MONESTIER, FRANCE
APRIL 26, 2012

THE USE OF NON-CONVENTIONAL MICROORGANISMS IN WINEMAKING

19

LALLEMAND

MONESTIER, FRANCE, APRIL 26, 2012

THE USE OF NON-CONVENTIONAL MICROORGANISMS IN WINEMAKING

PROCEEDINGS
OF

THE *XXIII*es *ENTRETIENS SCIENTIFIQUES LALLEMAND*

LALLEMAND

FOREWORD

At the *XXIII*[es] *Entretiens Scientifiques Lallemand*, researchers presented the most recent discoveries regarding the utilization of non-conventional yeast and bacteria in winemaking. This was the occasion to hand out the *Prix Michel Feuillat – Entretiens Scientifiques Lallemand* to Claire Brice from the INRA and author of the study entitled, "A genetic and genomic approach to the molecular basis of variations in the efficiency of nitrogen utilization in *Saccharomyces cerevisiae* yeast." The Lallemand – Institute of Masters of Wines research bursary was awarded to Matthew Forster of Great Britain, second-year student in the Master of Wine program, for his essay, "How could the diversity of yeast strains and bacteria species contribute to enhancing varietal aroma and bring complexity to wine?" The winners of the ML Wine competition (Madrid, 2012) also received their awards.

One of the greatest researchers in the field of the wine microbiology, Professor Aline Lonvaud from the Université Bordeaux Ségalen, opened the *XXIII*[es] *Entretiens Scientifiques Lallemand* with a presentation on the importance of the complexity of biodiversity, the potential of microorganisms in wine, as well as the influence of the environment on the performance and the metabolism of wine yeast and bacteria to the quality of the wine.

Another remarkable figure in the research community, Professor Amparo Querol (IATA-CSIC, Spain), a specialist in oenological yeasts, presented her most recent work on hybrid yeasts of natural origin – as natural as the selected yeasts. The research on these natural hybrid yeasts is of great interest.

Over the past four years, *Torulaspora delbrueckii* yeast has been making an important place for itself as a tool for fermentation. One of the pioneers in the study of this microorganism, Dr. Jean-Michel Salmon (INRA, France), presented the findings that led to the development of sequential inoculation with *T. delbrueckii* 291 and its complementary yeast, *S. cerevisiae* 734. The wines resulting from this fermentation method have all been described as more complex, rich and very aromatic.

Continuing on the subject of sequential inoculation, Professor Debra Inglis (CCOVI, Brock University, Canada) described the positive impact of this yeast pair in wines high in sugar, especially ice wines, where the level of acetic acid – strictly controlled – must be limited. On the sensory level, these wines were richer and more aromatic.

Dr. Angelica Ganga of the Universidad de Santiago de Chile presented *Metschnikowia pulcherrima*, a non-conventional yeast selected and characterized as part of her research, which has been added to the portfolio of active dry yeasts (ADY). The *M. pulcherrima* LAMAP L1781 yeast is particularly promising for its α-arabinofuranosidase enzyme activity, which stimulates the liberation of thiols and terpenes in white wines.

Of course, the diversity of microorganisms is also seen among lactic bacteria. Dr. Sergi Ferrrer of the Universitat de València in Spain studied the oenological interest of 24 species of *Lactobacillus* bacteria inoculated into wine, alone or combined with *Oenococcus oeni*. These homofermentative microorganisms can influence the aromatic profiles of wines, especially the fruity notes.

The utilization of inactive yeasts is increasingly common in the winemaking industry. Dr. Antonia Morata, from the Universidad Politécnica de Madrid, Spain, spoke on the results of research directed by Dr. José Antonio Suárez-Lepe, which show the interest of inactive non-*Saccharomyces* yeasts. Indeed, the biomass from *Schizosaccharomyces pombe* and *Saccharomyces ludwigii* is rich in polysaccharides from cell membranes, which can potentially intensify the perception of volume in the mouth.

To conclude the *XXIII*[es] *Entretiens Scientifiques Lallemand*, Dr. Pierre Strehaiano of the Université de Toulouse, France, made a presentation on the links and complex interactions between different organisms, such as yeasts-yeasts and yeasts-bacteria, and their impact on the final product.

As shown at these scientific meetings delving into the diversity of microflora, research into non-conventional microorganisms is creating multiple possibilities for fermentation in controlled conditions and is facilitating the work of the winemaker.

CONTENTS

THE USE OF NON-CONVENTIONAL MICROORGANISMS IN WINEMAKING

WINEMAKING: MASTERING A COMPLEX MICROBIAL SYSTEM

Aline LONVAUD-FUNEL

Université Bordeaux Segalen, Faculté d'œnologie, Institut des Sciences de la Vigne et du Vin,
33882 Villenave d'Ornon CEDEX, France

1. Introduction

Winemaking, like all other processes for the preparation of fermented beverages and foods, is based on the growth of a large number of microorganisms. The grapes off the vine are naturally contaminated by fungi, yeast and bacteria, which form a complex biological system notable for the diversity of partners and the interactions among them. Originally, fermentation was a way to conserve raw fruit juice, grains, vegetables and milk, which are particularly unstable. When the grape is crushed, the biofilm – formed on the surface of the berry during maturation – comes in contact with the juice that becomes the new environment for the microorganisms. Gradually, through a complex set of interactions, including both inhibitions and stimulations, the yeast and bacteria transform the grape must into wine. The process has obviously been the same since ancient times, but since the end of the 19th century empiricism has been replaced by winemaking rules founded on the progress of the knowledge of microorganisms. Cagniard de Latour (1838) and Pasteur (1860 and 1867) are the authors of the most spectacular advances in establishing the existence of the phenomenon of fermentation and its role in the production of wine. The countless studies on the microorganisms of the grape and wine, their nature and metabolisms, have led to the fundamental rules for better control of the transformations and their impact on the quality of the wine. Since the period that began with the work of Pasteur through until the 1960s, the knowledge of microbiology – the knowledge most basic and immediately applicable to oenology – has multiplied with discoveries in the methods of cultivating microorganisms and conducting chemical analyses. Enhancements and innovations continued, taking a decisive leap when the molecular methods, which had become more affordable, were introduced in the early 1990s.

Now, the evolution of the composition of the grape must in fermentation, and even the wine after vinification, is interpreted in the light of more accurate microbiological analyses. In addition to the main reactions of alcoholic fermentation and malolactic fermentation on the aroma and taste of wine, is the more discrete but observable impact often characteristic of genera, species and even strains. The precise and specific analysis of genomes now provides a new basis to continue investigating the microbiology of winemaking. The stakes today are a more accurate knowledge of the indigenous microbial system and the improved exploitation of its diversity.

2. Microbial Diversity and Ecology

Through microscopic observation, the diversity of microorganisms in the must and wine was appreciated by Pasteur, notably in his "Études sur les maladies des vins" (studies on the diseases of wines) published in 1865. The microscope remains the instrument of choice for the wine microbiologist, in the first analysis. However, the accuracy of the information is rapidly insufficient. Culturing on agar nutrients, based on the work of Koch (1881), was a major discovery in microbiology, making it possible to isolate microorganisms in order to identify and investigate their properties. The diversity of oenological microbial systems was investigated by isolating and purifying the clones of yeasts and bacteria, before growing them to ob-

tain the biomass necessary for analysis. The keys to identifying genera and species include numerous tests that are often complicated and sometimes ambiguous or unreproducible. Characterizing the strains of a species presents similar difficulties. Nevertheless, very important findings on the microflora of musts and wines were described by several research teams in the years from 1920 to 1960. They are the basis of winemaking progress. Castelli (1954) showed a distribution of different yeast species in Italy, and in 1967 he produced a highly documented review of the numerous results already available on the ecology of wine yeasts in several countries. Today, in almost all the grape-growing and wine-producing regions of the world, these studies are repeated with other methods. Current data make us appreciate the quality of the work of our precursors, confirming and completing their findings with even greater accuracy.

3. Analytical Methods

Regarding the genome (and not the expression of the genome), the methods of DNA analysis provide more certainty and accuracy to long-awaited answers. Identification is based on nucleic acid sequences signaling the affiliation of the organism to a specific genus and species. In addition, the analysis of specific targets guarantees the distinction between strains of the same species. With bioinformatic analysis, the data suggest the mechanisms of the evolution of the strains within the species, particularly their adaptation to ecological niches. With their extreme precision, these methods are very rapid and most are ultimately less expensive than conventional methods. They continue to improve and are better suited to the analysis of large samples. Isolation is no longer necessary before identification, which expands access to non-cultivable microorganisms. Complex mixtures can be studied. The specificity and sensitivity of the detection of species or strains, including spoilage strains, have never been so great. Analyses are supported by the genome sequences of species known to wine microbiologists. Other analyses vastly expand inventories, but as long as the sequences are part of the hundreds of thousands available in databanks, any microbial DNA extracted from a grape or wine sample can be identified, or detected, in a mixture. The first applications of molecular biology for oenological identification were DNA probes for wine bacteria, and the mitochondrial DNA restriction profiles. Since then, the protocols have evolved and are constantly being improved. The polymerase chain reaction (PCR) protocol, with its specificity and sensitivity, has become indispensable. Depending on the question, its specificity is adjusted: universal primers to amplify regions where the sequence indicates the species, primers to amplify the DNA specific to a species, amplification profiles, or primers that detect the presence of genes encoding specific functions of strains. Mixtures of DNA amplified together, but of different sequences, are resolved by gel or liquid electrophoresis. The analysis of the diversity of the mixtures of microorganisms is more detailed than the identification of clones isolated on agar media. By combining the best choice of primers and the best choice of methods for analyzing the amplifiers (electrophoresis after enzymatic or non-enzymatic restriction, denaturing gels, capillary electrophoresis, and so on), the scope of the analysis is very broad.

3.1 The microorganisms on the grape

Studies of the microbial community of the grape berry have benefited greatly from this progress. Analysis of the DNA extracted from the water used to wash the grapes has already shown the great diversity of species that contaminate the grapes from *véraison* to maturity (Renouf et al. 2005). More recently, analysis has shown an incidence of the mode of conduct of the vine (Cordero-Buesa et al. 2011, and Martins et al. 2012). Dozens of species of yeasts and bacteria have been identified. The existence of many of them had so far escaped identification because they were not grown or poorly grown in traditional regions. After the harvest, many of these species are eliminated as soon as the grape is crushed as the very particular environment of the biofilm covering the skin is destroyed. The microbial system of the fermenting grape must is much less complex and continues to simplify itself throughout the process.

3.2 The microorganisms in the wine

Saccharomyces cerevisiae and *Oenococcus oeni* are the dominant species during winemaking, and they provide much of the basic reactions of alcoholic fermentation (AF) and malolactic fermentation (MLF). But they live with other species of yeasts and lactic acid bacteria whose physiological requirements and metabolic functions are different. In addition, each species, starting with *S. cerevisiae* and *O. oeni*, is represented by several strains, whose characteristics are also varied. Throughout AF, then MLF, the species successively replace one another based on their adaptation to the changing conditions of the environment (Zhang et al. 2008). For each species, as has been demonstrated for *S. cerevisiae* and *O. oeni*, the diversity of the strains remains high. But the diversity can be momentarily decreased by the massive intake of a strain in a concentrated form that dominates the system.

Knowledge of the diversity of strains in a potentially usable species, like yeast strains, is an important issue. It has long been studied and used for the selection of *S. cerevi-*

siae strains, and most recently for *O. oeni*. The genomic tools provide the strain typing without difficulty. The quality of the collections, which are the basis of selection or genetic improvement by crossbreeding, is guaranteed. Moreover, the bioinformatic analysis of gene sequences, genomic regions or even entire genomes is a powerful tool in the study of the evolution of species in their ecological niche, their domestication and their spatiotemporal spread. They reveal the genetic mechanisms that lead to the acquisition of the most appropriate mechanisms to survive and multiply in association with other species, or to dominate them. They show the frequent existence of natural hybrid strains of several yeast species, and the interest for these strains that follows (Erny et al. 2012). The analysis of genotypes for more than 250 strains of *O. oeni* showed that this species evolves in two major phylogenetic groups; subgroups appear to be linked at the origin of isolates (type of wine, cider or region) (Bridier et al. 2010). On the species level, the work of Makarova and Koonin (2007) shows how, through successive losses and gains of genes, lactic acid bacteria (LAB) have evolved from their common ancestor.

Diversity is not limited to "technological" species, whose family has been growing in recent years with the participation of several non-*Saccharomyces* and *Lactobacillus plantarum* species. The wine also contains spoilage yeast and bacteria that come from the vineyard or sometimes the winery. Several species of yeasts (including *Brettanomyces bruxellensis*, *Zygosaccharomyces bailii* and *Saccharomycodes ludwigii*) and acetic spoilage bacteria are part of the system, as well as strains of LAB of every kind characterized by particular metabolisms ("wine diseases," and biogenic amines). These spoilage microorganisms are usually in the minority during active phases of fermentation and have no impact. But if they are present, their growth is still possible after winemaking because the wine remains an appropriate nutrient medium. Scarce or even unnoticed during fermentation, certain species multiply when the activity of the dominant yeast and LAB stops, if basic precautions are not taken.

3.3 Selected yeasts and lactic acid bacteria

A bit of history

The addition of yeasts to the grape must, to control AF, was directly inspired by the observations of Pasteur, who demonstrated their role in winemaking. In his research, published in 1876, Pasteur made the following comment, which appears obvious to us today, "This is a proof, to say in passing, that ordinary wine, its taste and its qualities certainly depend, in large part, on the specific nature of the yeasts that develop during the fermentation of the grape harvest. One must think that, if the same grape must were submitted to the action of different yeasts, the result would be wines of various kinds. From the point of view of practical applications, new research should be undertaken in this direction."[1] Pasteur also proposed protocols for the purification of yeasts: "The purification of yeasts can be done by a variety of methods, whether it is a mixture of yeasts or whether the primary goal is to remove the ferments of disease..."[2] and he filed a patent in 1873. The first to exploit these findings was Jacquemin (1900), who prepared selected yeasts for the first time. "This work of selecting is extremely long and requires at least three months to be completed... This is how I managed to own a collection of yeast races from all the good wines in Europe, and from a very large number of lesser-known wines."[3] He developed a method for packaging and shipping yeasts, and exported them to America and Australia (Patent No. 1891, "Process for the industrial shipping of pure yeasts"). The utilization of selected strains spread, arousing enthusiasm or skepticism. The boom came much later, in the 1970s, when the delicate problem of storing and shipping the yeasts was solved by drying. They are now easy to use and succeed most of the time triggering AF. Objectives change, however, and the selection now includes new, increasingly precise standards, notably the impact on the aroma of wines.

The history of malolactic starters is more recent, and more complex, first, because the importance of MLF came to be understood only recently and then very slowly; next, because the culture, selection and multiplication of oenological bacteria on an industrial scale – and especially their survival – are difficult. The earliest references are probably those of the experiments conducted in 1955 by Peynaud and Domercq (1959), "The question arose whether the malic acid fermentation could be caused by inoculation with pure cultures of bacteria, in the same way yeast cultures cause alcoholic fermentation… To conclude, it seems possible that the use of malolactic bacteria starters carefully selected for their activity and homofermentative character, i.e., they do not produce volatile acids from sugars, is already feasible in the practice of winemaking."[4] Subsequently, the fermentative character was not long regarded as essential. Other oenologists were experimenting elsewhere in the world, and began to compare the performance of different strains, often *Leuconostoc* (later *O. oeni*), and also *Lactobacilli*. Faced with unexpected difficulties, addition protocols prolifer-

1 Our translation.

2 Our translation.

3 Our translation.

4 Our translation.

ated. However, everywhere the results remained random and hard to explain, but a consensus quickly formed on the fact that added bacteria do not survive in the wine as easily as added yeasts in grape must. Peynaud (1967) summarized the situation: "We have moved progressively from seeding the grape harvest and fermentation vats, to inoculation when the wines are still running slightly sweet, to find the only possible technique is the implantation of lactic acid bacteria in finished wines, which no longer have any fermentable residual sugar... We believe that the success of empirical testing while not knowing enough about the nature of the bacteria, their properties and their needs is the result of random chance only and is not likely to be reproducible."[5] In the 1970s, the first malolactic cultures (all *O. oeni* except one *L. brevis*) were produced on an industrial scale as concentrated frozen or lyophilized preparations. But failures were still numerous until the work of Lafon-Lafourcade (1983), who described a process for reactivating the industrial biomass for the improved survival of the inoculum in the wine. Then in 1996 the development of a preparation for direct seeding (Nielsen et al. 1996) simplified operations. A renewed interest for malolactic starters followed, as well as increased activity to select strains.

The contributions expected from understanding genomes

Easy access to genotypes and complete genomes lets us hope for more accurate, reliable and rapid approaches for the selection of *S. cerevisiae* and *O. oeni* strains. Similarly, a future is foreseeable for strains obtained from specific inter or intra *Saccharomyces* crosses. Classically, selecting and collecting strains is based on the study of the physiology, certain metabolisms and, more generally, behaviour under oenological conditions (must for yeasts and wine for LAB). This involves heavy lab work prior to proceeding to testing in pilot conditions then in the field. Although the experience gained over decades has led to more effective starters, there is still more progress to be made. With a reasoning that may be too simplistic, researchers hope to be able to provide the industry with specific tools, in the form of easy-to-find molecular markers. But the great challenge remains to find the links between genomes and phenotypes. The essential characters are the growth and adaptation to the oenological environment, on one hand, and their metabolisms for their effect on the quality of the wines, on the other. The goal is to find simple correlations between the presence/absence of genes, groups of genes or genomic regions and the technological quality of the strains. These genes would be then used as markers to sort collections, but at least two drawbacks must be considered. First, the physiological and metabolic properties of a microorganism are the result of functioning in networks of genes that are more or less complex. Then, the scope of the phenotypic character remains difficult. If it is only about the utilization of a certain substrate, or the production of a compound, not really subject to environmental conditions, the situation is simple. But most of the time, it is due to multifactorial characters regulated by the environment, and errors are predictable.

Based on quantitative trait loci (QTL) mapping, advances have been described for the *S. cerevisiae* yeast where genetic regions involved in technological properties have been identified, and utilized as markers in strain-crossing programs (Marullo et al. 2007, 2009). Much more recent data from genomics research comparing strains of *O. oeni* have not yet brought the tools expected. However, genomic regions appear to be statistically linked to groups of strains (Bridier et al. 2010), but the link with the main quality – the survival of the bacterium after inoculation in the wine – has not yet been made.

4. The Utilization of Non-*Saccharomyces* Yeasts and Non-*Oenococcus* Bacteria as Oenological Starters

The first oenological microbiologists (Pasteur and Jacquemin) advocated the selection of strains to prepare starters. This was also the opinion of Hermann Müller-Thurgau (1894-1897), deducted from the observation of the spontaneous phenomenon. The author noted that AF starts with apiculate yeasts and ends with ellipsoidal yeasts. He showed that the products of *Kloeckera apiculata*, with a low fermentation capacity, inhibited *Saccharomyces cerevisiae* var. *ellipsoideus*. The addition of pure cultures of *S. ellipsoideus* was therefore necessary, but the winemaking books of the time report controversy between the supporters and the opponents of selecting, and the generalization of the utilization of pure cultures.

Since then, the commonly utilized starters are pure preparations of yeast or bacteria. The starters prepared with mixtures of strains pose the problem of interactions among them that are unpredictable and a function of environmental conditions. It is less risky to seed with a pure strain, which must also undergo interactions with indigenous microflora.

