* and *Fiano* wines.
The HS–SPME technique utilized allowed the detection of trace aroma compounds permitting to pick up the molecules involved in the characterization of qualitative aroma profile of the wines under study.
Moreover, by Panel Test analysis, the *Fiano* wine was considered characterized by a marked acidic taste and an intense, aromatic and pleasant flavour, rich of grapefruit and fruity notes, while the *Aglianico* was considered an astringent and acidic young wine, characterized by an intense flavour rich in cherry, viola and berries notes.
These properties enhanced the flavour and taste of both wines when compared with the homologues produced with conventional starter cultures.

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P-3-11

A fast and reliable preparation method of template DNA from must and wine suitable for PCR analysis in order to differentiate grape (*Vitis vinifera* L.) cultivars

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The identification of *Vitis vinifera* L. cultivars directly from wine is an important issue because wines is also identified, in several countries, by the variety name. Characteristics such as production yield, alcoholic level, acidity and anthocyanin levels vary among grape varieties and are usually highly correlated with the final wine quality. Particularly relevant is the control of the quality and authenticity of monovarietal grapewine.
Molecular markers, such as random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), microsatellite and amplified fragment length polymorphism (AFLP) are used on *Vitis vinifera* autochthonous, in several studies for to discriminate among grape cultivars. The main difficulty for the traceability molecular is the extraction of a pure and DNA from complex wine matrixes. During the fermentation processes, the temperature and pH are the main factors that influence the stability of DNA. The primary structure of the DNA is deteriorates with the chemical reactions of hydrolysis and oxidation that cause the rupture of the DNA filament. Another important factor is the pH value. Acidic foods, such the wine and all fermentation food products present high degradation of the DNA as a consequence of acid catalysis. This reaction leads to the rupture of the filament and the breakage of the DNA. Furthermore, co-existence of PCR inhibitors, such as polyphenols might be a limiting in step in DNA analysis.
In this study, we developed a fast and simple protocol for DNA extraction from must and wine. Protocol has been validate on several wines made from typical monovarietal grapes commonly grown in Puglia (Italy) and it has been observed to be suitable for qualitative and quantitative PCR analysis. Moreover, purified DNA were also used in PCR experiments for wine traceability.

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Description of new species of acetic and lactic acid bacteria from winemaking process

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Wine final quality is the consequence of the effects produced by particular microorganism successions during the fermentation. Apart from different fungi and yeast species, we can find lactic acid bacteria (LAB) and acetic acid bacteria (AAB) species during winemaking process.
The role of LAB in winemaking could be beneficial, as they could perform malolactic fermentation which decreases excessive acidity of wine, but also detrimental as they can cause several alterations: production of acetic acid, off-flavours, ropiness and biogenic amines. Many of those effects are species specific, so the correct identification of wine microorganisms is very important for wine quality.
AAB are found in wine when production and storage conditions are not correctly controlled, reducing considerably its quality. AAB oxidize wine sugars and alcohols resulting in an accumulation of several detrimental concentrations of acetic acid, acetaldehyde and ethyl acetate or gluconic acid. For these reasons, AAB presence in wine must to be avoided and its fast detection and correct identification is greatly recommended.
Several studies have been done in recent years in order to know the bacterial diversity associated to winemaking, and as a consequence, some new species have been described lately. The description of a new species is a very important goal for taxonomy but it is also important for improving the knowledge of wine bacterial diversity and their possible effects in wine. The main goal of our work was the identification of LAB and AAB present in different stages of the winemaking process by a polyphasic approach.
As a result from this study, *Lactobacillus harbinensis, Lactobacillus pantheris, Lactobacillus satsumensis*, *Acetobacter ghanensis, Acetobacter syzygii* and *Kozakia baliensis* are reported in wine for the first time. We have also described the presence of three novel species from wine, for which the names *Lactobacillus bobalius* (Mañes-Lázaro et al., 2008a), *Lactobacillus uvarum* (Mañes-Lázaro et al., 2008b) and *Lactobacillus oeni* (Mañes-Lázaro et al., 2009) have been chosen.
It is expected than more new species associated to winemaking could be detected from extensive characterization studies.

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Session IV
Methods for detection of micro-organisms affecting wine safety and quality

Poster presentations

Aroma analysis by GC/MS and electronic nose dedicated to *Negroamaro* and *Primitivo* typical Apulian wines.

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The concept of wine quality and safety is closely linked to its aroma. For this reason Oenology is involved in the study of wine aroma characteristics and composition. At present, inside Apulia wine industry, it's strongly felt the need to develop innovative methodologies able to provide a characterization of Apulian typical wines based on the composition of their aroma.
In the present work, an innovative analytical technique as an Electronic Nose (e-nose), together with a standard chromatographic method as gas chromatography-mass spectrometry (GC/MS), were used to analyse the volatile fraction of Apulian wines of different denominations of origin. In particular, two of the most utilized Apulian grape varieties in wine-making, i.e. *Primitivo* and *Negroamaro*, were considered and some *Negroamaro* and *Primitiv*o based monocultivar wines were used. Such work aims, on one side, to start a systematic characterization of Apulian monocultivar wines by building a registry of "aroma identity cards" with the complete wine odour profile by a GC/MS analysis, and on the other side, to calibrate an Electronic Nose to the recognition of some chemical compounds correlated to sensorial descriptors and considered negative markers of quality if exceeding a concentration threshold. This will allow to identify the volatile compounds of the considered monocultivar red young wines (vintages 2008) with major influence on wine aroma within their perception threshold values.
As regards GC/MS analysis a specific Solid Phase Extraction method previously described was followed (Lopez et al., 2002). The identification and quantification of the volatile compounds was carried out with a GC-MS AGILENT. A DB-WAX capillary column (60 m· 0.25 mm i.d. and 0.25 μm film thickness) was used. As regards e-nose, an array of micromachined gas sensors based on metal oxides and a sampling method based on a dynamic stripping of the headspace of wine samples by an air flow, were used (Lozano et al., 2007). The sensor array was exposed to the headspace of different wine samples added with increasing concentrations of markers (as acetic acid, hydrogen sulphide, 4-ethylfenol, 4-vinylguaiacol, isoamyl alcohols, etc.). By the application of suitable data analysis techniques, the system was trained to analyse wine quality by identifying a quality wine area in a multi-dimensional space of quality descriptors according to a classification criteria.

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Proteome analysis of *Oenococcus oeni* ATCC BAA-1163

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Oenococcus oeni is the most important Lactic Acid Bacteria (LAB) in the wine industry, because its predominant role for the deacidification of the wine, a process termed malolactic fermentation (MFL), that usually follows the alcoholic fermentation (AF). Therefore, it is of industrial interest the knowledge of proteins involved in metabolic pathways and transport systems of this bacteria. To this end, in silico predictions inferred from the genome sequence could be complemented by proteomic data. In this work, we report on the characterization of the proteome of *O. oeni* ATCC BAA-1163. This strain was chosen, because it has been completely sequenced, and thus it is possible to tentatively identify its encoded proteins. Protein preparations of subcellular fractions from LAB have been standardized. The *O. oeni* ATCC BAA-1163 membrane and cytosolic protein preparations have been subjected to two-dimensional gel fractionation, by use of a no linear range of pH 3.0-11.0 during electrofocusing. A comparative analysis of both proteomes was performed with the aim to establish whether the identified proteins are present in both or only one. Protein spots of interest were excised from the gel and digested with trypsin for further analysis by MALDI-TOF spectroscopy. In the course of the study we have identified 115 different polypeptides, which have been classified by their putative function and subjected to bioinformatics analysis for prediction of subcellular location.

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Screening of biogenic amines production by commercial yeast starter cultures

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Biogenic amines (BA) are undesirable toxic compounds that can be found especially in foods and beverages from fermentative processes. Although the levels of BA in wine are rather low in comparison to other foods, the presence of BA in wine is particularly hazardous because ethanol and acetaldehyde can enhance their toxic effects on human metabolism by inhibiting the amine-oxidases responsible for their catabolism (Rosi et al, 2009). The main factors affecting the formation of BA during vinification are concentrations of BA-precursor amino acids in musts and the presence of microorganisms able to decarboxylate these amino acids. Generally, it was observed that higher amounts of amino acids in must produce higher amounts of BA after malolactic fermentation (Ancìn-Azpilicueta et al, 2008). However, the role of *Saccharomyces* starter cultures in amine biogenesis has been rarely studied, even if a few reports have correlated the BA formation to the specific yeast strain that predominates during alcoholic fermentation (Ancìn-Azpilicueta et al, 2008). The aim of this study was to evaluate the potential of 38 commercial yeast starter cultures to produce the BA histamine, tyramine, phenylethylamine and putrescine in wine. For this purpose, pure active dry wine yeasts, *Saccharomyces cerevisiae* and *S. bayanus*, were inoculated in Chardonnay white must supplemented with tyrosine or histidine; a third sample was also used without precursors. Fermentations took place in controlled, laboratory-scale conditions and the produced wines were evaluated for the presence of BA by means of reverse-phase high performance liquid chromatography using postcolumn derivatization with o-phthalaldehyde and fluorescence detection (Beljaars, 1998). Although a certain variability was observed among the cultures, in general all the yeasts tested produced very low or non-detectable amounts of the target BA at the end of the fermentation process. The maximum level of total amines was 5.3 mg/L, and putrescine was the most prevalent BA produced. The enrichment of must by the precursor amino acids did not produce an increase of BA during alcoholic fermentation. On the basis of these results, the commercial yeast starters analyzed show low aminogenic capability, thus indicating that these microorganisms are not responsible for the production of the most hazardous BA in wine.