In recent years, there has been a revival of interest in yeast from genera other than *Saccharomyces*, and still more recently in *Lactobacillus plantarum* for LAB. In the latter case, the reason is mainly to enjoy the better survival

5 Our translation.

rates of different strains of this species. As for yeast, the interest is mainly to take advantage of metabolic transformations specific to other species. It is well recognized that non-*Saccharomyces* yeasts participate fully in transforming the must. Their populations remain high during AF, even if inoculaton with *Saccharomyces* yeast is practiced (Zott et al. 2010), and they produce the taste and the aroma compounds of wine. In high sugar wines, the addition of *Torulaspora delbrueckii* or *Candida zemplinina* reduces volatile acidity (Bely et al. 2008, and Rantsiou et al. 2012). New fields of research and experimenting are open in laboratories and wineries. In the lab, the priority is to evaluate the diversity of strains in species less well known than *S. cerevisiae*. According to the strain, interactions with other yeasts and the environment, as well as metabolisms, can be very different (Renault et al. 2009). In the winery, the experiments should result in proposing simple and reliable addition protocols so the associations between non-*Saccharomyces* and *S. cerevisiae* yeasts are carried out correctly.

5. Conclusion

Today, the tools and methods for the study of wine microorganisms – whether on the genetic, physiological or metabolic level – promise interesting developments in the mastery of the microbial community of wine-making. The principle is to first explore the diversity of the microflora in order to utilize it. Genomic analyses, at all levels of investigation, are reliable, fast and less and less expensive. Beginning in the vineyard – the first source of microorganisms – ecological research enlightens us on the distribution of strains and species in relation to environmental parameters (climate, vine management, etc.). At the species level, it is essential to identify the diversity of strains. There may be more differences among strains of the same species than among strains of different species. This fundamental work is based on amassing collections whose quality now can be better controlled.

After the advent of pure starter cultures, the renewed interest in non-*Saccharomyces* yeasts takes into account the reality of the interactions between microorganisms and their impact on the quality of the wine. But ultimately this is not so new; the Italian oeonological microbiologist Castelli wrote in the proceedings of the 2nd International Oenology Symposium in 1967: "…*Torulaspora rosei*... is interesting for its very low formation of acetic acid in the fermentation products. Since 1942, we have counselled [oenologists] to prepare fermentation starters with particular strains of *Torulaspora rosei*, especially to produce white wines of average alcoholic degree. The utilization of *Torulaspora rosei* with *Saccharomyces ellipsoideus* or other species, either in pure or staggered fermentations, is now so widespread it seems unnecessary to talk about it more."[6]

But undoubtedly more likely than with pure cultures, the results of mixed inoculations will maintain a good share of uncertainty. The great unknown comes from interactions whose complexity is difficult to assess, and their force. Simplified laboratory models provide a large part of the answer, but their application in the winery is still far from obvious.

References

Bely, M., P. Stoeckle, I. Masneuf-Pomarède, and D. Dubourdieu. 2008. Impact of mixed *Torulaspora delbrueckii-Saccharomyces cerevisiae* culture on high-sugar fermentation. *Int. J. Food Microbiol.* 122:312-320.

Borneman, A. R., E. J. Bartowsky, J. McCarthy, and P. J. Chambers. 2010. Genotypic diversity in *Oenococcus oeni* by high-density microarray comparative genome hybridization and whole genome sequencing. *Appl. Microbiol. Biotechnol.* 86:681-691.

Bridier, J., O. Claisse, M. Coton, E. Coton, and A. Lonvaud-Funel. 2010. Evidence of distinct populations and specific subpopulations within the species *Oenococcus oeni*. *Appl. Environ. Microbiol*. 76:7754-7764.

Cagniard de Latour, Charles. 1838. Mémoire sur la fermentation vineuse presented to the Académie des Sciences June 12, 1837. *Annales de Chimie et de Physique*. 68:206-222.

Castelli, T. 1954. Les Agents de la Fermentation Vinaire. *Archiv fur Mikrobiologie*. 20:323-342.

Castelli, T. 1967. Écologie et systématique des levures de vin. In *Fermentations et vinifications*. 2e Symposium international d'œnologie. Ed. INRA Paris. I:89-106.

Ciani, M., F. Comitini, I. Mannazzu, and P. Domizio. 2010. Controlled mixed culture fermentation: a new prospective on the use of non-*Saccharomyces* yeasts in winemaking. *FEMS Yeast Res.* 10:123-133.

Cordero-Bueso, G., T. Arroyo, A. Serrano, J. Tello, I. Aporta, M. D. Vélez, and E. Valero. 2011. Influence of the farming system and vine variety on yeast communities associated with grape berries. *Int. J. Food Microb*. 145:132-139.

Erny, C., P. Raoult, A. Alais, G. Butterlin, P. Delobel, F. Matei-Radoi, S. Casaregola, and J. L. Legras. 2012.

6 Our translation.

Ecological success of a group of *Saccharomyces cerevisiae/Saccharomyces kudriavzevii* hybrids in the northern European wine-making environment. *Appl. Environ. Microbiol*. 78:3256-3265.

Jacquemin, G. 1900. *Les fermentations rationnelles (vins, cidres, hydromels, alcools)*. Malzéville-Nancy.

Lafon-Lafourcade, S., et al. 1983. Induction de la fermentation malolactique des vins par inoculation d'une biomasse industrielle congelée de *L. oenos* après réactivation. *Conn. Vigne Vin.*

Martins, G., C. Miot-Sertier, B. Lauga, O. Claisse, A. Lonvaud-Funel, G. Soulas, and I. Masneuf-Pomarède. 2012. Grape berry bacterial microbiota: Impact of the ripening process and the farming system. *Int. J. Food Microb*. 158:93-100.

Marullo, P., C. Mansour, M. Dufour, W. Albertin, D. Sicard, M. Bely, and D. Dubourdieu. 2009. Genetic improvement of thermo-tolerance in wine *Saccharomyces cerevisiae* strains by backcross approach. *FEMS Yeast Res.* 9:1148-1160.

Marullo, P., G. Yvert, M. Bely, M. Aigle, and D. Dubourdieu. 2007. Efficient use of DNA molecular markers to construct industrial yeast strains. *FEMS Yeast Res.* 7:1295-1306.

Makarova, K. S., and E. V. Koonin. 2007 Evolutionary Genomics of Lactic Acid Bacteria. *J. Bacterio.* 189(4):1199-1208.

Nielsen, J. C, C. Prahl, and A. Lonvaud-Funel. 1996. Malolactic fermentation in wine by direct inoculation with freeze-dried *Leuconostoc oenos* cultures. *Am. J. Enol. Vitic*. 47:42-48.

Pasteur, L. 1860. *Mémoire sur la fermentation alcoolique*. Imprimerie de Mallet-Bachelier. Paris.

Pasteur, L. 1876. *Études sur la bière, ses maladies, causes qui les provoquent, procédé pour la rendre inaltérable; avec une théorie nouvelle de la fermentation*, Masson, Paris.

Peynaud, E., and S. Domercq. 1959. Sur la possibilité de provoquer la fermentation malolactique en vinification à l'aide de bactéries cultivées. *C. R. Acad. Agric*. 45:355-358.

Rantsiou, K., P. Dolci, S. Giacosa, F. Torchio, R. Tofalo, S. Torriani, G. Suzzi, L. Rolle, and L. Cocolin. 2009. *Candida zemplinina* can reduce acetic acid produced by *Saccharomyces cerevisiae* in sweet wine fermentations. *Appl. Environm. Microbiol*. 78:1987-1994.

Renault, P., C. Miot-Sertier, P. Marullo, P. Hernández-Orte, L. Lagarrigue, A. Lonvaud-Funel, and M. Bely. 2009. Genetic characterization and phenotypic variability in *Torulaspora delbrueckii* species: potential applications in the wine industry. *Int. J. Food Microbiol*. 134:201-210.

Renouf, V., O. Claisse, and A. Lonvaud-Funel. 2005. Understanding the microbial ecosystem on the grape berry surface through numeration and identification of yeast and bacteria. *Aust. J. Grape Wine Res*. 11:316-327.

Zott, K., C. Miot-Sertier, O. Claisse, A. Lonvaud-Funel, and I. Masneuf-Pomarede. 2008. Dynamics and diversity of non-*Saccharomyces* yeasts during the early stages in winemaking. *Int. J. Food Microbiol*. 125:197-203.

Zott, K., C. Thibon, M. Bely, A. Lonvaud-Funel, D. Dubourdieu, and I. Masneuf-Pomarede. 2011. The grape must non-*Saccharomyces* microbial community: Impact on volatile thiol release. *Int. J. Food Microbiol.* 151:210-215.

OENOLOGICAL INTEREST OF NON-CONVENTIONAL *SACCHAROMYCES* SPECIES AND THEIR INTERSPECIES HYBRIDS

Amparo QUEROL
Departamento de Biotecnología, Instituto de Agroquímica y Tecnología de los Alimentos (IATA), CSIC
Avda/Agustín Escardino Benlloch, 7, 46980 Paterna (Valencia), Spain

Abstract

The fermentation of wine is a complex process produced as a result of the activities of a succession of microorganisms, with *Saccharomyces* yeasts (mainly *S. cerevisiae*) being responsible for alcoholic fermentation. Although *S. cerevisiae* is the most frequent species in wines, and the subject of most studies, *S. bayanus* var. *uvarum* and *S. paradoxus* strains, as well as natural hybrids between such *Saccharomyces* species as *S. cerevisiae* x *S. kudriavzevii*, and *S. cerevisiae* x *S. bayanus* var. *uvarum*, are also involved in wine fermentation and can be preponderant in certain wine regions.

Studies performed in our laboratory comparing the properties of natural wine hybrids with their parent species, showed that strains of non-conventional *Saccharomyces* species, such as *S. bayanus* var. *uvarum* and *S. kudriavzevii*, exhibit physiological properties of potential interest in oenology because they can respond to new demands of the wine industry, including their ability to ferment at low temperatures, their increased production of glycerol, their lower ethanol yield and their higher assimilation of fructose.

Several of these characteristics, e.g. low sugar/alcohol rate and an incremental difference in glycerol production, might be of special interest to solve oenological problems caused by climate change. The increase of glycerol can conceal the astringency caused by tannins in younger grapes, and lowering the sugar/alcohol ratio is already a requirement of the sector, independent of climate change.

1. Introduction

From an oenological point of view, the transformation of must into wine is a complex process where yeast, fungi and wine bacteria play significant roles. However, only yeasts from the *Saccharomyces* genus (mainly *S. cerevisiae*) are responsible for alcoholic fermentation (Pretorius 2000). Although *S. cerevisiae* is the yeast species most frequently found in wine fermentations and, until recently, the subject of primary studies (Pretorius 2000, Barrio et al. 2006), the participation of other *Saccharomyces* species, including *S. bayanus* var. *uvarum* and *S. uvarum* (Naumov et al. 2000), and natural yeast hybrids between species of the genus *Saccharomyces*, such as *S. cerevisiae* x *S. kudriavzevii* (González et al. 2006, González et al. 2008, and Peris et al. 2011) and *S. cerevisiae* x *S. bayanus* var. *uvarum* (Masneuf et al. 1998), can be also present along the fermentative process.

2. Categorization of New Yeast Species in Oenology

Saccharomyces cerevisiae is the predominant species in most of the industrial fermentative processes, e.g., bread production, brewing, winemaking, cider and sake production, as well as traditional fermented beverages around the world (pulque, masato, chicha, sorghum beer, palm wine, etc.). The metabolic activities of *S. cerevisiae* have been exploited by humans since the development of agriculture, and from an economic point of view this yeast can be considered the most important among microorganisms.

S. bayanus is a complex of strains grouped into two varieties: *S. bayanus* var. *bayanus* and *S. bayanus* var. *uvarum*, although several authors consider them authentic species. Both varieties are characterized by two physiological traits unique among the *Saccharomyces* genus: 1) The active fructose transport system, and 2) The cryophilic character (Tronchoni et al. 2009), i.e., the ability to grow at lower temperatures than *S. cerevisiae*. Both characteristics are of maximum interest for oenologists. The variety *uvarum* has been isolated in wine and cider only. Characterized by a lower fermentative capacity than *S. cerevisiae*, this variety is more tolerant to low temperature fermentation and produces less acetic acid and amylic alcohol, although it produces more glycerol, succinic and malic acids, and higher alcohols (Sipiczki 2002), and some strains even exhibit pectinolytic activity (Naumov et al. 2001) producing more aromatic wines.

S. uvarum is the predominant yeast in wine regions of continental climate in Europe, especially in low temperature wine fermentations, such as Txacolí in Basque country. Although this yeast variety has been described in wines, a few strains have been commercialized. Recently, our group patented one *S. uvarum* (BMV58) in collaboration with the Murviedro winery.

S. kudriavzevii was isolated in Japan from decaying leaves and soil. The physiological characterization of *S. kudriavzevii* (Naumov et al. 2000, Belloch et al. 2008, and Arroyo-López et al. 2009) indicates very good growth at low temperatures, even at 4°C, high cellulolytic activity, inulin (a fructose polymer) hydrolysis, galactitol utilization, synthesis of starch-like polymers, and problems for anaerobic growth. Despite these distinctive characteristics, *S. kudriavzevii* can ferment grape must with 200 g/L sugar (González et al. 2007). This yeast could be a good model for studies on yeast adaptation to growth at low temperatures and poor sugar environments. *S. kudriavzevii* is also involved in the origin of several natural yeast hybrids that appear as predominant in wine fermentations in Central Europe wine regions (González et al. 2006, and Lopandić et al. 2007), and in the production of several ale beer types (González et al. 2008). Recently, through the selection of yeasts growing at low temperatures, *S. kudriavzevii* has been isolated from oak trees in Portugal (Sampaio and Gonçalves 2008) and in Spain by our group (Lopes et al. 2010). Data from our group indicate that these strains, though similar to the Japanese strains, are genetically and physiologically different from them and more similar to the parental strain of the *S. cerevisiae* x *S. kudriavzevii* hybrids, indicating that the origin of the wine hybrids is in Europe (Peris et al. 2012).

3. Results

3.1 Oenological characterization of non-conventional *Saccharomyces* species

We have investigated the fermentation dynamics, as well as metabolite and aroma production, of several cryophilic *S. cerevisiae*, *S. uvarum*, *S. kudriavzevii* and natural hybrids between these species at temperatures of 12°C and 28°C (Gamero et al., submitted, and Tronchoni et al. 2009). Most of the cryophilic strains used in our study have been isolated from wine fermentations at low temperatures (see table 1). The comparison of fermentation days between

Table 1. List of strains used in this study. Duration of microvinifications with Tempranillo must at 12°C and 28°C

Species	Yeast strains	Origin	Days 12°C	Days 28°C
S. cerevisiae	T73	wine, Spain	21	6
	FCry[b]	wine, France	17	3
	FRCh[c]	sparkling wine, France	15	4
S. bayanus	BMV58	wine, Spain	21	6[a]
	CECT 12600	wine, Spain	17	4
	CECT 1969	red currant, Holland	24[a]	4[a]
S. kudriavzevii	IFO 1802	decayed leaves, Japan	11	11
S.c. x S.b.	Lalvin S6U	wine, Italy	14	6
S.c. x S.k.	Lalvin W27	wine, Switzerland	14	5
	AMH[d]	wine, Germany	20	11
	HA 1841	wine, Austria	21	7
	VIN7	wine, South Africa	23	6
S.c. x S.b. x S.k.	CBS 2834	wine, Switzerland	25	8

[a] Stuck fermentations; [b] Fermol Cryophile; [c] Fermol Reims Champagne; [d] Assmanhausen

fermentations at both temperatures revealed that all strains fermented faster at 28°C than at 12°C, except *S. kudriavzevii* IFO 1802 (see table 1). This strain was the slowest at 28°C and the fastest at 12°C, therefore it could be considered an authentic cryophilic strain and, consequently, the remaining strains could be considered cryotolerant. Nevertheless, most of the *Saccharomyces* strains and hybrids were able to consume all reducing sugars in the must at both fermenting temperatures. Regarding *S. bayanus* var. *uvarum* strains and hybrids, we also observed that it is another cryotolerant *Saccharomyces* species and would not be inhibited by ethanol at moderate or intermediate fermentation temperatures (see table 1).

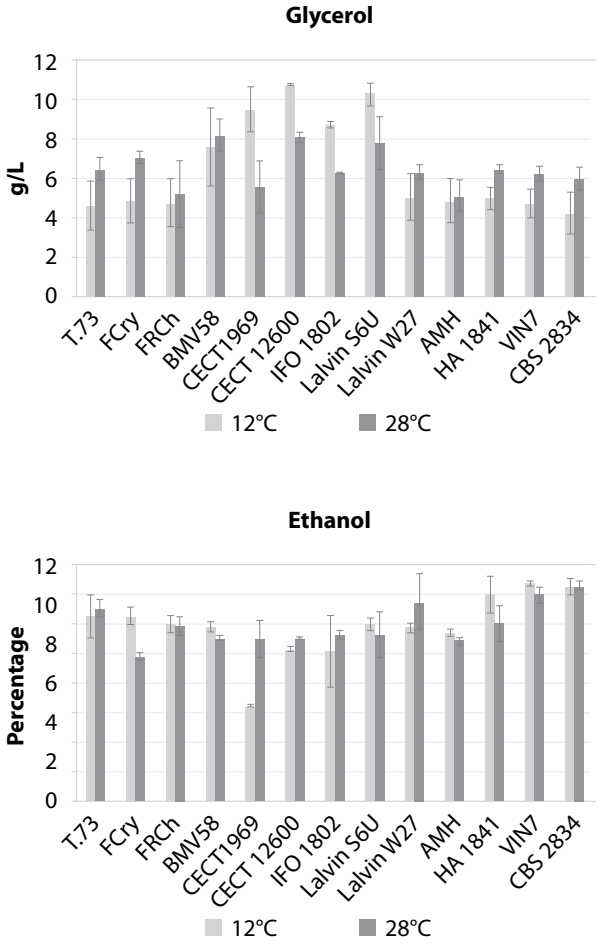
Figure 1 shows the results of the glycerol and ethanol content, comparing the fermentation at 12° and 28°C. As can be seen, the cryotolerant *S. bayanus* var. *uvarum* and *S. kudriavzevii* and their hybrids produced higher amounts of glycerol at both temperatures, compared to *S. cerevisiae*. Glycerol is one of the main metabolites produced in wine fermentation, contributing to wine quality by providing slight sweetness, smoothness and fullness, and by reducing wine astringency. Our results suggest that cryotolerant *Saccharomyces* species well adapted to growth at low temperatures, such as *S. uvarum*, *S. kudriavzevii* and double hybrid *S. cerevisiae* x *S. uvarum* Lalvin S6U and *S. cerevisiae* x *S. kudriavzevii*, were significantly the highest glycerol producers at 12°C, as demonstrated by ANOVA analysis. Moreover, previous reports from our group comparing glycerol production by non-cryotolerant *S. cerevisiae*, cryophilic *S. kudriavzevii* and cryotolerant *S. uvarum* and *Saccharomyces* hybrids support our findings (Arroyo-López et al. 2010).

3.2 Generation of artificial hybrids in *Saccharomyces* genus for winemaking

Although *S. uvarum* and *S. kudriavzevii* showed interesting oenological properties, including better capacity to grow at low temperatures and higher production of glycerol, flavours and aromas, they are not always dominant in wine fermentation. Arroyo et al. (2011) demonstrated that temperature plays an important role in the competition between *S. cerevisiae* and *S. kudriavzevii*. In this way, *S. kudriavzevii* was less affected at 17°C, but *S. cerevisiae* was clearly the best competitor at 31°C, preventing the growth of *S. kudriavzevii*. Population levels of *S. kudriavzevii* always significantly decreased in the presence of *S. cerevisiae*. For this reason, the only way to utilize the good properties of the non-*S. cerevisiae* species in wine fermentation is to utilize artificial hybrids.

We evaluated the usefulness of three different hybridization methods – spore-to-spore mating, rare-mating and protoplast fusion – for the generation of intra- and interspecific stable hybrids, being the first report to compare different methods to obtain artificial hybrids to be used in fermentations (Pérez-Través et al. 2012). Spore-to-spore mating is an easy but time-consuming method; hybrids generated with this technique could lack some of the industrially relevant traits present in the parent strains because the segregation occurred during meiosis and spore generation prior to hybridization. Hybrids obtained by protoplast fusion get the complete information of both parents, but they are currently considered to be genetically modified organisms (GMOs). Hybrids obtained by rare-mating are easily obtained by the optimized methodology described in this work; they originally contain a complete set of chromosomes from both parents and they are not considered GMOs. Based on these results, it became evident that a last crucial aspect to be considered in every hybridization program is the genetic stabilization of recently generated hybrids that guarantee their invariability during future industrial utilization.

Figure 1. Glycerol and ethanol production by the different strains in the fermentations at 12°C and 28°C



Applying rare-mating techniques, we developed inter-artificial hybrids between *S. cerevisiae* species and intra-artificial hybrids between *S. cerevisiae* and *S. uvarum* or *S. kudriavzevii* that were stable after industrial production and have the oenological properties of both parents (Pérez-Través, manuscript in preparation).

Acknowledgements

This work was supported by grants AGL2009-12673-CO2 from the Spanish Government to A. Querol and E. Barrio.

References

Arroyo-López, F. N., S. Orlić, A. Querol, and E. Barrio. 2009. Effects of temperature, pH and sugar concentration on the growth parameters of *Saccharomyces cerevisiae*, *S. kudriavzevii* and their interspecific hybrid. *Int J Food Microbiol*. 131(2-3):120-127.

Arroyo-López, F. N., R. Pérez-Torrado, A. Querol, and E. Barrio. 2010. Modulation of the glycerol and ethanol syntheses in the yeast *Saccharomyces kudriavzevii* differs from that exhibited by *Saccharomyces cerevisiae* and their hybrid. *Food Microbiol.* 27(5):628-637.

Arroyo-López, F. N., L. Pérez-Través, A. Querol, and E. Barrio. 2011. Exclusion of *Saccharomyces kudriavzevii* from a wine model system mediated by *Saccharomyces cerevisiae*. *Yeast.* 28(6):423-435.

Barrio, E., S. S. González, A. Arias, C. Belloch, and A. Querol. 2006. Molecular mechanisms involved in the adaptive evolution of industrial yeasts. In: A. Querol, G. H. Fleet (Eds.), *Yeasts in Food and Beverages*. Springer-Verlag, Berlin. 153-174.

Belloch, C., S. Orlić, E. Barrio, and A. Querol. 2008. Fermentative stress adaptation of hybrids within the *Saccharomyces sensu stricto* complex. *International Journal of Food Microbiology.* 122:188-195.

González, S. S., E. Barrio, J. Gafner, and A. Querol. 2006. Natural hybrids from *Saccharomyces cerevisiae*, *Saccharomyces bayanus* and *Saccharomyces kudriavzevii* in wine fermentations. *FEMS Yeast Research*. 6:1221-1234.

González, S. S., L. Gallo, M. D. Climent, E. Barrio, and A. Querol. 2007. Enological characterization of natural hybrids between *Saccharomyces cerevisiae* and *S. kudriavzevii*. *Int. J. Food Microbiol.* 116(1):11-18.

González, S. S., E. Barrio, and A. Querol. 2008. Molecular characterization of new natural hybrids between *Saccharomyces cerevisiae* and *Saccharomyces kudriavzevii* from brewing. *Applied and Environmental Microbiology*. 74:2314-2320.

Lopandić, K., W. Tiefenbrunner, H. Gangl, K. Mandl, S. Berger, G. Leitner, G. A. Abd-Ellah, A. Querol, R. C. Gardner, K. Sterflinger, and H. Prillinger. 2008. Molecular profiling of yeasts isolated during spontaneous fermentations of Austrian wines. *FEMS Yeast Res*. 8(7):1063-1075.

Lopes, C. A., E. Barrio, and A. Querol. 2010. Natural hybrids of *Saccharomyces cerevisiae* x *Saccharomyces kudriavzevii* share alleles with European wild populations of *S. kudriavzevii*. *FEMS Yeast Res*. 10:412-421.

Masneuf, I., J. Hansen, C. Groth, J. Piškur, and D. Dubourdieu. 1998. New hybrids between *Saccharomyces sensu stricto* yeast species found among wine and cider production strains. *Applied and Environmental Microbiology*. 64:3887-3892.

Naumov, G. I., E. S. Naumova, I. Masneuf, M. Aigle, V. I. Kondratieva, and D. Dubourdieu. 2000. Natural polyploidization of some cultured yeast *Saccharomyces sensu stricto*: Auto- and allotetraploidy. *Systematic and Applied Microbiology.* 23:442-449.

Naumov G. I., H. V. Nguyen, E. S. Naumova, A. Michel, M. Aigle, and C. Gaillardin. 2001. Genetic identification of *Saccharomyces bayanus* var. *uvarum*, a cider-fermenting yeast. *Int. J. Food Microbiol*. 65:163-171.

Pérez-Través, L., C. A. Lopes, E. Barrio, and A. Querol. 2012. Evaluation of different genetic procedures for the generation of artificial hybrids in *Saccharomyces* genus for winemaking. *Int J Food Microbiol.* 156(2):102-111.

Peris, D., C. Belloch, K. Lopandić, J. M. Alvarez-Pérez, A. Querol, and E. Barrio. 2012. The molecular characterization of new types of *Saccharomyces cerevisiae* x *S. kudriavzevii* hybrid yeasts unveils a high genetic diversity. *Yeast*. 29(2):81-91.

Peris, D., C. A. Lopes, C. Belloch, A. Querol, and E. Barrio. 2012. Comparative genomics among *Saccharomyces cerevisiae* x *Saccharomyces kudriavzevii* natural hybrid strains isolated from wine and beer reveals different origins. *BMC Genomics*. 13:407.

Pretorius, I. S. 2000. Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. *Yeast*. 16:675-729.

Sampaio, J. P., and P. Gonçalves. 2008. Natural populations of *Saccharomyces kudriavzevii* in Portugal are associated with oak bark and are sympatric with *S. cerevisiae*

and *S. paradoxus*. *Appl Environ Microbiol*. 74(7):2144-2152.

Sipiczki, M. 2008. Interspecies hybridization and recombination in *Saccharomyces* wine yeasts. *FEMS Yeast Res*. 8(7):996-1007.

Tronchoni, J., A. Gamero, F. N. Arroyo-López, E. Barrio, and A. Querol. 2009. Differences in the glucose and fructose consumption profiles in diverse *Saccharomyces* wine species and their hybrids during grape juice fermentation. *Int J Food Microbiol*. 134(3):237-243.

THE USE OF *TORULASPORA DELBRUECKII* IN MIXED YEAST CULTURES

J.-M. SALMON
NRA, UE999, Unité expérimentale de Pech Rouge, 11430 Gruissan, France

1. Introduction

The differences between wine fermentations carried out with pure cultures and those performed with indigenous yeasts have long been debated. In the field of oenology, the use of pure yeast cultures offers undeniable advantages in terms of the ease of control and the homogeneity of fermentations, but may reduce the flavour complexity of wines. Considering this, Lallemand and INRA, in a common research project in 2004, evaluated the utilization of novel and desirable non-*Saccharomyces* yeasts, in conjunction with a highly fermentative *Saccharomyces cerevisiae* yeast to ensure the completion of fermentation, to see whether these novel yeasts could be applied effectively in winemaking for the optimization of the wine bouquet. Indeed, the impact of non-*Saccharomyces* species on the sensory profiles of wines was largely underestimated, because it was in fact unknown (Ciani 1997, and Ciani and Maccarelli 1998).

Not only are these yeasts proving to be very useful to correct certain analytical defects in wines, they also intensify and improve their sensory properties (Ciani and Picciotti 1995, Ciani 1997, Ciani and Maccarelli 1998, Ciani and Ferraro 1998, Ciani et al. 1996, and Ferraro et al. 2000). The *Torulaspora delbrueckii* (Ciani and Picciotti 1995, Martinez et al. 1990, Mauricio et al. 1991, and Moreno et al. 1991) and *Candida stellata* species in particular have been studied (Ciani and Ferraro 1998, Ciani et al. 1996, Ferraro et al. 2000, and Soden et al. 2000) for their organoleptic contributions during alcoholic fermentation. The succession of yeast populations, with the alternating dominance of "exotic" yeasts and *Saccharomyces* during alcoholic fermentation, has emerged as a cornerstone of the aromatic complexity of wines (Ciani 1997, Ferraro et al. 2000, Plata et al. 2002, and Rojas et al. 2003).

2. Screening of Non-*Saccharomyces* Candidates

First, a wide screening of several non-oenological *Saccharomyces* and non-*Saccharomyces* yeasts was performed to determine their contributions to the sensory properties of the final wine, and for their ability to survive until a fermentation progress of 0.5 dCO_2/dt (g/L/h).

During the fermentation of an actual grape must (a "neutral" variety – Maccabeu, INRA Pech Rouge, France) at 24°C, some tested yeasts present good fermentative capacities (high fermentation rates, short fermentation duration), but two of them present no efficient fermentation kinetics: a specific strain of *Candida stellata* presents a lag phase of about 50 hours and a very low maximal fermentation rate (0.6 g CO_2/h/L), even after oxygen and nitrogen additions, while *Candida parapsilosis* presents a total fermentation duration of more than 500 hours. Other non-*Saccharomyces* yeasts tested present good fermenta-

tive capacities that could cope with winemaking requirements, including *T. delbrueckii* TD291, *Hansenula anomala* and *C. stellata*.

An expert panel tasted the final wines in order to characterize initially whether the wines could be pooled or not, and to highlight their main potential sensory characteristics. The main characteristics are as follows.

• *Candida stellata*: The corresponding wine presents a good and powerful bouquet (banana and white flowers). Smooth and well-balanced mouthfeel. This wine is also characterized by a light bitterness at the end. This wine presents good overall sensory results.

• *Candida stellata* (different strain from above): The nose of this wine is less aromatic, with a small impression of reduction. In the mouth, it feels like a non-balanced wine, with bitterness, astringency and a rough and tart taste.

• *Torulaspora delbrueckii* TD291: This wine bouquet resembles *C. stellata* (powerful bouquet and banana aroma). A spicy- and toasty-flavoured wine. In the mouth, the taste is very fatty and looks like milk bread.

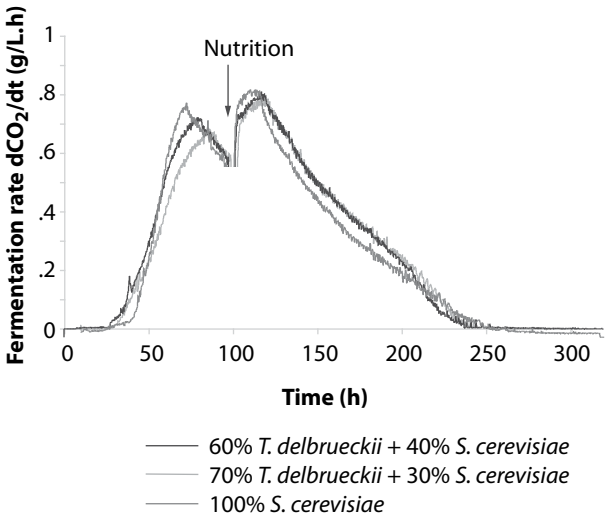
• *Hansenula anomala*: A not intense but fine bouquet characterizes this wine. It presents a light toasty flavour. A well-balanced wine.

Thanks to these organoleptic analyses, we demonstrated that some yeasts tested, such as *C. stellata* and *T. delbrueckii*, seem to enhance the aromatic properties of wines. However, the differences observed are not sufficient to enable a non-expert jury to train themselves to compare and describe wines. For these reasons, the *T. delbrueckii* was selected for its potential interest in mixed yeast cultures, as *T. delbrueckii* was known as initially present in some grape musts, and which did not confer organoleptic defects on the wines (Ciani and Picciotti 1995, Ciani 1997, Martinez et al. 1990, Mauricio et al. 1991, and Plata et al. 2002).

3. Extemporaneous Mixed Inoculation with *Saccharomyces cerevisiae* and *Torulaspora delbrueckii*

We wished to test the following premise: That inoculation with a mixed starter containing the *T. delbrueckii* yeast and an oenological *Saccharomyces* (EC1118) could directly generate complexity and intensity in the sensory profiles of wines. To validate this hypothesis, different extemporaneous co-inoculations were tested on a "neutral" grape variety (Maccabeu, INRA Pech Rouge, France), with a potential alcohol of 12.5% vol. The fermentation temperature was 20°C, and various co-inoculations were tested (figure 1). Standard analyses of the wines thus obtained are summarized in table 1. A significant decrease of the volatile acidity in the wines produced from mixed inoculations was noted (Fleet 1990, and Heard and Fleet 1985).

Figure 1. Fermentation kinetics



Surprisingly, the co-inoculation with *T. delbrueckii* and *S. cerevisiae* did not affect the aromatic complexity of the wines when compared to the wine fermented by a pure *S. cerevisiae*. The contrary was observed for the ex-

Table 1. Standard analysis of the final wines

Mixed inoculations	Alcohol (%, v/v)	pH	Total acidity (g/L H_2SO_4)	Volatile acidity (g/L H_2SO_4)	Free SO_2 (mg/L)	Total SO_2 (mg/L)	Residual sugars (g/L)
70% *Torulaspora delbrueckii* + 30% *Saccharomyces cerevisiae*	12.35	3.45	2.95	**0.10**	8	51	< 2
60% *T. delbrueckii* 60% + 40% *S. cerevisiae*	12.30	3.47	2.95	**0.16**	7	51	< 2
100% *S. cerevisiae*	12.20	3.48	3.10	**0.27**	15	70	< 2

temporaneous mixed inoculations: As the persistence of *T. delbrueckii* is important, great is the risk of an aromatic imbalance. In our case, this imbalance was characterized by the predominance of isoamyl acetate in the wine flavour profile. To confirm this orientation of the flavour profile of wines highlighted during chemical analysis, the wines were submitted to a sensory panel. This panel was deliberately composed of 20 non-professional tasters who are therefore more representative of current consumers. The impact of the amylic note was extremely strong, thus masking the presence of other esters in the wines produced by *S. cerevisiae*. However, the panel was unable to detect significant differences between the wines obtained after co-inoculations and the wine issued from fermentation by the pure *S. cerevisiae*.

According to these results, although co-inoculation between species has been seen as a way to increase the intensity of certain aromatic notes in wines, the risk of accentuating the aromatic imbalance and then depleting the aromatic complexity of the wines was certain (Plata et al. 2002, and Rojas et al. 2003). Furthermore, the assumption that co-inoculation with an "exotic" yeast and a *Saccharomyces* yeast would directly generate complexity and intensity in the resulting sensory profiles was not confirmed in our study. This could be attributed to the potential synergies and antagonisms between the yeasts tested, inherent to the metabolic characteristics of each species.

We therefore developed a new strategy based on the successive inoculation of both yeasts. With this approach, we had the ambition of replicating the ecology of the environment of natural spontaneous alcoholic fermentations by:

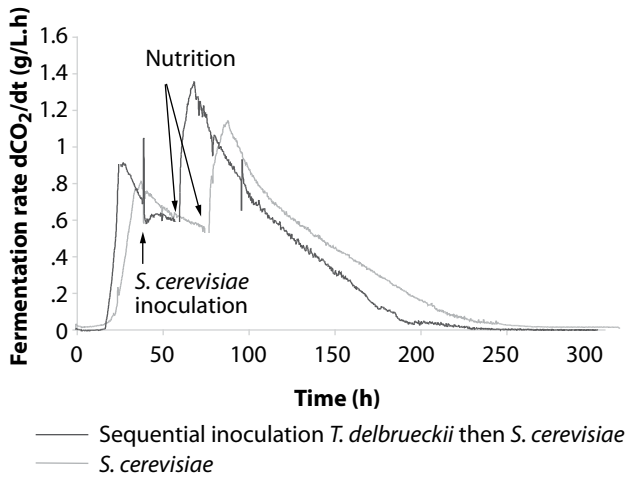
- Promoting the growth of the "exotic" yeasts during the first third of the alcoholic fermentation, which is a crucial time for the elaboration of the balance and aromatic intensity of wines
- Then promoting the development of the *Saccharomyces* yeast to secure the completion of fermentation.

Such a sequential inoculation avoids the competition between the fermentation and the organoleptic qualities of each species (Ciani and Ferraro 1998, Ciani et al. 1996, Ferraro et al. 2000, and Zironi et al. 1993).

4. Sequential Mixed Inoculation with *Saccharomyces cerevisiae* and *Torulaspora delbrueckii*

Under temperature and nutrition conditions identical to those described above, we worked on a Maccabeu grape variety with an alcohol potential of 13.6%/vol. We tried to reproduce the succession of natural yeast populations by first inoculating with *T. delbrueckii*, and, after 35 hours of fermentation, inoculating with *S. cerevisiae* EC1118 (figure 2). Standard analyses of the resulting wines are summarized in table 2. A strong decrease in volatile acidity was already observed in the mixed culture compared to the *S. cerevisiae* monoculture.

Figure 2. Fermentation kinetics



The resulting aroma profile of the sequential development of *T. delbrueckii* and *Saccharomyces* revealed a harmonious increase of ester concentrations (figures 3 and 4): four of the six esters assayed were present in higher concentrations than those observed in the wine from the pure *Saccharomyces* fermentation. Only isoamyl acetate, which exerted a significant dominant contribution on the flavour profile of the control wine, decreased after sequential inoculation.