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Different regulation of the tyrosine decarboxylase and the agmatine deiminase genes in *Lactobacillus brevis* IOB 9809

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The fermented beverages are complex systems with a wide number of factors influencing the metabolic activities of micro-organisms. A large host of factors has been observed to affect the wine bacterial metabolism, including, temperature, pH, alcohol content, organic acid and sugar concentration and the time of bacterial survival. The aim of this work was to establish the influence of different wine factors on the regulation of tyrosine decarboxylase and agmatine deiminase genes in *Lactobacillus brevis* using a quantitative reverse transcription polymerase chain reaction (RT-qPCR) approach. The expression of the tyramine decarboxylase (*tdc*) and agmatine deiminase (*agd*) genes, analysed over a complete culture cycle, revealed that early growing cells contain substantial amounts of *tdc* mRNA, which rapidly decline at the entry into stationary phase. In contrast, *agd* mRNA was detectable either during log phase or in early and late exponential phase. Furthermore, different stress conditions such as cold shock (18 °C), acid shock (pH 3) ethanolic shock (12% v/v) and sulphite stress (15 g/hl) were imposed and genes expression analysed after 3 min, 5 min and 10 min. Moreover, the expression of *tdc* and *agd* genes was also valuated by imposing multiple stress conditions such as ethanol and cold stress, ethanol and sulphite stress, ethanol and pH stress, cold and ethanol stress, cold and pH stress. The results reported in this work suggest that, in *L. brevis*, tyrosine decarboxylase and agmatine deiminase genes are differently expressed during cells growing and respond differentially to abiotic stress commonly encountered in wine.

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BiAMFOOD: controlling biogenic amines in traditional food fermentations in European regions

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Biogenic amines are naturally occurring low molecular weight compounds in humans. They are involved in natural biological processes such as synaptic transmission, blood pressure control, allergic response and cellular growth control. On the other hand, biogenic amines may cause human health problems such as headache, palpitations, flushing or vomiting when high concentrations are present, a problem usually caused by the ingestion of fermented foods containing high concentrations of these compounds. High levels of BA in foods may be the consequence of spoilage, but also be inherent to the food production process. Lactic Acid Bacteria (LAB) are the main source of biogenic amines in foods. In bacteria, biogenic amines are the end products of amino acids catabolic pathways. LAB grow on meat, vegetables and fermented foods, which are rich in amino acids that are converted into biogenic amines to produce metabolic energy for the microorganism and to provide resistance against acid stress. The BiAMFOOD project, funded under the EU 7th Framework, focuses on microorganisms in the food chain that produce BAs with the main goal to improve the quality of traditional fermented food by reducing their BAs content. The project focuses on three different fermentation processes in four different regions of Europe and the whole food chain is considered. At the beginning of the food chain, the potential of microorganisms to form BA is analyzed; during the fermentation process the physiological conditions that result in BA formation are controlled and at the end of the chain the survival of microorganisms producing BA in the digestive tract are analyzed and their effect on the consumer considered.

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Viability of the use of commercial enzymatic preparations on the vinification of Bordô grape

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The transfer of phenolic compounds from grapes to the must is influenced by the technological process used in vinification. The application of appropriate pectinases may cause the hydrolysis of polysaccharides from the cell wall, favoring the release of these compounds in the must and improving visual characteristics (color and lightness) of wines. This study evaluated the use of commercial enzymatic preparations Pectinex XXL and Novozym 3309 (Novozyme) in Bordô grapes during artisanal wine making in São Paulo State, Brazil. Hundred g of fruit were bleached using saturated vapor and softly crushed, receiving the addition of 5 mL of enzymatic preparations, which were adjusted to the same total protein content (0.15 mg). Samples were incubated at 37 °C under agitation (100 rpm), at must's natural pH (3.3), for 120 minutes and, afterwards, pressed to obtain the must. Two controls were carried out, one sample in natural and one bleached, both with addition of 5 mL of water. Experiments were carried out in duplicate. Samples were evaluated regarding color parameters, (color indices, T coloration, composition of red color intensity) and methanol content. The results showed that samples enzymatically treated presented increase in red color without differences among them. This suggests that the application of enzymatic preparations contributed to the release of anthocyanins from the grape's peel. The must treated with Pectinex XXL presented significantly lower methanol content (146.00 ± 3.54 mg/mL) than the ones found in must treated with Novozym 3309 (343.50 ± 7.07 mg/mL) and did not exceed the maximum limit allowed by the brazilian legislation (350 mgL^{-1}). These results encourage the use of Pectinex XXL during pre-maceration of Bordô grapes for the development of better quality wines without causing damages to human health as established by the law.

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Development and validation of a sensitive and reliable real-time PCR direct assay in order to quantify *Brettanomyces* in wine: survey in wines of the Rhône Valley.

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The yeast Brettanomyces is responsible for the production of spoiled wines because it results mainly in volatile phenols production, but also in volatile acidity. The non controlled accumulation of such molecules (mainly 4-ethylphenol and 4-ethylguaiacol) in wines leads to sensory defects (horse stable, ink, band aids, animal…) which compromise the wine quality. The need for a quick method, specific, sensitive and reliable in order to detect this spoilage yeast increased during the last decade. Real time PCR fits with all of these criteria. We propose here some improvements of existing methods (Phister and Mills, 2003; Delaherche et al., 2004) in order to increase the robustness of the analysis. Six different protocols were evaluated to perform DNA extraction from wine and three different PCR mix were examined. The best consensus method which relies on the use of PVPP to remove PCR inhibitors was adopted. In addition, an internal control (the yeast *Yarrovia lipolytica*, a microorganism that is not naturally present in wine) was developed in order to avoid false negative results due to failure in isolation and/or amplification of DNA. Among the validation criteria, the specificity, the linearity, the repeatability and the reproducibility of the method were evaluated. The limits of detection and quantification were determined as 5 and 31 CFU/ml respectively. This method could thus be applied for the quick and reliable enumeration of *Brettanomyces* during early steps of winemaking, but also just before bottling. Some routine analysis using qPCR were performed and also compared to the culture method (Petri dish containing cycloheximid/actidion), results about specificity and viable but non cultivable physiological status (VBNC) are also discussed.

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Evaluation risk of biogenic amine content

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Our purpose was to develop a method which can help the winemaker to better control its biogenic amine production with a rationalized approach of selected starter strain using.
An obligation of the wine industry is to control critical technological factors to produce wines with low levels of biogenic amines (BA). The most regulated, histamine, is present due to the histidine decarboxylase activity of lactic acid bacteria (LAB) during malolactic fermentation. Not only does the production of BA in wine depend on the presence of biogenic amine producing bacteria, but also it depends on other parameters of wine such as amino acid precursors content, pH or MLF duration (Martin-Alvarez, Marcobal et al., 2006). Then, Lonvaud-Funel *et al.* (1994) noted that histamine production by *Oenoccoccus oeni* bacteria depends particularly on histidine concentration in the medium.
The development of a specific PCR method targeted on genes of the histidine decarboxylase pathways of LAB allowed to enumerate bacteria producing histamine in wine (Lucas, Claisse et al., 2008). In parallel, a new practical method for simultaneous analysis of BA and amino acids was recently developed. It consists of reversed phase separation by HPLC and UV-vis detection of the aminoenones formed by the reaction of amino acids and BA with the derivatization reagent diethyl ethoxymethylenemalonate (DEEMM) (Gomez-Alonso, Hermosin-Gutierrez et al., 2007).
In order to prevent biogenic production during winemaking, PCR tests coupled with precursor detection could be performed at the end of alcoholic fermentation and give information about the BA and precursor content along with the presence of histidine producing bacteria. First approach was realized with lab experiments to determine risk zones. The second step will consist in testing the method with real cases.

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Biogenic amine content of Rhône valley wines: change in amine concentration during sample conservation

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The present paper reports the development of a new method for simultaneous analysis of 8 biogenic amines (Histamine, Methylamine, Ethylamine, Tyramine, Putrescine, Cadaverine, Phenethylamine, and Isoamylamine). It is based on a method developed by Gomez-Alonso et al. (2007). The proposed analytical method has the following advantages: easy derivatization of wines, quantification of biogenic amines, and complete degradation of excess derivatization reagent during sample preparation to preserve column. It consists of reversed phase separation by HPLC and UV-vis detection of the aminoenones formed by the reaction of amino acids, biogenic amines, and ammonium ion with the derivatization reagent diethyl ethoxymethylenemalonate (DEEMM).
The technique was confirmed with an alternative oenological analysis method for the validation, quality control and uncertainty assessment (OIV Oeno 10/2005). The reliability of the method was satisfactory in terms of linearity (from 0.5 to 50 $mg.L^{-1}$), precision (relative standard deviation below 5%), recovery (from 99.7 to 101.1%), and sensitivity (detection limit below 0.1 $mg.L^{-1}$).
As a specific application of the proposed method, the biogenic amine content of Rhône valley wines was investigated. A total of 100 red wines obtained from wineries in the region of Rhône valley (France) were evaluated in terms of 8 different biogenic amines. Moreover, according to results which found a variation in the concentration of biogenic amine during storage time (Vidal-Carou et al., 1991, Jimenez Moreno et al., 2003), a same sample was analyzed at the time of preparation, after 1, 2, and 4 days and after 1, 2, and 4 weeks. Different storage temperatures were studied. To determine the stability of the derivatives, the same experiment was done with and without derivatization. As can be seen, the compounds produced by the derivatization reaction were perfectly stable, instead of wine conserved which showed a variation in the concentration of amines, particularly histamine, over time, by way of example due to a probable degradation. The results indicated that storage time was an important factor affecting biogenic amine content.