Table 2. Standard analysis of the final wines

Mixed inoculations	Alcohol (%, v/v)	pH	Total acidity (g/L H_2SO_4)	Volatile acidity (g/L H_2SO_4)	Free SO_2 (mg/L)	Total SO_2 (mg/L)	Residual sugars (g/L)
Torulaspora delbrueckii* then *Saccharomyces cerevisiae	13.25	3.58	3.05	**0.36**	8	38	**< 2**
***S. cerevisiae* alone**	12.20	3.48	3.10	**0.27**	15	70	**< 2**

Figure 3. Summary of the impact of sequential inoculation on the ester profiles of the wines

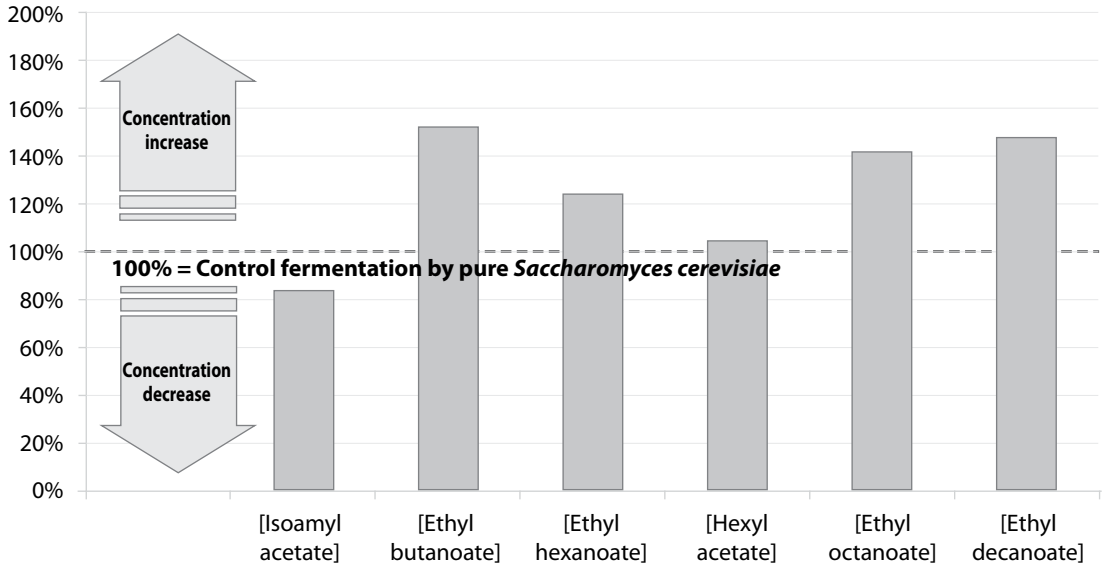
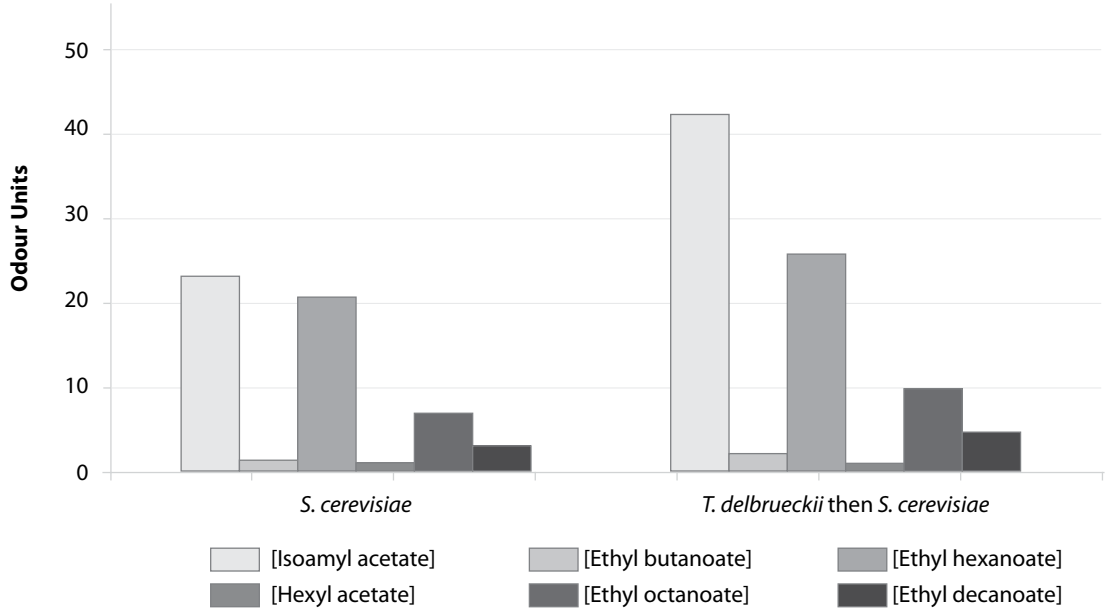


Figure 4. Aromatic ester profiles of the wines

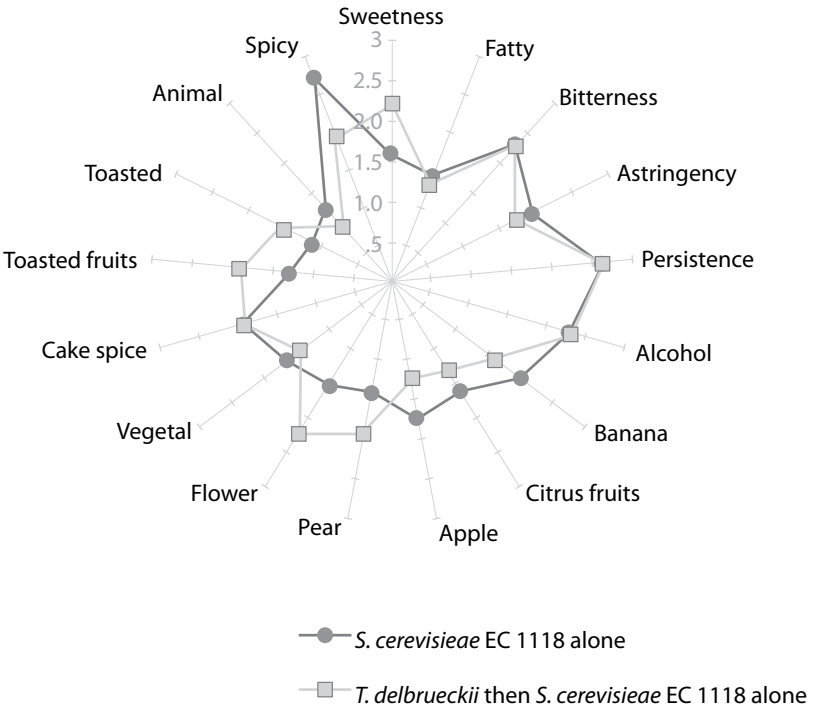


The wines obtained were tested for differentiation and characterization by sensory analysis. Statistically significant differences at the level of 5% were found between the wines from the sequential inoculation of *T. delbrueckii* and *S. cerevisiae* and the control wine resulting from the inoculation of the pure *S. cerevisiae*. Sensory profiles obtained by descriptive analysis showed an intensification of certain descriptors such as floral, cooked fruit and gingerbread, supported by a more intense sweetness on the palate. More common notes, such as apple, banana, spicy and animal, were clearly diminished (figure 5).

5. Conclusions

Our results clearly showed that the predominance of *Torulaspora delbrueckii* in the initial part of fermentation is a key factor for a homogeneous increase of esters in wines produced with mixed cultures. Very similar results to those presented here with *T. delbrueckii* were also obtained with a *Candida stellata*, thus reinforcing our decision to develop sequential mixed non-*Saccharomyces* and *Saccharomyces* starters, rather than extemporaneous inoculations.

Figure 5. Comparison of the sensory profiles obtained



Several important conclusions could be drawn:

- Yeast (non-*Saccharomyces*) selection could be realized by simple experiments based on sequential inoculation (fermentation kinetics)
- It is impossible to control the remanence of non-*Saccharomyces* yeasts during mixed cultures in alcoholic fermentation by varying the inoculum ratio (the effect of vitamins, oxygen and nitrogen)
- In term of sensorial (and chemical) impact on the final wine, sequential inoculation results in much more interesting wines than extemporaneous inoculation
- Long remanence of non-*Saccharomyces* yeasts during the fermentation is not necessary to obtain a sensorial impact.

The choice of a sequential inoculation with an "exotic" yeast followed by the later inoculation of the *Saccharomyces* yeast remains the only way to increase the aromatic complexity of wines. After all, by using a sequential inoculation of non-*Saccharomyces* and *Saccharomyces* – a natural succession of yeast flora – oenologists are only mimicking nature!

References

Ciani, M., and G. Picciotti. 1995. The growth kinetics and fermentation behaviour of some non-*Saccharomyces* yeasts associated with wine-making. *Biotech. Letters*. 17:1247-1250.

Ciani, M. 1997. Role, enological properties and potential use of non-*Saccharomyces* wine yeasts. *Recent Res. Dev. Microbiol.* 1:317-331.

Ciani, M., and F. Maccarelli. 1998. Oenological properties of non-*Saccharomyces* yeasts associated with wine-making. *World J. Microbiol. Biotechnol.* 14:199-203.

Ciani, M., and L. Ferraro. 1998. Combined use of immobilized *Candida stellata* cells and *Saccharomyces cerevisiae* to improve the quality of wines. *J. Appl. Microbiol.* 85:247-254.

Ciani, M., L. Ferraro, and F. Fatichenti. 1996. Enhanced glycerol content in wines made with immobilized *Candida stellata* cells. *Appl. Env. Microbiol.* 62:128-132.

Etievant, P. 1991. Wine. In: *Volatile compounds in food and beverages*. Ed. H. Maarse. Marcel Dekker Inc. New York. 483-546.

Ferraro, L., F. Fatichenti, and M. Ciani. 2000. Pilot scale vinification process using immobilized *Candida stel-*

lata cells and *Saccharomyces cerevisiae*. *Process Bioch*. 35:1125-1129.

Fleet, G. H. 1990. Growth of yeast during wine fermentations. *J. Wine Res*. 3:211-223.

Heard, G. M., and G. H. Fleet. 1985. Growth of natural yeast flora during the fermentation of inoculated wines. *Appl. Environ. Microbiol*. 50:727-728.

Martinez, J., F. Toledano, C. Millan, and J. M. Ortega. 1990. Development of alcoholic fermentation in non-sterile musts from Pedro Ximenez grapes inoculated with pure cultures of selected yeasts. *Food Microbiol*. 7:217-225.

Mauricio, J. C., S. Guijo, and J. M. Ortega. 1991. Relationship between phospholipids and sterol contents in *Saccharomyces cerevisiae* and *Torulaspora delbrueckii* and their fermentation activity in grape musts. *Am. J. Enol. Vitic*. 42:301-308.

Mora, J., J. I. Barbas, and A. Mulet. 1990. Growth of yeast species during the fermentation of musts inoculated with *Kluyveromyces thermotolerans* and *Saccharomyces cerevisiae*. *Am. J. Enol. Vitic*. 41:156-159.

Moreno, J. J., C. Millan, J. M. Ortega, and M. Medina. 1991. Analytical differentiation of wine fermentations using pure and mixed yeast cultures. *J. Ind. Microbiol*. 7:181-190.

Plata, C., C. Millan, J. C. Mauricio, and J. M. Ortega. 2002. Formation of ethyl acetate and isoamyl acetate by various species of wine yeasts. *Food Microbiol*. 20:217-224.

Rojas, V., J. V. Gil, F. Pinaga, and P. Manzanares. 2003. Acetate ester formation in wine by mixed cultures in laboratory fermentations. *Int. J. Food Microbiol*. 86:181-188.

Soden, A., I. L. Francis, H. Oakey, and P. Henschke. 2000. Effects of co-fermentation with *Candida stellata* and *Saccharomyces cerevisiae* on the aroma and composition of chardonnay wine. *Austr. J. Grape Wine Res*. 6:21-30.

Zironi, R., P. Romano, G. Suzzi, F. Battistutta, and G. Comi. 1993. Volatile metabolites produced in wine by mixed and sequential cultures of *Hanseniaspora guilliermondii* or *Kloeckera apiculata* and *Saccharomyces cerevisiae*. *Biotech. Letters*. 15:235-238.

UTILIZING NON-TRADITIONAL YEASTS IN WINEMAKING SEQUENTIAL CULTURES: *METSCHNIKOWIA PULCHERRIMA/ SACCHAROMYCES CEREVISIAE*

Maria Angelica GANGA[1], Alvaro ARAVENA[1], Hector REBOLLEDO[1], Felipe FONSECA[1], Edith QUEZADA[1], Claudio MARTINEZ[1, 2], Pedro CARRILES[3], José Maria HERAS[4] and Anne ORTIZ-JULIEN[5]

[1] Department of Science and Food Technology, Laboratory of Biotechnology and Applied Microbiology (LAMAP), University of Santiago, Chile

[2] Study Center in Food Science and Technology, University of Santiago, Chile

[3] Lallemand Chile, Av Ricardo Lyon 400, Dept 68, Providencia, Santiago, Chile

[4] Lallemand Spain C/ Zurbano, 71 Officina 6, 28010 Madrid, Spain

[5] Lallemand France, 19, rue des Briquetiers, 31702 Blagnac, France

1. Introduction

In wine production, yeasts are responsible for transforming the sugar present in grape must into ethanol, carbon dioxide and hundreds of secondary products that collectively contribute to the various qualities of the product (Fleet 2003). These microorganisms can therefore have a positive or negative effect on the sensory qualities of the wine. Initially, the must is dominated by non-*Saccharomyces* yeasts because the concentration of *Saccharomyces* yeasts is low, but the *Saccharomyces* yeasts gradually take over during alcoholic fermentation (AF) (Pretorius et al. 2000). Although non-*Saccharomyces* yeasts were long considered harmful to the sensory characteristics of wine, in recent years it has become clear that utilizing these yeast starter cultures under controlled conditions may provide more complex sensory characteristics, thus improving the quality of the final product (Ciani and Ferraro 1998, and García et al. 2002). Experiments carried out by Ciani and Picciotti (1995) on white musts, utilizing six non-*Saccharomyces* yeasts independently, showed that the AF of natural must with *Candida stellata* produced wine with a higher concentration of glycerol compared with other microorganisms utilized, including *Saccharomyces cerevisiae*.

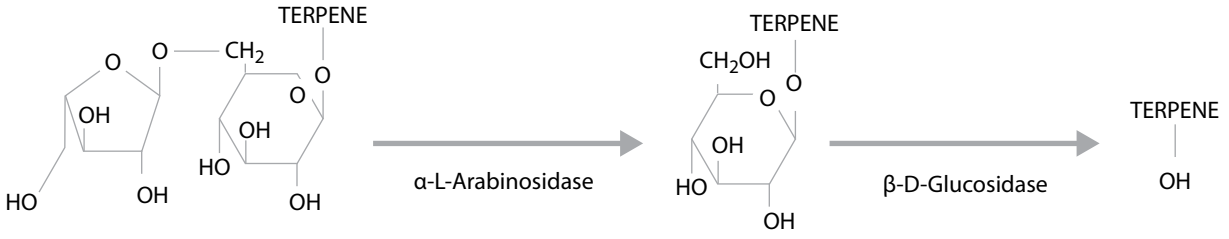
On the other hand, the *Hanseniaspora uvarum* and *Kloeckera apiculata* yeast species tested showed similar values to those produced in the fermentation with *S. cerevisiae* only. As well, the mixed culture produced high amounts of ethyl acetate and acetic acid, which would exclude these yeasts from winemaking. In a similar experiment, Ciani and Maccarelli (1998) observed that when utilizing *C. stellata* the resulting product showed a high concentration of glycerol and succinic acid compared to the other species tested. When testing Chardonnay must with *Candida membranaefaciens*, Garcia et al. (2010) obtained wines that were more complex on an organoleptic level, with a notable increase in esters.

For some years, our working group has been working on defining the potential of non-*Saccharomyces* yeasts for utilization in winemaking by studying the enzymes (xylanases, cellulases and glycosylases, etc.) they secrete into the medium (Ganga and Martinez 2004). We have therefore been able to determine that a strain of *Metschnikowia pulcherrima* (L1781 or known as MP346), isolated from the Maule region of Chile (between the 35°S and 36°S parallels), secretes an enzyme with α-arabinofuranosidase activity into the culture medium.

Why is an enzyme with α-arabinofuranosidase activity important in winemaking?

One of the factors that most influences the aroma characteristic of a given grape varietal is the ripening stage. Both free and bound compounds accumulate in grapes during this period (Günata et al. 1985, and Sánchez et al. 2007). Many aromatic compounds are present in grapes, including alcohols, aldehydes, ketones, esters, acids and terpenes (Aznar et al. 2001). However, terpenes are the

Figure 1. Sequential action of hydrolytic enzymes on aromatic precursors (Günata et al. 1988)



main components responsible for the characteristic fruity aroma (Vilanova and Sieiro 2006). A large portion of the terpenes are found bound to sugars preventing them from forming part of the aroma of the product (Günata et al. 1988). These glycosylated terpenes can be transformed into their free form by the hydrolytic action of glycoside enzymes (figure 1) (Günata et al. 1988). The most outstanding terpenes are linalool, geraniol, nerol, citronerol and α-terpineol (Marais 1983, and Günata et al. 1985). Most of the glycosidic residues accompanying the aroma precursors are of the arabinofuranosidase and glycoside types (Yanai and Sato 2000). That is why enzymes with α-arabinofuranosidase and β-glucosidase activity are extremely important in the release of volatile aromatic compounds. As shown in figure 1, the first enzyme catalyzes the hydrolysis of the link between arabinose and glucose, releasing the substrate for the action of the second enzyme, which is capable of hydrolyzing the bond between the glucose and the terpene, the latter forming part of the aroma (Günata et al. 1988).

Based on the above, the objective of this study was to determine whether the drying process of the *M. pulcherrima* L1781 yeast affects the production of α-arabinofuranosidase activity described in the original isolate, and to examine the effect of its utilization on the sensory characteristics of wines produced with sequential cultures (*M. pulcherrima* + *S. cerevisiae*).

2. Materials and Methods

2.1 Preliminary studies

Microorganisms: For the study, we used the strain *M. pulcherrima* L1781 (or MP246 or its commercial name Flavia®) – part of the culture collection at the Laboratory of Biotechnology and Applied Microbiology, Universidad de Santiago, Chile (LAMAP-USACH) – produced in active dry form by Lallemand. *Saccharomyces cerevisiae* var. *bayannus* Lalvin QA23® (Lallemand) was also used. To inoculate with the active dry yeast provided by Lallemand, we followed the instructions on each package. A concentration of 25 g/hL was used in the tests.

RAPD analysis: For the random amplification of polymorphic DNA (RAPD) reaction, the following mixture was used: dNTPs 5 µL (1 mM); $MgCl_2$ 4 µL (25 mM); Taq 2 µL (1 U/µL); Primer (5`-CTGAAGCGCA-3`) 16 µL (10 µM) and DNA 2 µL (~0.5 µg/µL), totalling a final volume of 50 µL with distilled water. The amplification program consisted of the following: 95°C for 3 minutes, 94°C for 1 min, 35°C for 2 min, 72°C for 2 min for 44 cycles, then one cycle at 72°C for 5 min.

Growth medium with sugar beet cossettes: In order to define the α-arabinofuranosidase activity of the yeast isolates under study (*M. pulcherrima* L1781 dry yeast, as well as the original control strain obtained directly from the LAMAP-USACH strain collection), each yeast was grown in a culture medium with sugar beet cossettes – 3 g/L $(NH_4)_2S0_4$, 1 g/L KH_2P0_4, 0.5 g/L $MgS0_4$ x 7 H_2O, 20 g/L beet cossettes, 5 g/L yeast extract, adjusted to a pH of 5.2 (Labbé and Pérez 2003, and De Ioannes et al. 2000).

Quantification of the α-arabinofuranosidase activity: Once the microorganism has grown on the medium inducing the sought-after activity, 10 mL of culture medium was freeze-dried by lyophilization. The amount of protein present in the dry extract was obtained according to the protocol described by Bradford (1976), using bovine serum albumin (BioLabs, United States) as the standard. With the lyophilized samples, the enzyme activity was determined in accordance with the protocol described by Günata et al. (1990). One unit (U) of enzyme activity was defined as the amount of enzyme that 11 µmol of p-nitrophenyl-α-L-arabinofuranoside (pNPA) consume per minute.

2.2 Fermentation and analysis

Fermentations in natural must: Fermentations were carried out at the laboratory and pilot levels. In both cases, tests were conducted with *M. pulcherrima* L1781 / *S. cerevisiae* Lalvin QA23® sequential cultures, and as the control test, fermentation with only *S. cerevisiae* Lalvin QA23® was used. In the fermentations, GoFerm Protect® (Lallemand) and Fermaid® K (Lallemand) nutrients were used in accordance with the supplier's instructions.

At the end of the fermentations, the wines were left to stand for 21 days at 12°C, adding Lallzyme® Beta enzyme (Lallemand) at a concentration of 5 g/hL.

Testing at the laboratory level: A quantity of 3.2 L of natural must of the Muscat of Alexandria (Chile) varietal was fermented in reactors with a maximum capacity of 5 L. To inoculate the must, culture starters were prepared with *S. cerevisiae* Lalvin QA23® and *M. pulcherrima* L1781 active dry yeasts at a concentration of 100 g/L. After obtaining each starter culture, in the case of the *M. pulcherrima* each must was inoculated at a concentration of $1x10^7$ CFU/mL. After 48 hours, a concentration of $1x10^8$ CFU/mL of *S. cerevisiae* was added to the must. To determine the viability of *M. pulcherrima*, must samples were taken every six hours during the first two days and then every 24 hours until its disappearance; for the quantification of *S. cerevisiae*, samples were collected every 48 hours. As the control test, fermentation was performed using a monoculture (*S. cerevisiae* only) at a concentration of $1x10^8$ CFU/mL. The fermentation processes were followed by quantification of the reducing sugars (Somogyi 1952), taking samples every two days until the end of the fermentation. The trials were conducted in triplicate.