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Full value tracing of wine chain

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There is a growing interest in both academics and industrial communities to know, manage and analyses data, information and knowledge about related to biodiversity and its applications. With this in mind a group of private and public organizations in Italy started a strategic and large initiative, known as Molecular Biodiversity Laboratory (MBLab – www.mblabproject.it) and received the support of the Italian Minister of Research. MBLab aims to build novel bioinformatic industrial systems that leverage biodiversity information and knowledge, both applied to human health – in order to monitor safety and risks - and to agro-industrial, to trace products along food production and supply chains. Nowadays, the improvement of food quality and safety has become an enthralling challenge for industry and research. Recent escalation of food and product contaminations and recalls originating from both industrialized and emerging countries as well as confusion over marketing claims has eroded trust in Food Consumer Product manufacturers. A new generation of food traceability systems are expected which are able to collect and communicate transparent and trustworthy information about a product from farm to fork and ensure food and product safety and reliability. Industry need traceability systems with much more the breadth, depth and precision of ones current adopted to fit the strategic and operational needs of their food processing steps, thus assuring and promoting the genuineness and authenticity of their product. In this context consider the full spectrum of information along a food production and supply chain is fundamental. We need to trace and combine several information layers ranging from traditional business, logistic but also biological environmental. Biological traceability refers to the identification, characterization and recording of all the biological information existing along a food supply chain (grape cultivars, toxigenic/pathogenic fungi, virus, yeasts, lactic bacteria etc.) and the discovery of a molecular fingerprint that can be used as a biological signature to trace a food product, from the very beginning of its production to the last marketing step. The aim of the talk is to present Molecular Biodiversity Laboratory (MBLab) research initiative and the preliminary results and approach of one of its task that aims to develop a Full Value Tracing of Wine chain that leverage molecular biodiversity data collected from wine ecosystem.

Characterization of oenologic yeast-cell wall fractions by reducing cysteine content

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Thiols can perform several activities in must and wine. The antioxidant activity of glutathione is well known: it can bind with the o-quinones so preventing browning of must and wine (Salgues et al., 1986). Glutathione can also reduce the loss of thiol aromas and it slows down the atypical aging of white wines (Dubourdieu and La Vigne-Cruège, 2004). Similar behaviour could be exerted by the reduced cysteine of yeast cell-wall and it can also bind molecules responsible of reduced odours.
The main yeast cell-wall fractions are represented by hulls, lysates and mannoproteins and they can be used in winemaking in order to improve wine properties such as prevent protein haze, improve foaming properties, affect mouth feel and protect wine from oxidations while barrel aging. The oenological properties of mannoproteins can be influenced by yeast strains, yeast growth conditions, drying process and extraction method since strong heat treatments can reduce the content of cysteinyl residues.
An analytical method based on the reaction between thiols and p-benzoquinone was developed for the evaluation of the reduced cysteine content. Different commercial samples of yeast cell-wall fractions and active dried yeasts were characterized by the content of reduced cysteine (free and protein bound) and glutathione. Moreover, the intensity of the Maillard reaction was determined in order to better understand the effect of the different industrial preparations.
Samples of yeast cell-wall fractions showed very heterogeneous contents of reduced cysteine: some of then can behave as antioxidant in must and wine, while some samples can decrease the free thiol content in wine since they can bind such molecules so depleting wine from its varietal flavours or removing mercaptans responsible for the reduced odours. The active dried yeast samples were characterized by glutathione and protein cysteine contents in the range from 0.39 to 0.92 mmol/100 g of product and from 0.76 to 1.28 mmol/100 g of product, respectively.
The production of yeast cell-wall fractions having higher reduced protein cysteine contents and lower heat damage should be pursued.

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OTA adsorption kinetic by *Saccharomyces cerevisiae* strains during fermentation of must obtained from dry grapes "Moscato of Saracena", a southern Italy passito-wine (Calabria Region)

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Ochratoxin A (OTA) is the most abundant and the most toxic among ochratoxins. The International Agency for Research on Cancer has classified the ochratoxin A as a possible human carcinogen (category 2B).
The occurrence of OTA in wine samples has been reported in various studies predominantly dealing with European wines but also with wines of other regions. Generally, passito and red wines seem to contain a higher amount of OTA than white or rosé wines, and some results suggest that, at least for European and North African cultivation areas, southern regions are more affected by the contamination problem.
Different researchers evaluated the potential of oenological *Saccharomyces* (*S.*) and non-*Saccharomyces* yeast strains in reducing OTA contents present in synthetic and natural grape juice, white must and red must during fermentation.
The aim of the present work was to investigate the performance of 16 *S. cerevisiae* strains, previously isolated from Moscato of Saracena passito-wine samples, to detoxify OTA during the alcoholic fermentation of must obtained from dry grapes (passito). Fermentations were performed in 250 mL Erlenmeyer flasks containing 100 mL of Moscato of Saracena must (pH = 3.30, 38 °Brix, provided by local producer) artificially contaminated with 10 ppb of OTA. The flasks were closed with a Müller valve, previously filled with sulfuric acid. The fermentations were carried out at 16-18 °C in triplicate. Carbon dioxide production (fermentation activity) was measured by monitoring weight loss during fermentation. Residual concentration of OTA, the toxin concentration adsorbed by yeasts on the cells wall, and the OTA amount internalized in the cells during fermentation were evaluated by HPLC analysis of samples collected at 0, 7, 14 and 21 days of fermentation.
Three different behaviours were recorded by analyzing residual concentration of OTA in the musts and wines. In all cases the major part of OTA disappear during the first (medium decrease 33%, range 24-45%) or the second (medium decrease 38%, range 31-47%) week of fermentation thus corresponding to the yeast maximum rate of growth. During the following week the OTA content of wine can remain unchanged, slightly decrease or increase depending on strain (medium decrease 35%, range 27-44 %). After 21 days of fermentation a medium of 1.25 ppb (range 0.78-1.96 ppb) of OTA is adsorbed by yeast cell wall and 1.40 ppb (range 0.50-2.28 ppb) is internalized in the cell. The different response showed by strain analysed during this study suggests that opportune selected starter cultures can be profitably used for the reduction of OTA in wines. In fact during this study strains, named SC183, SC-M16 and SC-M32, showing a significant OTA reduction (more than 40 %) after 21 days of fermentation were individuated.

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Microbiological quality and pesticide residues of bottled wines

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At this time, quantitative and qualitative data about microbiological risks and pesticide residues in traded wines are rare. Proper microbiological wine quality means the presence of yeast and bacteria cells below the concentration prescribed/desired for a certain wine. The limits of acceptable concentrations of individual groups of microorganisms in wine depend on the chemical and microbiological parameters of the wine (i.e. pH value, concentrations of ethanol, free and total SO_2, reducing sugars and malic acid), internal control, and the control of trade chains as well as on the type of transport, storage and selling of wine (Loureiro and Malfeito-Ferreira, 2003). A study of pesticide residues in Italian wines from vineyards with a known history showed that 64 wine samples from six Italian regions contained the residues of five active ingredients at very low concentrations: benomyl (0.05 mg/L), dimethoate (0.02-0.06 mg/L), iprodione (0.02-0.07 mg/L), metalaxyl (0.04-0.14 mg/L) and vinclozolin (0.02 mg/L) (Cabras et al., 1995). In the study we determined the microbiological quality and pesticide residues of bottled wines. The microbiological analysis was performed using membrane filtration. Pesticide residues in wine were analysed using the multiresidual method with GC-MS, the multiresidual method with LC-MS-MS and the method for determination of dithiocarbamate residues with GC-MS. Furthermore, the effect of bentonite and the combined fining agent on the concentration of boscalid in wine was tested. The results of microbiological quality indicate that no less than one quarter of bottled wines on our store shelves is microbiologically unstable. Nine pesticide residues were determined in the samples. More than 50% of wines belonged to the category of wines with only up to two pesticide residues. The most frequently determined pesticide in wines was boscalid (76% of samples) followed by fenhexamid (44%). The highest concentrations of pesticide residues in wines were determined for cyprodinil (0.44 mg/L) and fludioxonil (0.21 mg/L). The combined fining agent was more efficient in lowering the concentration of boscalid in wine if compared with bentonite.

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Production of biogenic amines in Valtellina DOCG red wines

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The presence of biogenic amines (BA) in food and beverages is undesirable because they could cause negative physiological effects on the consumers. The BA detected in wine are more than 20 and the most frequent are putrescine, tyramine and histamine (Lehtonen, 1996). Among the BA, histamine is the most toxic, while putrescine and cadaverine can intensify the negative effects of histamine, tyramine and phenylethilamine (ten Brink et al., 1990). BA are produced mainly by enzymatic decarboxylation of amino acids; this activity can be endogenous, deriving from raw materials, and exogenous, deriving from microorganisms growing during the food processing and/or storage. In wine, BA production was attributed to malolactic bacteria by the observation of negative correlation between L-malic acid concentration and BA content after alcoholic fermentation. In particular, a relationship among the presence of BA, histamine, and bacteria belonging to *O. oeni* species has been found (Coton et al., 1998). The object of this work was to monitor the production of BA during vinification experiments of Valtellina Superiore DOCG red wine carried out in four different cellars. The experiments were done in duplicate and the samples were taken in three steps along wine-making process: at the beginning and at the end of MLF, and during the storage of wine in bottle. Throughout the malolactic fermentation (MLF) different *O. oeni* strains were isolated and typed by PFGE analysis confirming that a genetic variability in the evolution of *O. oeni* populations during MLF can take place (Renouf et al., 2009). In terms of chemical investigation, 12 BA were accumulated: among them putrescine was produced in the highest amount (min. 0.06 mg/L; max. 4.54 mg/L) and histamine was found in all the analysed batches (min. 0.06 mg/L; max. 1.71 mg/L). A significant increasing in the BA production was detected during the wine storage in bottle indicating that a residual decarboxylation activity was still present and/or an amination/transamination of aldehydes and ketones occurred.