Laboratory trials in oenological conditions (1.1 L):

- **Medium**. We used the synthetic medium described by Bely et al. (1991) containing 460 mg/L of nitrogen, 220 g/L of sugar, with no aerobic factor. Its amino acid composition simulates the nitrogen level of a standard grape must.
- **Fermentations**. Fermentations were performed under constant stirring at 20°C, in small fermenters (1.2 L) with fermentation locks.

  - Monitoring and control of fermentations:

  - **CO_2.** The amount of CO_2 released was determined by automatic measurements of fermenter weight loss every 20 minutes. The validity of this technique for estimating sugar and alcohol concentrations has been previously described.
  - **CO_2 production rate (dCO_2/dt).** The CO_2 production rate was calculated by polynomial smoothing of the last 11 evolved CO_2 values. The frequent acquisitions of CO_2 release and the precision of the weighing (0.1 or 0.01 g) allowed for the calculation of the CO_2 production rate with good precision and repeatability: variation coefficient of (dCO_2/dt) max = 0.8%.

Testing at the pilot level: The tests were conducted at a winery in Chile, where 900 L of Muscat of Alexandria must was inoculated with a concentration of 25 g/hL of *M. pulcherrima* L1781 active dry yeast (Lallemand). After 48 hours of incubation, it was then inoculated with *S. cerevisiae* Lalvin QA23® at a concentration of 25 g/hL. The fermentation was followed by decreasing the density. As a control test, fermentation was also conducted using *S. cerevisiae* only.

Physicochemical and sensory analyses, and aroma profile of the wines: Upon completing the maceration time on lees, the wines were filtered and the physicochemical analyses were performed (concentration of reducing sugars, alcohol concentration, pH, free and total acidity) using the protocols indicated by the OIV (Bordeu and Scarpa 1998). The analyses were conducted in duplicate. In addition, the wines were evaluated by a panel of winemakers (Flanzy et al. 2003, and Anzaldúa-Morales 1994). The aroma profile of the wines was performed by the Centro de Aromas de la Pontificia Universidad Católica de Chile (the aroma centre of the pontifical Catholic university of Chile), using gas chromatography-mass spectrometry (GC-MS); HP6890, MSHP972, Hewlett-Packard, Palo Alto, CA.

Statistical analysis: All the results were analyzed by analysis of variance (ANOVA), using the computer program Statgraphics Centurion XV, version 15.2.05.

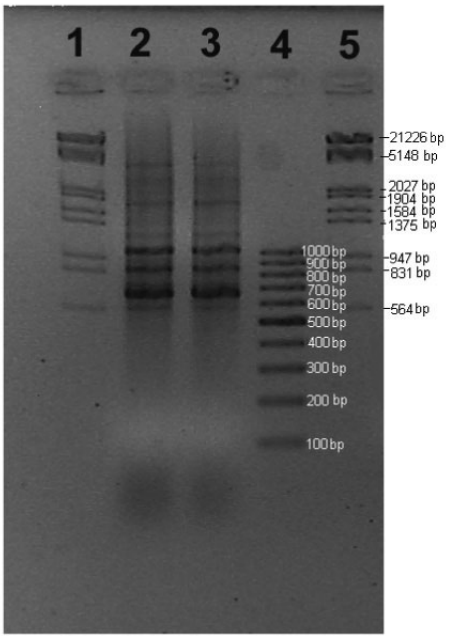
3. Results and Discussion

3.1 Preliminary studies

3.1.1 Comparison of the LAMAP-USACH *Metschnikowia pulcherrima* L1781 isolate and the dry product obtained by Lallemand

In order to verify that the dry isolate obtained by Lallemand has the characteristics of LAMAP-USACH (Fonseca 2008) a molecular analysis to compare the genetic profiles of both isolates was carried out. Based on this, the DNA from each sample (the dry yeast by Lallemand and the one obtained directly from the LAMAP-USACH strain collection) was subjected to a random amplified polymorphic DNA (RAPD) analysis (see figure 2). This figure shows that both isolates have the same electrophoretic pattern, confirming the isolates are the same.

Figure 2. Electrophoresis in 1% agarose gel. Tracks 1 and 5: marker phage digested with EcoRI + HindIII; track 2: isolate from *Metschnikowia pulcherrima* L1781 of LAMAP; track 3: *Metschnikowia pulcherrima* L1781 dry yeast supplied by Lallemand; Cariil 4: 100 bp marker



3.1.2 Quantification of α-arabinofuranosidase activity in the LAMAP-USACH L1781 isolate

In order to determine if the drying of *M. pulcherrima* L1781 affected the secretion of α-arabinofuranosidase activity, tests were carried out with the dry isolate and the control isolate (LAMAP-USACH strain collection). For this, both yeasts were grown in a medium with beet cossettes as a carbon source, in order to induce enzyme activity. Table 1 shows the results obtained.

Table 1. Determination of α-L-arabinofuranosidase activity

Metschnikowia pulcherrima L1781	Specific activity [U/mg]
Dry (Lallemand)	0.22[a]
Control yeast (LAMAP-USACH strain collection)	0.23[a]

One or more letters between the values in the same column indicates that there are no statistically significant differences at a confidence level of 95%.

The growth of yeasts was performed in duplicate. The enzymatic activity of each sample was determined in triplicate. U = the amount of enzyme that 1 µmol of pNPA consumes per minute.

When contrasting the activities obtained from both isolates, one can see there are no statistically significant differences between the activity of the dry isolate and the original microorganisms. Therefore the production of α-arabinofuranosidase activity by dry *M. pulcherrima* was not affected by the drying process.

3.2 Vinification in natural must

Testing at the laboratory level: The tests were conducted on must from the Muscat of Alexandria varietal, as described in the MATERIALS AND METHODS section. This varietal has been described as one of the richest in aroma compounds and precursors, specifically monoterpenes, in their free form (1513 µg/L) or linked to glycosides (4040 µg/L) (Ribérau-Gayon et al. 2000). A first test was conducted consisting of fermentations with sequential cultures of *M. pulcherrima* L1781 and then *S. cerevisiae* Lalvin QA23®. The non-*Saccharomyces* isolate was incubated for 48 hours and then *S. cerevisiae* was added. At the same time, a second test was conducted where only *S. cerevisiae* (in a monoculture) was used. AF was followed by the quantification of reducing sugars, and the viability of the yeasts was also determined throughout the process.

- **Growth of microorganisms:** To verify that the *M. pulcherrima* yeast grew in the inoculated must, samples were taken every six hours over a period of two days, which were inoculated on slides with a culture medium. *M. pulcherrima* has the ability to produce a reddish pigment in the culture medium, resulting in a pinkish halo around the colonies. This facilitates differentiation (Sipiczki 2006). The results showed that *M. pulcherrima* managed to remain in the must during the 48 hours of incubation. The yeast population remained virtually constant, with no significant growth observed. After inoculating the must with *S. cerevisiae*, the pink yeast population disappeared and *S. cerevisiae* (white colonies) prevailed entirely. *S. cerevisiae* is known to be a high-fermenting yeast (Moreno and Polo 2005), which suggests that once inoculated into the must the production of alcohol increases rapidly, thus causing the death of yeasts less tolerant to alcohol. It has been described that *M. pulcherrima* would remain in a culture medium until reaching 6% v/v of ethanol (Parapouli et al. 2010). From an oenological point of view, this is extremely important, because the must would be safe once it is inoculated with *S. cerevisiae* and the *M. pulcherrima* disappears and they do not become a problem in the production process.

- **Fermentation follow-up:** The monitoring of the fermentation processes tested (sequential cultures and monoculture) was carried out through the consumption of reducing sugar. Sugar consumption started slowly in the

sequential cultures, with sugar levels remaining practically constant until the third day. This concurs with the fact that *M. pulcherrima* was maintained during the first 48 hours before adding the *S. cerevisiae* culture. It is known that *M. pulcherrima* has a low fermentation capacity (Ciani and Maccarelli 1998) and therefore a low rate of consumption of glucose/fructose under anaerobic conditions. However, after inoculation with *S. cerevisiae*, a sharp drop in sugar levels was observed, indicating the sugar was being rapidly consumed. This behaviour was maintained until approximately the tenth day, and from that point on the sugar consumption became slower and more gradual. This decrease can be explained by the increase in the concentration of ethanol in the medium, which leaves a large portion of metabolically inactive yeasts and could affect the metabolism of glucose (Holm et al. 2001). In the control fermentation (*S. cerevisiae* monoculture), rapid sugar consumption was obtained from the start of fermentation until about the eighth day, and then it showed a slow decrease in the behaviour of reducing sugar, similar to what was observed in the sequential cultures.

- **Physicochemical analysis of the wines:** Once AF was completed and after the enzyme preparation was added, the wines were left to stand for 21 days at 12°C. The physicochemical analyses were then carried out on the wines. Table 2 shows the results.

To analyze ethanol concentration, a box-and-whisker plot was made, as the data did not show a normal distribution.

In the table, it is possible to observe that for all the tests the concentration values of pH, ethanol and volatile acidity are within the typical values for the wines (Bordeu and Scarpa 2000). As for the residual sugar, a lower quantity was noted in the test made with sequential cultures than in the monoculture. Similar results were obtained by Jolly et al. (2003) by using mixed cultures of non-*Saccharomyces/S. cerevisiae*. Nevertheless, from a winemaking perspective both wines achieved successful fermentation (dry wines).

- **Analysis of wine aromas:** To understand the aroma characteristics of the wines produced, they were submitted to a tasting panel, which gave the highest score to the wine made with sequential cultures (*M. pulcherrima* + *S. cerevisiae*). The oenologists described the wine as having more intense aromas, emphasizing fruity and floral esters. As noted above, during its stay in the must *M. pulcherrima* secreted an enzyme with α-arabinofuranosidase activity that acted on the terpene glycosides found in the must (Günata et al. 1998). Subsequently, with the help of the β-glucosidase enzyme (delivered in this test by Lallzyme® Beta), it allowed these terpenes to form part of the aromatic fraction of wine. The presence of free terpenes gives wines marked floral (geraniol and linalool) and citrus aromas (citronerol) (Günata et al. 1985, Marais 1983, and Vilanova and Sieiro 2006).

3.3 Characterization of *Metschnikowia pulcherrima* 346 in oenological conditions with sequential inoculations

To characterize the fermentative behaviour of *M. pulcherrima* (available commercially as Flavia®) in oenological conditions, a series of fermentations were performed in synthetic must first and then validated on Sauvignon Blanc must.

The strategy chosen was a sequential inoculation to allow the full expression of the non-*Saccharomyces* species from the earliest stage of the fermentation, especially its enzymatic activities, followed by a *S. cerevisiae* whose role was to secure the fermentation. Flavia® was first inoculated into the must or grapes at 25 g/hL (in active dry form) and then 48 hours later, the *S. cerevisiae* was inoculated into the must at 25 g/hL.

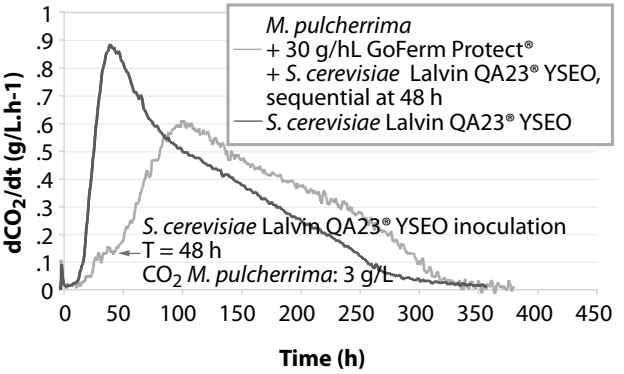
The fermentation kinetics are shown in figure 3 and compare the fermentation rate of the single inoculation of the *S. cerevisiae* with the sequential inoculation of *M. pulcherrima* at the beginning followed by the *S. cerevisiae* 48 hours later.

Table 2. Physicochemical analysis of wines from the Muscat of Alexandria variety utilizing sequential cultures or monoculture

Test	Residual sugar [g/L]	pH	Total acidity [g H_2S0_4/L]	Volatile acidity [g acetic acid/L]	Ethanol [% v/v]
1	1.33^{a}	3.82^{b}	1.95^{d}	0.04^{c}	11.80^{ab}
2	1.41^{c}	3.74^{a}	1.76^{c}	0.02^{a}	11.08^{a}

Test 1: Fermentation with *M. pulcherrima* L1781 + *S. cerevisiae* Lalvin QA23® in sequential cultures.
Test 2: Fermentation with *S. cerevisiae* Lalvin QA23® monoculture.
One or more letters between the values of a column indicate no statistically significant differences at a confidence level of 95%, according to the multiple range test.

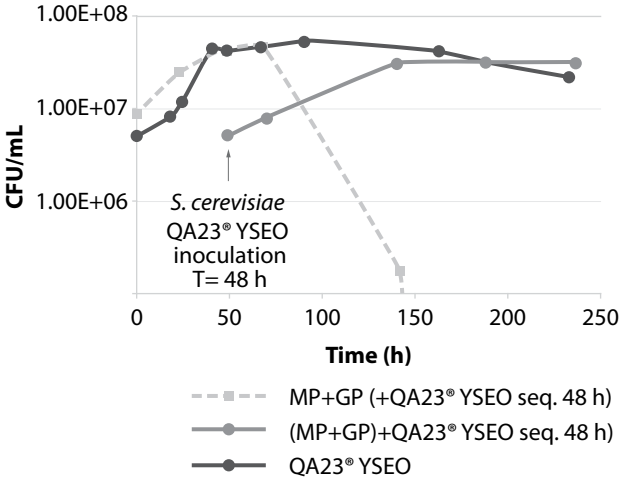
Figure 3. Fermentation kinetics of *Metschnikowia pulcherrima* and *Saccharomyces cerevisiae* compared to *S. cerevisiae* alone in Sauvignon Blanc



The kinetic profiles are different with a lower maximum speed of fermentation for the sequential inoculation, and a delayed onset of fermentation because of the lower fermentative activity of the *M. pulcherrima*. However, the fermentation lengths are quite similar and both fermentations are dry.

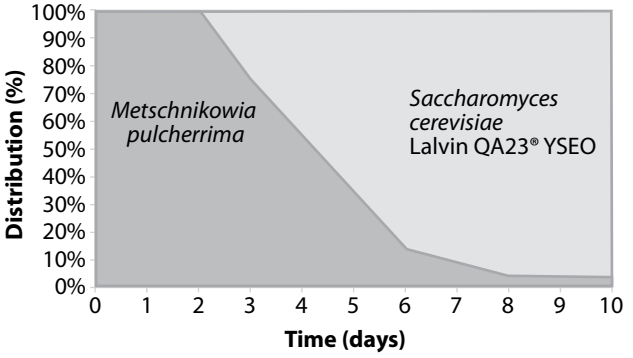
Figure 4 represents the follow-up of the population for both fermentations (sequential and single) of the two species in the case of sequential inoculation.

Figure 4. Populations of *Metschnikowia pulcherrima* and *Saccharomyces cerevisiae* compared to *S. cerevisiae* alone in Sauvignon Blanc



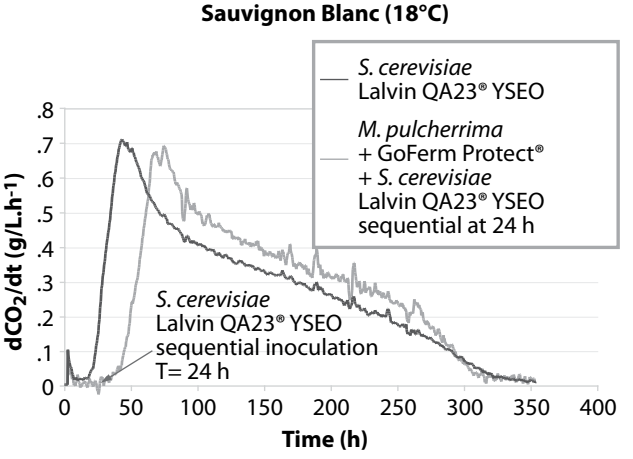
It's interesting to note the good multiplication of the *M. pulcherrima* during the two first days of AF. Once the medium is inoculated with *S. cerevisiae*, we observe one day of cohabitation of both species before a drastic die off of the *M. pulcherrima*, allowing the complete colonization of the medium by *S. cerevisiae*. The distribution of the viable population during the alcoholic fermentation is presented in figure 5.

Figure 5. Distribution of viable populations during alcoholic fermentation measured by flow cytometry



To study the interactions between both populations, the same experiments were carried out on Sauvignon Blanc varietal grapes. The only difference was the inoculation of the *S. cerevisiae* 24 hours after the inoculation with Flavia®, to avoid any risk of starting fermentation with non-selected *S. cerevisiae*. Indeed, Flavia® is highly sensitive to SO_2 so it is necessary to limit the SO_2 addition before inoculation with M. pulcherrima (figure 6).

Figure 6. Fermentation kinetics of *Metschnikowia pulcherrima* and *Saccharomyces cerevisiae* compared to *S. cerevisiae* alone in Sauvignon Blanc



S. cerevisiae was inoculated into must 24 hours after *M. pulcherrima*

In a trial done with Flavia® and Lalvin QA23® in Muscat of Alexandria must (2010), where a concentration of 25 g/hL (approximately 1 x 10^7 cells/mL) of Flavia® was inoculated into the must and then, after 48 hours of growth, the must was inoculated with *S. cerevisiae* Lalvin QA23® (25 g/hL). The finished wines were submitted to a tasting panel, where 80% of the oenologists expressed greater preference for the wine produced with the sequential cultures. This wine was characterized by an intense aroma emphasizing flowers, pineapple and mercapto-pentanone. To

correlate the results of the tasting panel with the aromas of the wines, the aroma profiles of the wines were analyzed by gas chromatography. This showed that the concentration of free terpenes was greater in the sample obtained from the sequential yeast cultures compared to the control wine, highlighting a greater intake of ß damascenone, linalool and geraniol, which are responsible for the tobacco, lavender and rose notes, respectively (Jackson 1998). Along with the increase of these volatile terpenes, there was an increase of 4-methyl 4-mercapto 2-pentanone (4MMP) and 3-mercaptohexanol (3MH), which deliver aromas of herbs, tomato leaves and grapefruit.

In 2010 and 2011, fermentations were carried out in other wine-producing countries (Portugal, Italy and Spain) using Flavia® In these cases, various varietals (Sauvignon Blanc, Alvariño, Garganega and Colombard) were utilized, showing similar results, i.e., greater complexity in the aroma of wines fermented with sequential cultures, with a predominance of aromas related to terpenes and thiols.

4. Conclusions

This study demonstrated the potential of non-*Saccharomyces* yeasts in the field of oenology. Although it is known that the presence of these yeasts at the start of alcoholic fermentation results in more complex aromas in the finished product, their utilization in the winery is difficult due to the lack of control. However, the utilization of pure cultures that can be controlled by a natural biocontrol process, like the production of ethanol by *S. cerevisiae*, would allow these yeasts to be utilized without restriction.

The utilization of *Metschnikowia pulcherrima* L1781 active dry yeast (Flavia®) in sequential fermentations has produced wines that are more complex on the organoleptic level. This yeast would act first on the glycosylated terpenes naturally present in the grapes, and would be capable of releasing volatile thiols while it remains in the must. Both volatile compounds deliver fruity and complex aromas, a quality sought after by the most demanding consumers.

References

Anzaldúa-Morales, A. 1994. *La evaluación sensorial de los alimentos en la teoría y la práctica*. Edit. Acribia, Zaragoza, España.

Aznar, M., R. Lopez, J. Cacho, and V. Ferreira. 2001. Identification and quantification of impact odorants of aged red wines from Rioja. GC-Olfactometry, quantitative GC-MS, and odor evaluation of HPLC fractions. *J. Agric. Food Chem*. 49:2924-2929.

Bely, M., J. M. Sablayrolles, and P. Barre. 1991. Automatic detection of assimilable nitrogen deficiencies during alcoholic fermentation in enological conditions. *J Ferm Bioeng*. 70:246-252.

Bordeu, E., and J. Scarpa. 1998. In: *Análisis químico del vino*, Textos Universitarios Facultad de Agronomía e Ingeniería Forestal. Ediciones Universidad Católica de Chile, Santiago, Chile.

Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Bioch*. 72:248-254.

Ciani, M., and L. Ferrano. 1998. Combined use of immobilized *Candida stellata* cells and *Saccharomyces cerevisiae* to improve the quality of wines. *J. Appl. Microbiol.* 85:247-254.

Ciani, M., and G. Picciotti. 1995. The growth kinetics and fermentation behaviour of some non-*Saccharomcyes* yeast associated with wine-making. *Biotechnol. Lett.* 17:1247-1250.

Ciani, M., and F. Maccarelli. 1998. Oenological properties of non-*Saccharomyces* yeasts associated with winemaking. *World J. Microbiol. Biotech.* 14:199-203.

De Ioannes, P., A. Peirano, J. Steiner, and J. Eyzaguirre. 2000. An α-L-arabinofuranosidase from *Penicillium purpurogenum*: Production, purification and properties. *J Biotec*h. 7:253-258.

Flanzy, C., A. López, J. Quevedo, V. Madrid, and A. Madrid. 2003. *Enología: Fundamentos científicos y tecnológicos*. Mundi-Prensa Libros. Madrid.

Fleet, G. H. 2003. Yeast interactions and wine flavour. *Int J Food Microbiol*. 86(1-2):11-22.

Fonseca, F. 2008. *Estudio de la producción de enzimas glicosídicas en levaduras vínicas nativas y su potencial uso en la industria*. Ph.D. Thesis. Facultad Tecnológica. Universidad de Santiago de Chile.

Ganga, M. A., and C. Martínez. 2004. Effect of wine yeast monoculture practice on the biodiversity of non-*Saccharomyces* yeasts. *J. Appl. Microbiol.* 96:76-83.

Garcia, A., C. Carcel, L. Dulau, A. Samson, E. Aguera, and E. Agosin. 2002. Influence of a mixed culture with *Debaryomyces vanriji* and *Saccharomcyes cerevisiae* on the volatiles of a muscat wine. *J. Food. Sc.* 67:1138-1143.

García, V., H. Vásquez, F. Fonseca, P. Manzanares, F. Viana, C. Martínez, and M. A. Ganga. 2010. Effects of using mixed wine yeast cultures in the production of Chardonnay wines. *Rev. Argen. Microbiol*. 42:226-229.

Günata, Y. Z., S. Bitteur, J. M. Brillouet, C. L. Bayonove, and R. Cordonnier. 1988. Sequential enzymic (sic) hydrolysis of potentially aromatic glycosides from grape. *Carbohydrate Research*. 184:139-149.

Günata, Z., C. Bayonove, R. Baumes, and R. Cordonnier. 1985. Aroma of grapes. I. Extraction and determination of free and glycosidically bound fraction of some white grape varieties. *J. Chromatogr.* 331:83-90.

Günata, Z., J. M. Brillouet, S. Voirin, R. Baumes, and R. Cordonnier. 1990. Purification and some properties of an α-L-arabinofuranosidase from *Aspergillus niger*. Action on grape monoterpenyl arabinofuranosylglucosides. *J. Agric. Food Chem*. 38:772-776.

Holm, E., P. Nissen, P. Sommer, J. Nielsen, and N. Arneborg. 2001. The effect of oxygen on the survival of non-*Saccharomyces* yeasts during mixed culture fermentations of grape juice with *Saccharomyces cerevisiae*. *J. Appl. Microbiol.* 91:541-547.

Jackson, R. 1994. *Wine Science: Principles and Applications*. Academic Press. USA.

Marais, J. 1983. Terpenes in the aroma of grapes and wines: a review. *South Afr. J. Enol. Viticul*. 4:49-58.

Moreno, V., and M. Polo. 2005. Winemaking Biochemistry and Microbiology: Current Knowledge and Future Trends. *Critic. Reviews Food Sc. Nutri*. 45(4):265-286.

Parapouli, M., E. Hatziloukas, C. Drainas, and A. Perisynakis. 2010. The effect of Debina grapevine indigenous yeast strains of *Metschnikowia* and *Saccharomyces* on wine flavour. *J. Ind. Microbiol. Biotechnol.* 37:85-93.

Pretorius, I. 2000. Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. *Yeast*. 16:675-729.

Ribéreau-Gayon, P., Y. Glories, A. Maujean, and D. Dubourdieu. 2000. Varietal Aroma. *Handbook of Enology: The Chemistry of Wine Stabilization and Treatments*, Vol. 2. Chichester, UK, John Wiley and Sons Ltd.

Sánchez, E., M. Díaz-Maroto, M. González, A. Soriano-Pérez, and M. Pérez-Coello. 2007. Aroma profile of wines from Albillo and Muscat grape varieties at different stages of ripening. *Food Control*. 18(5):398-403.

Somogyi, M. 1952. Notes of sugar determination. *J. Biol. Chem*. 195:19-23.

Vilanova, M., and C. Sieiro. 2006. Determination of free and bound terpene compounds in Albariño wine. *J. Food Compos. Anal*. 19:694-697.

Yanai, T., and M. Sato. 2000. Purification and characterization of a novel α-L-arabinofuranosidase from *Pichia capsulata* X91. *Bioscience, Biotechnology and Biochemistry*. 64:1181-1188.

THE NEW GENERATION OF WINE LACTIC ACID BACTERIA AND THEIR APPLICATION BEYOND BIOLOGICAL DEACIDIFICATION

Isabel PARDO and Sergi FERRER

ENOLAB, Departament de Microbiologia, Universitat de València, Spain

Abstract

The number of described species of lactic acid bacteria (LAB) in wines is increasing day to day. There are reports of existing LAB not previously isolated in wines, and there are new species never before described. This has arisen through the use of new methodologies (that are mainly but not only molecular methodologies) for the identification of LAB. Furthermore, climate change and new viticultural and oenological practices have an impact on the chemical and biological conditions of wines, influencing the population dynamics of microorganisms. The biodiversity of LAB for wine will be better understood in the coming years, leading us to establish new ecological and phylogenetic relationships among wine microorganisms. In any case, these new species will bring new properties and interactions, some beneficial and some detrimental to the quality of the wine. As these bacteria can harbour certain metabolic traits that could influence the composition of the wine and, in some cases, spoil it, it is important to understand the metabolisms of these LAB and how to manage them.

On the other hand, there are new uses for former technologies, such as thermovinification, and microorganisms, such as early inoculation, co-inoculation of yeasts and bacteria, or of two different bacterial species, and the immobilization of LAB) that are bringing new approaches for future wines.

The scenario for wine LAB is continuously changing, and we must be watchful – better to be proactive than reactive.

1. Introduction

1.1 What are lactic acid bacteria?

Lactic acid bacteria (LAB) are a group of Gram-positive, catalase-negative and non-spore-forming bacteria. At the morphological level they can take the form of cocci, rods or coccobacilli. They can be strictly anaerobic or microaerophilic organisms. All are chemoorganotroph and fermentative, characterized by the production of lactic acid as a major end product of the fermentation of carbohydrates (34). Some are acid-tolerant.

2. Lactic Acid Bacteria Populations

In grapes, the number of LAB is low (usually less than 10^3 CFU/mL). However, their numbers increase during the final days of ripening, depending mainly on weather conditions. During the first days of alcoholic fermentation (AF), the number of LAB generally increases to a maximum of 10^4 CFU/mL and then decreases to levels around 10^2 CFU/mL at the end of AF, due mainly to competition from yeast and the LAB's sensitivity to SO_2 and ethanol. After AF, the number of LAB increases and malolactic fermentation (MLF) begins when cells reach levels of 10^6 CFU/mL. When all the malic acid is degraded, the wine is generally stabilized with sulphite. Most LAB are eliminated with this treatment because of their sensitivity to SO_2, leaving small populations of LAB (≤1 to 10 CFU/mL). However, some bacteria can survive this treatment and grow in the wine to levels of 10^6 to 10^7 CFU/mL (18).

LAB species isolated from grapes include *Lactobacillus plantarum*, *Lactobacillus casei* and *Lactobacillus hilgar-*

dii. However, the grape must contains a great diversity of species, which may also include such species as *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus hilgardii*, *Lactobacillus brevis*, *Pediococcus damnosus*, *Pediococcus parvulus*, *Pediococcus pentosaceus*, *Leuconostoc mesenteroides* and *Oenococcus oeni* (17, 19, 23).

During AF, *O. oeni* and some species of *Lactobacillus* can survive, whereas *Pediococcus* and *Leuconostoc* gradually disappear or are drastically reduced in concentration (46). After AF, *O. oeni* become almost exclusive, as they are generally responsible for conducting MLF, being the species best adapted to wine. MLF is generally considered as positive (39), and LAB can produce other beneficial activities in wine, such as the freeing of aroma precursors and positive enzymatic activities (proteases, lipases, esterases, tannases, glycosidases, etc.) (4, 16, 28, 29, 44, 54, 73). Nevertheless, some strains of *Pediococcus*, *Lactobacillus* and *Leuconostoc* can survive, potentially becoming spoilage bacteria and producing *piqûre lactique*, mannitol taint, bitterness, tartaric acid degradation, mousiness, ropiness, biogenic amines, precursors of ethyl carbamate, geranium off-flavour, etc. (40, 45, 72). Many of these roles are species-specific, or even strain-specific, and the proper identification of these organisms is a must.

Although the group of LAB includes almost a dozen genera, most microorganisms found in wine are species of the genera *Lactobacillus*, *Leuconostoc*, *Oenococcus* and *Pediococcus*. All are microaerophilic and can therefore grow in the anaerobic conditions of fermenting wine (67).

Lactobacillus is one of the most important genera related to food microbiology, and many of the species included in it play an important role in the production, preservation and spoilage of food (10). These bacteria are Gram-positive, catalase-negative and non-spore-forming bacilli. They are found in a variety of habitats, such as beverages and fermented foods, mucous membranes and intestinal tracts of humans and animals, in sewage and plants, etc. (5).

The phylogenetic structure of this genus belongs to the phylum *Firmicutes*, is characterized by a low G+C content (mol %), is quite complicated and changing constantly due to the descriptions of new species. It is the largest genus of the order *Lactobacillales*. At present, there are around 180 described species (http://www.bacterio.cict.fr) which, according to Felis and Dellaglio (26), are grouped into 14 phylogenetic groups. It is a genetically diverse genus, with a G+C content ranging from 32 to 55 mol %, exceeding the limits for a genus, and is seemingly not well defined (62), as some studies of comparative genomics have demonstrated (9, 11).

2.1 New *Lactobacillus* species

There were 24 different species of *Lactobacillus* present during the winemaking process, and their ability to grow in must and wine is well documented (13, 14, 18, 40, 41, 52, 53, 70, 75). However, in recent years, changes in winemaking practices (some determined by climate changes) and the use of powerful genetic tools have led to the describing of new species of microorganisms isolated from wine; some among them belong to the genus *Lactobacillus*, such as *Lactobacillus bobalius* (49), *Lactobacillus kunkeei* (22), *Lactobacillus nagelii* (21), *Lactobacillus oeni* (50), *Lactobacillus uvarum* (51) and *Lactobacillus vini* (68).

New species from sluggish or stuck alcoholic fermentations

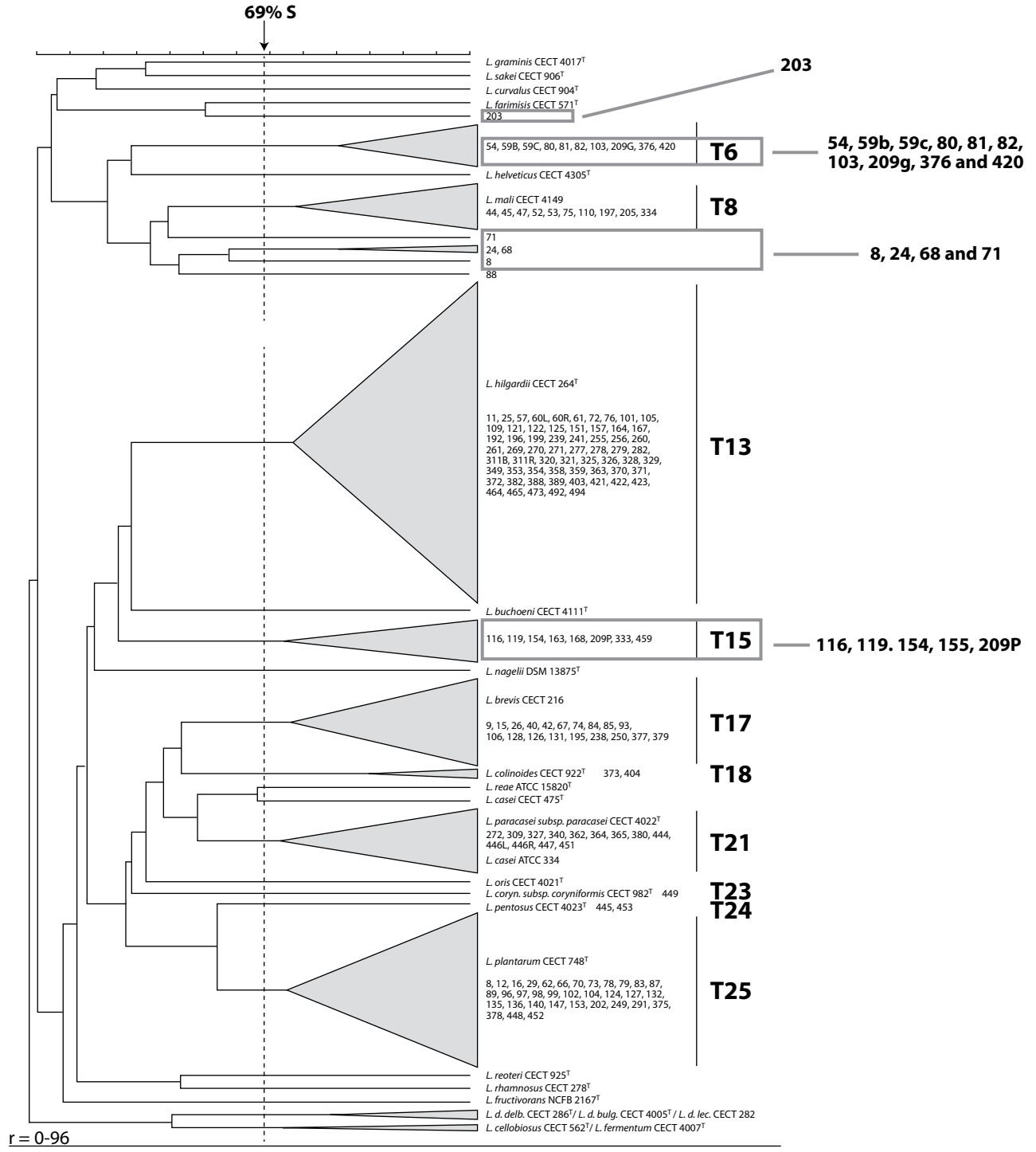
In the new millennium, Charles Edwards's research group has described two new species of *Lactobacillus* isolated from commercial grape wines undergoing sluggish/stuck AF: *Lactobacillus kunkeei* (22) and *Lactobacillus nagelii* (21). Are they the cause or the effect? These species appear to slow the AF of grape musts (32). Besides improper fermentation conditions or insufficient nutrients present in the grape must to support adequate yeast growth, the presence and growth of these spoilage bacteria must be taken into account in order to prevent alterations.

2.2 Characterization of new species: previous findings

In a preliminary study published in 2005 (70), our group, Enolab, isolated 178 different lactobacilli from wine, all characterized by a polyphasic approach. Strains were phenotypically identified at genus and species level by classic tests, including the analysis of cell morphology, homo- or heterofermentative character, sugar fermentation patterns, growth at different temperatures, and the optical nature of the isomer of lactic acid produced from glucose. Such molecular techniques as random amplification of polymorphic DNA (RAPD), amplified 16S rDNA restriction analysis (16S-ARDRA), PFGE-RFLP and ribotyping were utilized to characterize strains, and their potential for identification and/or typing was evaluated. The information obtained with these techniques was processed with BioNumerics software to analyze relationships existing between isolated strains and various reference species of the genus. Taxonomic dendrograms were then obtained, and this information allowed the proposal of molecular procedures suitable for the identification and typing of these wine microorganisms. The wine strains were identified as *Lactobacillus brevis* (19 strains), *Lactobacillus collinoides* (two strains), *Lactobacillus hilgardii* (71 strains), *Lactobacillus paracasei* (13 strains), *Lactobacillus pentosus* (two strains), *Lactobacillus plantarum* (34 strains) and *Lactobacillus mali* (10 strains).