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Determination of reduced glutathione content in must and wine

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The antioxidant activity of glutathione in must and wine is well known: it reduces the o-quinones preventing their polymerization, browning of wine and depletion of flavour-related thiols. Glutathione can hinder the formation of sotolone (3-hydroxy-4,5-dimethyl-2(5H)furanone) in wine, a major responsible of the atypical wine aging.
Glutathione content ranges from 56 μmol/kg to 372 μmol/kg in grape (Cheynier et al., 1989), depending on grape cultivar, environmental conditions and agronomic practises. The winemaking procedures before the alcoholic fermentation influence its concentration in must, since they can affect the activity of tyrosinase, the presence of oxygen and the integrity of grape skin.
The amount of glutathione decreases to 10-40 μM (Cassol and Adams, 1995) following winemaking according to the yeast strain and the aging conditions.
Determination of glutathione in grape, must and wine can permit to estimate the antioxidant potential and the shelf-life of wine.
The analytical method described for glutathione quantification is based on the derivatization reaction with p-benzoquinone. The S-glutathionyl-p-hydroquinone is separated by HPLC and detected spectrophotometrically at 303 nm. The detection limit and the lowest quantifiable amount are 0.42 μM and 1.41 μM, respectively. The analytical approach allows the cysteine content to be determined as well.
The method was applied to investigate 8 different winemaking processes performed under different conditions (5 grape cultivars, 7 yeast strains and 2 redox conditions).
The glutathione content ranged from 1.28 μM to 3.66 μM after crushing and racking when must was exposed to air. Higher contents (18.22 μM) could be obtained pressing the grape under controlled atmosphere. Glutathione concentration rapidly increased at the end of alcoholic fermentation and the final levels (0-92.50 μM) were affected by exposure of must to oxygen, yeast strain and nitrogen availability to yeast.
The cysteine was absent in many samples or its concentration did not exceed 13.51 μM.

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Regulation of *pks* genes expression involved in OTA production by *Saccharomyces cerevisiae*

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Aim of this study was to investigate the biocontrol activity of *Saccharomyces cerevisiae* wine strains on *Aspergillus ochraceus* and *Aspergillus carbonarius*, the main fungal species responsible for the accumulation of ochratoxin A (OTA) in grape and wine. Some of the *S. cerevisiae* strains tested were able to significantly reduce OTA content in the culture filtrates when co-cultivated with ochratoxigenic strains in YES medium. A selected strain of *Saccharomyces cerevisiae* (DISAABA 1182) was tested for its effect on OTA production and *pks* (polyketide synthase) gene expression in *A. carbonarius* and *A. ochraceus*. In order to examine a possible correlation between OTA production and the expression of *pks* genes regulated by the yeast antagonistic activity, *A. carbonarius* and *A. ochraceus* were co-cultured with *S. cerevisiae* 1182 and incubated in the presence of *S. cerevisiae* 1182 culture filtrate. *S. cerevisiae* 1182 was able to inhibit fungal growth and OTA production when co-cultured in YES. The transcription of the genes was monitored using a reverse transcription (RT)-PCR based approach, and OTA production was monitored in parallel by HPLC. Analysis of *A. carbonarius* and *A. ochraceus* polyketide synthase gene transcript levels confirmed that *pks* gene transcripts is tightly correlated with OTA production. Moreover, the pks gene was down-regulated in the presence of *S. cerevisiae* 1182 or *S. cerevisiae* 1182 supernatant. This suggests that genes involved in the polyketide biosynthesis are transcriptionally regulated in presence of *S. cerevisiae* 1182.

Biogenic amine production during vinifications on industrial scale

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Biogenic amines (BAs) including histamine, putrescine, cadaverine and tyramine are compounds that can be found in food and beverages mainly as a consequence of microbial decarboxylation of the precursor amino acids and that can cause toxic effects to humans. The presence of BAs in red wines is well documented and several studies support that in winemaking BAs are basically formed during the malolactic fermentation (MLF) by the action of lactic acid bacteria (LAB). However, quantitative data correlating the time courses of BA accumulation and growth of yeasts and LAB during the whole winemaking process, including the ageing stage, are lacking. Therefore, in this work several spontaneous vinifications, carried out in four different cellars in Tuscany, were taken into consideration with the aim to evaluate the BA production and the microbial population dynamics. At the end of the alcoholic fermentation in all cases, the highest amine concentrations were recorded for putrescine, an amine that is known to be accumulated in grapes of vines under stress conditions. With regard to the other BAs, histamine and tyramine were always below their detection limits, while cadaverine and spermidine concentrations ranged from values below the detection limits to values of about 3 mg/L and phenylethylamine and spermine never exceeding 1 mg/L. Hence, these findings confirmed that wine yeasts and alcoholic fermentation are not responsible for a significant presence of BAs in wine. After the alcoholic fermentation spontaneous MLF occurred in all wines and wild populations of *Oenococcus oeni* showed comparable dynamics. Moreover, as concerns the time courses of BA accumulation during MLF a quite similar pattern was observed with histamine, tyramine and putrescine always as the most abundant amines. After completion of MLF the wines entered the ageing stage and LAB population revealed a progressive loss of viability but BA formation continued with regularity. In order to obtain a possible generalization of the BA time course during and after MLF, all data concerning the amounts of each main amine produced during these stages were normalized to their respective maximum value, reached, in each industrial process, when viable bacterial cells were no more detectable. The normalized data demonstrated that the highest BA concentration was always reached during the ageing stage following the completion of MLF, at the end of the death phase of the bacterial population. In particular, histamine and putrescine were mostly released after MLF completion, whereas tyramine was produced both during and after MLF. In conclusion, these results demonstrate that BA accumulation, when it occurs, follows a general pattern in industrial vinifications and suggest the most appropriate times to perform treatments in order to minimize BA levels in wine.