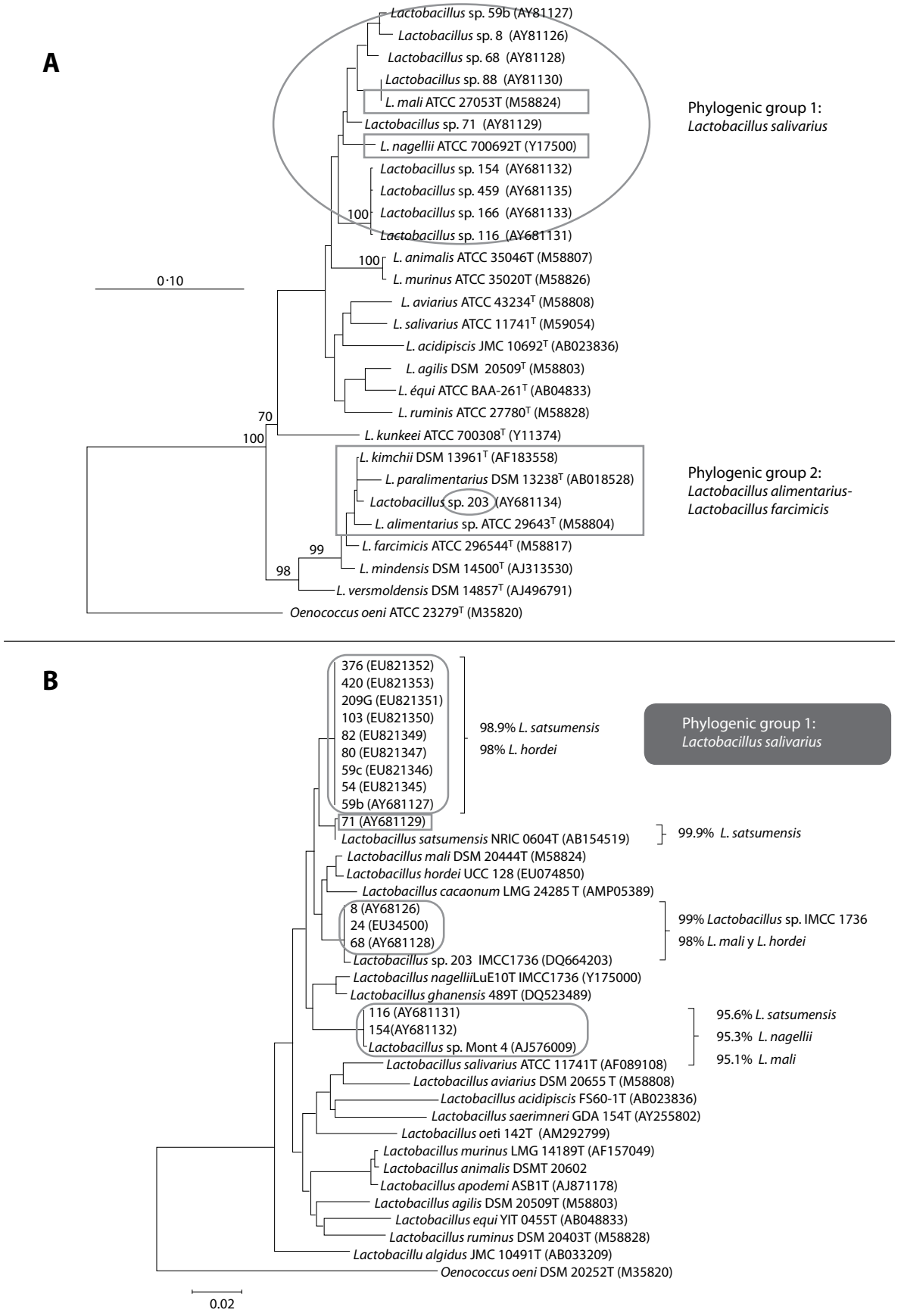
There remained a number of isolates that could not be ascribed to any reference strain, grouped into seven clusters: T5, T6, T9 through T12 and T15 (figure 1). We selected one representative strain from each cluster, except for the T15 cluster, from which four strains were chosen because preliminary results indicated this group was more distant from reference strains (<95% sequence similarity). The selected strains were 8, 59b, 68, 71, 88, 116, 154, 166, 203

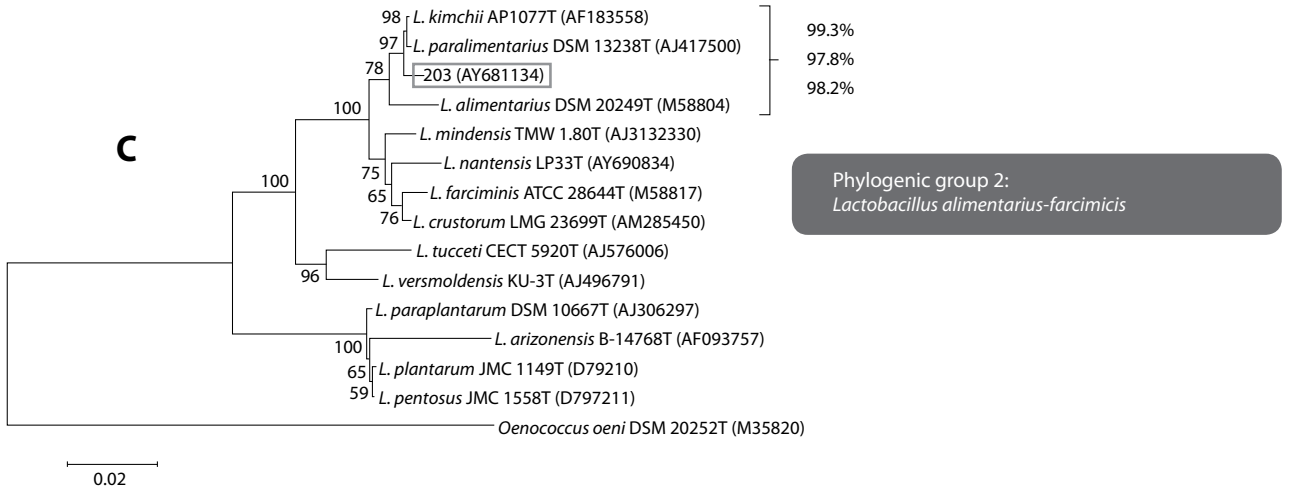
Figure 1. Dendrogram derived from the comparison of all the combined technique patterns obtained from wine lactic acid bacteria and reference strains.



Levels of similarity between patterns were calculated by using the similarity coefficient of each technique and the clustering is based on the UPGMA method. The vertical dotted line indicates the 69% similarity value for delineating clusters. The cophenetic correlation for this dendrogram was 0.96. Subspecies of *Lactobacillus delbrueckii* are abbreviated as *L. d. delb.* (subsp. *delbrueckii*), *L. d. bulg.* (subsp. *bulgaricus*) and *L. d. lac.* (subsp. *lactis*); *Lactobacillus coryniformis* is *L. coryn.*

Figure 2. Phylogenetic trees showing the positions of strains 8, 59b, 68, 71, 88, 116, 154, 166, 203 and 459, and some *Lactobacillus* species based on 16S rRNA gene sequences





Clustering is based on neighbour-joining. Bootstrap values (expressed as percentages of 1000 replicates) of 70% or greater are shown at branch points. GenBank accession numbers are given in parentheses. Bar, 10% (A), and 2% (B and C) nucleotide substitutions.

and 459: their almost complete 16S rRNA gene sequences (approximately 1,500 bp long) were subjected to similarity searches with the ARB database, and all strains were assigned to the genus *Lactobacillus*. The analysis sited them specifically within the heterogeneous *L. casei* group as defined by Collins et al. (12) and redefined into four groups by Schleifer and Ludwig (71). Figure 2 displays phylogenetic trees of *Lactobacillus* species, based on the neighbour-joining method, which includes the 16S rRNA gene sequence data of these 10 strains. Strain 203 was found in the *L. plantarum* group, which represents a cluster of related species consisting of *Lactobacillus alimentarius*, *Lactobacillus farciminis* and the recently described *Lactobacillus paralimentarius* (7), *Lactobacillus kimchii* (76), *Lactobacillus mindensis* (24) and *Lactobacillus versmoldensis* (38). With the exception of *L. kimchii*, all these species were previously isolated from sourdoughs, not wine. The closest relatives of strain 203 were *L. kimchii*

Figure 3. DNA-DNA hybridizations performed on each strain with the closest relatives

	71*	***Lactobacillus sp.* 71***	***Lactobacillus mali* 88***
59b	100.0	47.6	55.1
54	100.0		
***L. mali*, DSM, 20444^T**	30.7		
***L. nagelii*, CECT, 5983^T**	32.1		
***L. vini*, CECT, 5924^T**	34.2		
***Lactobacillus, sp.* 8**	35.5		

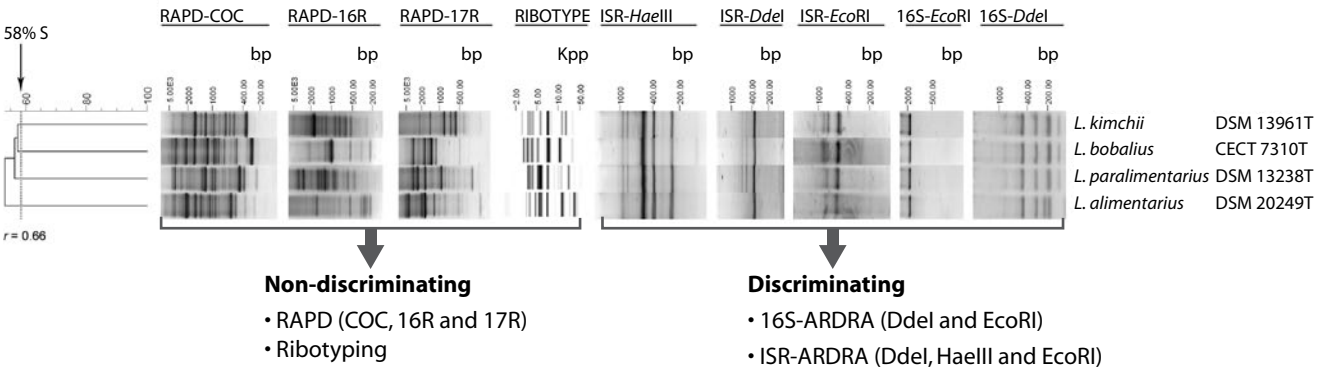
	Mont 4*	**116***
Mont 4	100	77
116	100	100
154	100	97
***L.mali*, CECT 4148**	49	31
L. nagelii, CECT 5983^T	52	38

	71*	***L. satsumensis*, DSM 16230^T***
71	100.0	52.0
***L. satsumensis*, DSM, 16230^T**	42.6	

	203 *	***L. kimchii*, DSM 13961^T***
203	100.0	52.0
***L. alimentarius*, CECT, 570^T**	42.6	
***L. kimchii*, DSM, 13961^T**	62.5	
***L. paralimentarius*, DSM, 13238^T**	464.2	

	8*	***L. mali* CECT, 4149***	***L. mali* 88***	***L. satsumensiss* CECT, 4149***	**IMCC 1736***
8	100.0	52.0	53.4	49.0	41.74
24	84.4				53.09
68	74.0				43.29
***L. mali*, 88**	43.1				33.72
IMCC 1736	41.49				100

Figure 4. Some rapid-discriminating and non-discriminating molecular tests between *Lactobacillus bobalius* and its closest relatives



(99.3%), *L. alimentarius* (98.2%) and *L. paralimentarius* (97.8%) (70). These three species were not included initially in our research because their habitats do not comprise wine, but rather kimchi and sourdough. In further studies, we compared strain 203 in depth with *L. kimchii*, *L. paralimentarius* and *L. alimentarius* by a polyphasic approach that included DNA-DNA hybridization.

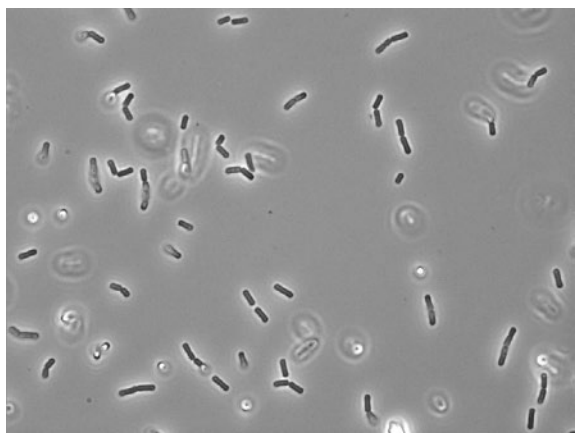
To ascertain whether some of these strains could constitute novel species or new descriptions of some *Lactobacillus* species not previously isolated from wine, DNA-DNA hybridizations were performed, each strain with the closest relatives (figure 3). Strain 71 hybridized with *L. satsumensis* with high values, indicating that it belongs to this species. However, other strains or groups of strains did not, demonstrating that they would constitute new species; grouped strains 59b with 54, Mont 4 with 116 and 154, 8 with 24 and 68, and 203 alone. With all this information, we were able to describe new species of *Lactobacillus* isolated from wines, and new descriptions of existing species not previously reported in wines.

Description of *Lactobacillus bobalius* sp. nov.

Lactobacillus bobalius (pertaining to the grape variety Bobal) (49) are Gram-positive, non-motile, non-spore-forming rods, measuring 0.71 to 1.03 µm wide by 1.65 to 3.41 µm long. Cells are found singly, in pairs and in short chains. Microaerophilic; colonies on MRS agar after four days incubation at 28°C are 1.8 to 2 mm in diameter, smooth, circular to slightly irregular, white and catalase negative. Growth occurs from 15 to 45°C, but not at 5°C. Facultative heterofermentative, no gas is produced from glucose. DL-lactate is produced as an end product from hexoses and pentoses. Ammonia is not produced from arginine, and mannitol is not produced from fructose. Dextran is not produced from sucrose. Citric and malic acids are utilized. Strain 203 ferments ribose, glucose, fructose, mannose, N-acetyl-glucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, sucrose, trehalose, melezitose, β-gentibiose and gluconate, and hydrolyzes aesculin. On the other hand, *Lactobacillus bobalius* does not ferment glycerol, erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, adonitol, methyl α-xyloside, galactose, L-sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl α-D-mannoside, methyl α-D-glucoside, lactose, melibiose, inulin, D-raffinose, starch, glycogen, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, 2-ketogluconate or 5-ketogluconate. The G+C content is 34.03 ± 0.77 mol%. The cell wall contains A4 α L-Lys-D-Asp peptidoglycan type.

Lactobacillus bobalius sp. nov. was proposed for strain 203, which was isolated in 1997 by A. M. Rodas from a Bobal grape must. The reference strain is 203^T (= CECT 7310^T = DSM 19674^T) (49). The differential characters between *L. bobalius* and its closest phylogenetic neighbours, and other phenotypic and genomic traits, can be retrieved from the literature (49). Some rapid-discriminating and non-discriminating tests can be seen in figure 4, and its morphology in figure 5.

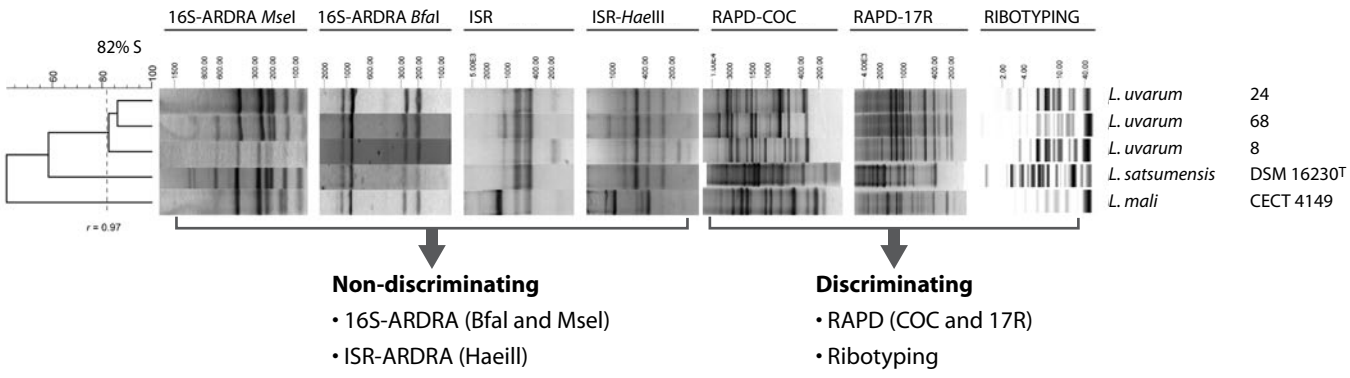
Figure 5. Cellular morphology of *Lactobacillus bobalius*



Description of *Lactobacillus uvarum* sp. nov.

Lactobacillus uvarum (51) are Gram-positive, motile, non-spore-forming rods 0.89 to 1.20 µm wide by 1.18 to 3.48 µm long. Cells are found singly, in pairs and in short chains. Aerotolerant; colonies on MRS agar after four days incubation at 28°C are 1 to 1.25 mm in diameter, white,

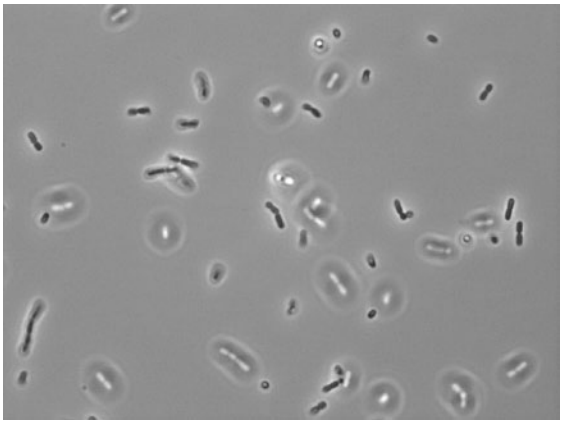
Figure 6. Some rapid-discriminating and non-discriminating molecular tests between *Lactobacillus uvarum* and its closest relatives



smooth, circular and with entire edges. Catalase negative. Growth occurs at pH 4.5 and 8, and in the presence of 5% (w/v) NaCl, but not at pH 3.3, nor with 10% (w/v) NaCl. Obligate homofermentative: gluconate and pentoses are not fermented and glucose is fermented but no gas is released. L-lactate is produced as the exclusive end product from hexoses. Ammonia is not produced from arginine, and mannitol is not formed from fructose. Exopolysaccharide is produced from sucrose. All strains ferment glucose, fructose, mannose, mannitol, methyl α-D-glucoside, N-acetyl-glucosamine, amygdalin, arbutin, salicin, maltose, sucrose, trehalose, β-gentiobiose and D-turanose, and hydrolyze aesculin. The isolates do not ferment glycerol, erythritol, D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, adonitol, methyl β-xyloside, galactose, rhamnose, dulcitol, inositol, methyl α-D-mannoside, lactose, melibiose, inulin, melezitose, D-raffinose, starch, glycogen, xylitol, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate or 5-ketogluconate. The G+C content is 36.07 ± 0.07 mol%.

The differential characters between *L. uvarum* and its closest phylogenetic neighbours, and other phenotypic and genomic traits, can be retrieved from the literature (51). Some rapid-discriminating and non-discriminating tests can be seen in figure 6, and its morphology in figure 7.

Figure 7. Cellular morphology of *Lactobacillus uvarum*



Description of *Lactobacillus oeni* sp. nov.

Lactobacillus oeni (50) are Gram-positive, non-spore-forming rods, measuring 0.63 to 0.92 μm wide by 1.38 to 3.41 μm long; 90% of strains are motile. Cells are found singly, in pairs and in short chains. Microaerophilic; colonies on MRS agar after four days incubation at 28°C are 0.8 to 1.2 mm in diameter, smooth, circular, regular and white. Catalase negative. Growth occurs from 15 to 45°C, but not at 5°C, at pH 4.5 and 8.0, but not at pH 3.3 or with 10% ethanol. They transform L-malic acid into L-lactic acid. Homofermentative, *Lactobacillus oeni* do not ferment gluconate or ribose. L-lactate is produced as the end product from hexoses. Ammonia is not produced from arginine, and mannitol is not produced from fructose. Exopolysaccharide is produced from sucrose. Acid is produced from glucose, fructose, mannose, L-sorbose, mannitol, sorbitol, methyl α-D-glucoside, N-acetyl-glucosamine and trehalose. On the other hand, acid is not produced from erythritol, D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, adonitol, methyl β-xyloside, galactose, rhamnose, dulcitol, inositol, methyl α-D-mannoside, amygdalin, arbutin, cellobiose, maltose, lactose, melibiose, sucrose, inulin, melezitose, D-raffinose, starch, glycogen, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate, and 5 ketogluconate. Aesculin is not hydrolyzed. Acid production from glycerol is strain-dependent; 90% of strains ferment salicin and β-gentiobiose. The cell wall contains peptidoglycan of the D-meso-diaminopimelic acid type. The G+C content is 37.17 ± 0.16 mol%.

Lactobacillus oeni sp. nov. was proposed for strains 54, 59b, 59c, 80, 81, 82, 103, 209G, 376 and 420, which were isolated from Bobal wine in 1997 by A. M. Rodas. The reference strain is 59b^T (= CECT 7334^T = DSM 19972^T) (50). The differential characters between *L. oeni* and its closest phylogenetic neighbours, and other phenotypic and genomic traits, can be retrieved from the literature (50).