Biogenic amine synthesis in high quality tempranillo wines. Relationship with lactic acid bacteria and vinification conditions

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Biogenic Amines are low molecular nitrogenous organic bases that appear often in fermented food (wine, cheeses, sausages, etc.) and have negative effects on the human health and the quality of food. From the amines present in wines, we can distinguish histamine and tyramine for their harmful effects on the human health (nausea, hypertension, throbs, reddening, headache, etc.) (Bauza et al., 1995), and putrescine for being the most frequent and abundant in wines. Putrescine and other amine like cadaverine, phenylethilamine and tryptamine have a negative effect on the aroma and flavour; besides promoting, together with the alcohol, the harmful effects on the health of histamine and tyramine (ten Brink et ., 1990). The presence of histamine in wines supposes a barrier to the exportation, countries like Switzerland have limits of 10 mg/L and other countries as France, Belgium and Germany consider diminishing to even 2 mg/L the allowed limits.

In the Tempranillo variety, biogenic amines can be formed during the alcoholic fermentation (AF), especially histamine; putrescine and cadaverine are formed on a smaller amount. This increase is possibly due to the activity of yeast, since the unique species of lactic bacteria that appear (*L. plantarum, L. hilgardii* and *P. parvulus*) are exclusively in the grape in very low concentration, and disappear during the alcoholic fermentation.

Biogenic amines suffer two large increases, one between AF and the end of malolactic fermentation (MLF), and another from final of MLF to 4 months of ageing, specially histamine and putrescine in this last period. There are no major changes in the concentrations of tyramine or cadaverine in aging.

When commercial *Oenococcus oeni* starters are employed (non-biogenic amine producers), a lower production of biogenic amines is observed, but late growth of spoiling bacteria (amine producers) can happen at the last ageing stages. Lysozyme can only help when low numbers of bacteria are present. Microoxigenation does not alter the lactic acid bacteria populations and the biogenic amines synthesis. The vat type seems not to have an influence.

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Determination of chloroanisoles in wine and cork stoppers by solid-phase microextraction (SPME) and gas-chromatography coupled with mass spectrometry (GC-MS)

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The presence of certain volatile organic compounds in wine causes the loss of wine freshness and natural aromas, giving rise to unpleasant mouldy odours. This effect is known as *cork taint* and causes important economic losses every year in the wine and cork industries. This problem is usually related to the migration of off-odour compounds from the cork stopper to the bottled wine. The primary compounds considered responsible for this defect are chloroanisoles, especially 2,4,6-trichloroanisole (TCA) (Buser et al., 1982; Châtonnet et al., 2004). Chloroanisoles usually arise from *O*-methylation of phenolic precursors, as a detoxification method, by different microorganisms, especially fungi, under particular conditions of temperature and water content. Chlorophenols are often present because of the packaging, the fungicides, herbicides or wood preservatives that are used in wineries or some cork stopper manufacturers' practices, such as using hypochlorite as a cork bleaching agent. These off-flavour compounds are present in cork stoppers at low levels (nanogram of compound per gram of cork); reported values for the sensory threshold for TCA in cork soaks varied from 4 to 10 ngL^{-1} (Vlachos et al., 2007). It is therefore necessary to develop analytical methods with enough sensitivity to determine these compounds directly in wine samples and cork stoppers.
A sensitive and selective method using SPME and gas chromatography with high-resolution mass spectrometry (GC-HRMS) was developed for the detection and quantification of TCAs in commercial Italian wine samples (Bianco et al., 2009). The thorough experimental testing for the extraction and pre-concentration of these compounds in wine samples indicates that the pure polymeric extracting phase of PDMS is more suitable and recommended for these types of samples. It was found that deterioration of DVB/CAR/PDMS fiber coating occurs with water–ethanol solutions, thus precluding its use for solid phase microextraction of cork-taint compounds in wine. Detection limits of TCA and related compounds, under optimized experimental conditions, ranged from 0.2 to 0.4 ng/L at a signal-to-noise ratio of 3 and quantification limits from 0.8 to 1.5 ng/L. The proposed method was successfully applied to commercially available Italian white and red wines using 2,4,6-TCA-d_5 as the internal standard. It is expected that this analytical methodology would be very useful for the quality control of musty taint in cork stoppers. Work is underway along this direction.

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