Figure 8. Some rapid-discriminating and non-discriminating molecular tests between *Lactobacillus oeni* and its closest relatives

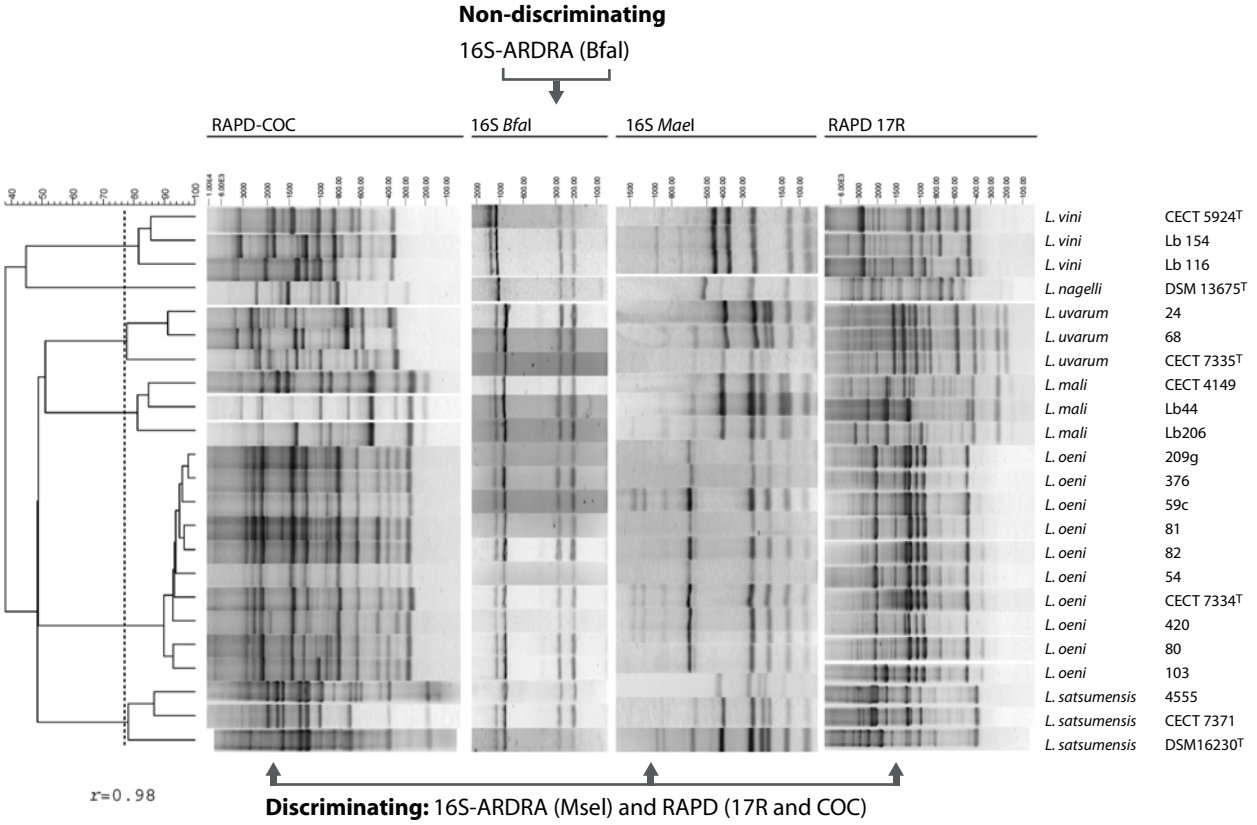
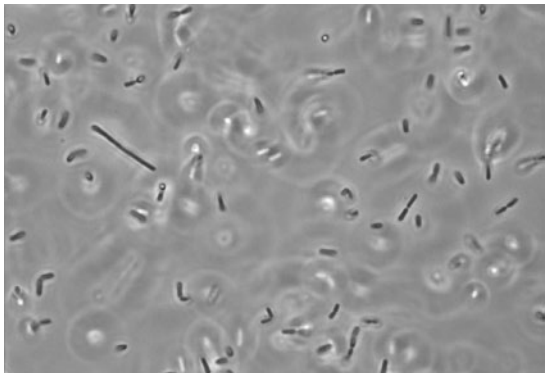


Figure 9. Cellular morphology of *Lactobacillus oeni*



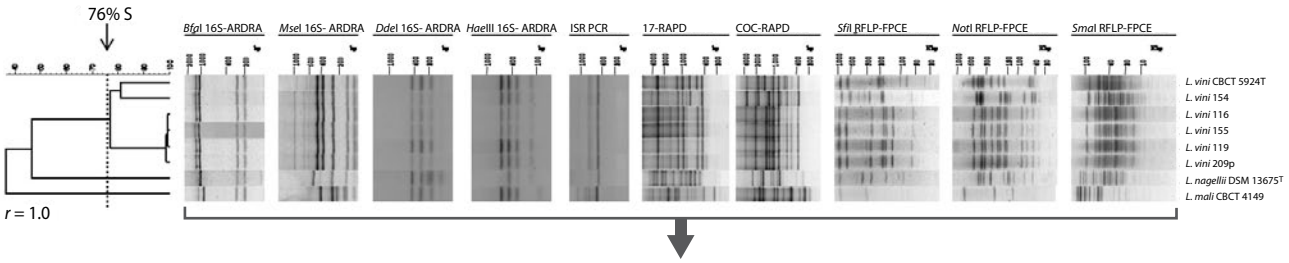
Some rapid-discriminating and non-discriminating tests can be seen in figure 8, and its morphology in figure 9.

Description of *Lactobacillus vini* sp. nov.

Lactobacillus vini (68) are Gram-positive, motile, non-spore-forming rods 0.49 to 0.82 µm wide by 1.36 to 2.8 µm long. Cells are found singly, in pairs and in short chains. Facultative anaerobic; colonies on MRS agar after four days incubation at 28°C are 0.7 to 1.5 mm in diameter, with entire edges, smooth, glistening and white. Catalase negative. Growth occurs from 25 to 45°C, but not at 15°C or less. Homofermentative, no gas is produced from glucose. Cells contain L-Lys-D-Asp in their peptidoglycan. DL-lactate isomer is exclusively produced as the end product from hexoses and pentoses. Ammonia production from arginine is variable since only strains 116 and 119 are positive. Mannitol is not produced from fructose. Exopolysaccharide is not produced from sucrose. Citric and malic acid are utilized. All strains fermented L-arabinose, D-glucose, D-fructose, D mannose, N-acetylglucosamine, amygdalin, salicin, cellobiose, maltose, sucrose, trehalose and β-gentibiose. Aesculin is hydrolyzed. None of them fermented glycerol, erythritol, D-arabinose, D-xylose, L-xylose, D-adonitol, methyl β-D-xyloside, L-sorbose, dulcitol, inositol, mannitol, sorbitol, methyl β-D-glucoside, lactose, melibiose, inulin, melezitose, D-raffinose, starch, glycogen, xylitol, D-turanose, D-lyxose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate and 5-ketogluconate.

In addition, the type strain shows the following traits: it does not produce ammonia from arginine, does ferments D-ribose, does not ferment D-galactose, methyl α-D-mannoside and D-tagatose; it is unable to hydrolyze arginine, and does not split arbutine. The mol% G+C content is 39.4. *Lactobacillus vini* sp. nov. is proposed for six strains: Mont 4T, 116, 119, 154, 155 and 209P. The type strain Mont 4T (DSMZ 20605T, CECT 5924T) was isolated in 1978 by P. Barre from high-temperature-fermenting grape must (2). The

Figure 10. Some rapid-discriminating and non-discriminating molecular tests between *Lactobacillus vini* and its closest relatives



Discriminating

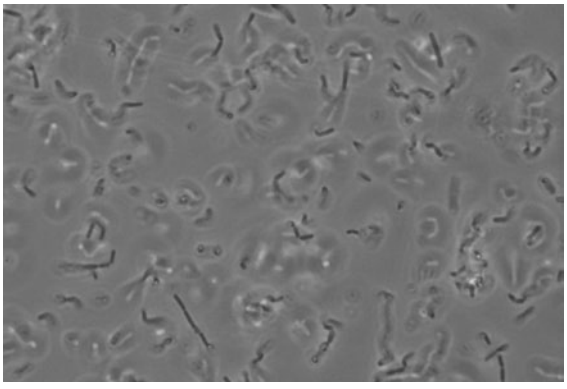
- 16S-ARDRA (BfaI, MseI, DdeI and HaeIII)
- ISR-PCR
- RAPD (17R and CFOC)
- Macrorestriction (SfiI, NotI and SmaI)

reference strains are 116 (CECT 7072) and 154 (CECT 7073), both isolated from Spanish fermenting grape musts (70).

This species shows a strange and unforeseen pathway for the fermentation of pentoses in *Lactobacillus*. Traditionally, LAB that ferment pentoses do so by using a phosphoketolase that splits them into glyceraldehyde-3-phosphate, that yields lactate and acetyl-phosphate, which is converted into ethanol or acetate. We could detect DL-lactate exclusively as the end product from pentoses in *L. vini* strains, with molar ratios of 1.57 to 1.70 (lactate/pentose) for all strains and pentoses, near the theoretical 1.67 molar ratio lactate/pentose value. These results agree with those obtained previously (2, 34, 63), and are explained by the existence of transaldolase and transketolase activities, permitting strains of *L. vini* to use pentose sugars via an inducible pentose phosphate pathway. This pathway, which yields lactate exclusively as a final product, is different from the 6-phosphogluconate pathway used by facultative heterofermentative lactobacilli (68).

The differential characters between *L. vini* and its closest phylogenetic neighbours, and other phenotypic and genomic traits, can be retrieved from the literature (68). Some rapid-discriminating and non-discriminating tests can be seen in figure 10, and its morphology in figure 11.

Figure 11. Cellular morphology of *Lactobacillus vini*



2.3 New descriptions for existing species not previously reported in wine

Some of the strains we isolated did not constitute new species, but rather new descriptions for existing species in wines not previously reported, as in the above described case of strain 71 that finally belongs to *L. satsumensis* (48). This organism had been initially isolated from *shochu*, a traditional Japanese distilled spirit made from fermented rice (25). A similar situation occurred for strains we isolated from grape juices and wines, and belong to *Lactobacillus harbinensis*, isolated previously from fermented vegetables (56), *Lactobacillus coryniformis*, previously isolated from dung (although that is not its only habitat) (69), *Lactobacillus vaccinostercus*, also previously isolated from cow dung (33), *Lactobacillus pantheris*, previously isolated from jaguar feces (43), and *Lactobacillus florum*, a fructophilic LAB now found in South African grape and wine samples and previously isolated only from peony and bietou flowers (57).

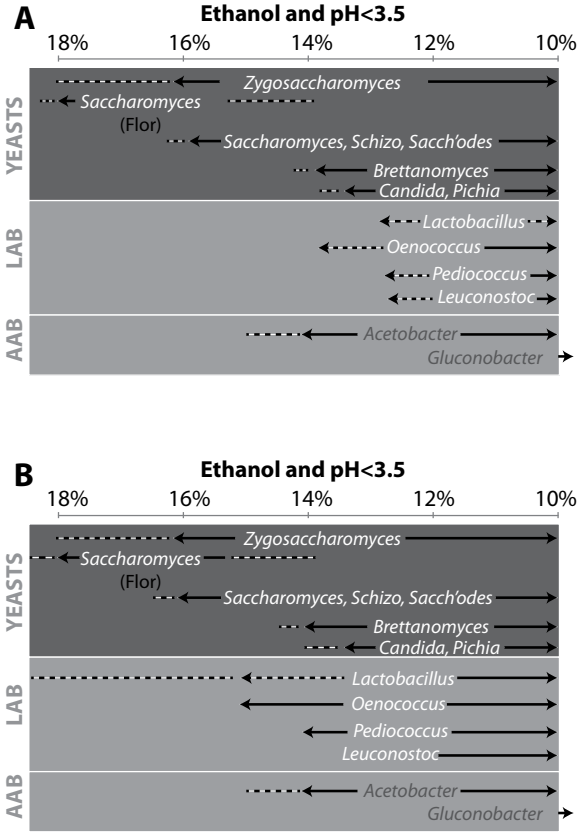
Why so many descriptions in recent years?

One reason is that we can now handle new laboratory methods (that are mainly but not only molecular), that are fast, reliable and especially informative. Great amounts of information can be obtained quite easily from the organisms that live in wines. These techniques obviously permit many applications to wine microbiology that were not possible only a few years ago. For instance, once quite difficult, it is now quite easy to follow the implantation of a commercial starter in a wine. A polyphasic approach has proved essential to revealing this phenomenon where new species or new descriptions have been made – the more information we recover, the more discriminative characters we find.

A second reason is derived from changes in viticultural and oenological practices. There is a general tendency to reduce the use of SO_2, sometimes by using new chemical alternatives, allowing greater opportunity for the survival of microorganisms. In some cases, macerating time can

be quite long nowadays, and contact with lees is also very popular for some wines, which can enrich the wine in nutrients and other components. Enzymes may be used, harbouring such activities as pectinases, proteases, laccases, etc., which will also change the composition of wines. Thermovinification, although not a completely new technology, is using new equipment and being tried once again by some winemakers, changing the population dynamics of microorganisms. So do other techniques, such as washing the grapes, utilizing different co-inoculation strategies or stabilizing the wine. Added growth factors and nutrients can be employed, as can oak chips, etc. All these technologies are changing the conditions for the propagation of microbes: new winemaking techniques are bringing new opportunities for the survival of microorganisms.

Figure 12. Growth ranges of yeasts, lactic acid bacteria and acetic acid bacteria at different ethanol contents in low pH (A) and high pH (B) wines (Loureiro 2007)

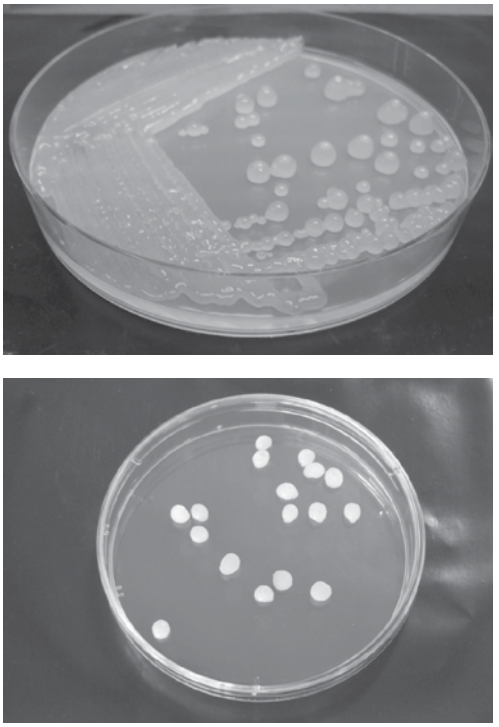


A third reason is derived from the changes in the climate: sugar content in grapes – and thus ethanol at the end – is higher now than years ago, while the pH is increasing. This implies a great change in the growth ability of microorganisms, mainly for LAB, under these circumstances, as can be observed in figure 12. At pH values <3.5, LAB can usually grow in up to 13% (v/v) ethanol content, whereas wines with pH >3.8 can grow in wines with 15% (v/v) of ethanol. This is only an example to illustrate that changes in the environment will bring changes in the population dynamics and composition of the microbiota of wines.

These new species bring new properties and interactions, some beneficial to the wine quality and some detrimental. Some of these bacteria harbour certain metabolic traits that can influence wine composition, both positively and negatively, and in some cases could spoil wines. They can synthesize interesting compounds such as polysaccharides and enzymes, but also biogenic amines, off-odours and noxious substances.

An example is the odd behaviour of *Lactobacillus vini*, which is able to ferment both hexoses and pentoses via an inducible pentose phosphate pathway by using transaldolase and transketolase activities (68). This pathway, which exclusively yields lactate as the final product, is different from the 6-phosphogluconate pathway used by facultative heterofermentative lactobacilli. And this metabolism will yield only lactic acid from any sugar, with no risk of volatile acid production.

Figure 13. Polysaccharides produced on agar plates by some new species of wine lactic acid bacteria



Some other bacteria can produce different types of polysaccharides as observed in figure 13. These compounds can influence the sensory properties of the wines, in one way or another, and these presumed changes have not yet been clarified. Further research on these new compounds and their impact on wine will be necessary in the future.

Which lactic acid bacteria can be employed as malolactic starters?

There are a number of LAB that have been utilized as MLF starters belonging to the species *Lactobacillus plantarum*, *Lactobacillus hilgardii*, *Lactobacillus brevis*, *Lactobacillus casei*, *Oenococcus oeni* (syn. *Leuconostoc oenos)*, *Pediococcus* sp. and *Pediococcus parvulus*. Each of them has demonstrated different properties, although these characteristics have been shown many times to be strain dependent. However, the species *O. oeni* has been the most utilized, because its ability to survive in the harsh wine conditions of high ethanol and low pH.

These starters have been prepared in a number of different forms, from frozen cultures to liquid suspensions, direct inoculation (MBR), build-up (1-Step) or traditional freeze-dried (table 1) (37).

However, without discarding *O. oeni* as the starter for our wines (long live the king!), a new generation of MLF cultures is rising. Certainly, these are not really new species on the scene, as many years ago *Lactobacillus plantarum* starters were employed in freeze-dried preparations (61, 65, 66). But new improvements, new strains and new strategies are being employed that can bring new approaches for the oenologists (15, 42, 55). Bou and Krieger described the use of lactic bacterial strains of the genera *Lactobacillus* and *Pediococcus* that were capable of initiating and carrying out complete MLF on direct introduction, in the dried, frozen or lyophilized state, without a previous acclimatization step (6). These bacteria were resistant to alcohol, with an excellent survival rate on inoculation and a rapid start to the fermentation activity.

New strains of *L. plantarum* are utilized with improved characteristics that have a greater impact on the sensory properties of wines, as they are able to produce a series of enzymes that can be beneficial to the wine, such as ß-glucosidases, proteases, esterases and decarboxylases (15, 31, 42, 55, 58, 59, 64, 74) (table 2). These activities are reflected in the characteristics of the final wines and the fruity characters that are enhanced after MLF performed by these organisms (figure 14) (3). Some strains of *L. plantarum* are even able to reduce ochratoxin A (OTA) in wines after direct inoculation (MBR) (27). And some strains of *L. plantarum* can perform MLF after AF faster than spontaneous strains or even inoculated *O. oeni* starters (20) (figure 15).

Table 1. Types and properties of malolactic fermentation starters in winemaking (37)

	Type of malolactic bacterial culture				
Property	**Frozen**	**Liquid suspension**	**Direct inoculation (MBR)**	**Quick build-up culture (1-Step)**	**Traditional freeze-dried (standard)**
Storage temperature and shelf life	Up to 120 days at -26°C or up to 1 year at -29°C in a non-defrosting freezer	Up to 2 days at room temperature or up to 2 weeks at 4°C	Up to 18 months at 4°C or to 30 months at -18°C	Up to 18 months at 4°C or to 30 months at -18°C	Up to 18 months at 4°C or to 30 months at -18°C
Open container	Once thawed *use immediately* do not refreeze	*Use immediately*	*Use immediately*	*Use immediately*	*Use immediately*
Time for starter preparation	48h before inoculation	10-fold expansion in 3-7days	0-15 min	18-24 h	3-14 days
Nutritional supplements	30g yeast extract to activation media	~1 g yeast extract per liter growth medium	Proprietary MLB nutrients recommended under more challenging MLF conditions	Proprietary activator. MLB nutrients recommended under more challenging MLF conditions	Proprietary MLB nutrients recommended under more challenging MLF conditions
Usage rates	Red wine ~1 g hL^{-1} White wine ~3-8.5 g hL^{-1}	2-5% inoculation volume or when using finished wine to prepare the starter, then 5-10% inoculation volume	~1 g hL^{-1}		~1 g hL^{-1}

Table 2. Enzymatic activities of *Oenococcus oeni* and *Lactobacillus plantarum* strains (A) and their respective roles in total ester production in wines (excluding ethyl acetate) (42) (B)

A

Enzyme	*L.plantarum 56*	*O. oeni S5*	Significance
β-D-glucosidase	+	-	Release of glycosidically bound aroma compounds
Phenolic acid decarboxylase	+	-	Metabolism of phenolic acids
Proline iminopeptidase	+	-	Release of free amino acids as aroma precursors
Citrate lyase α-subunit	+	-	Diacetyl production
Arginine deiminase	-	+	Ethyl carbamate production

B

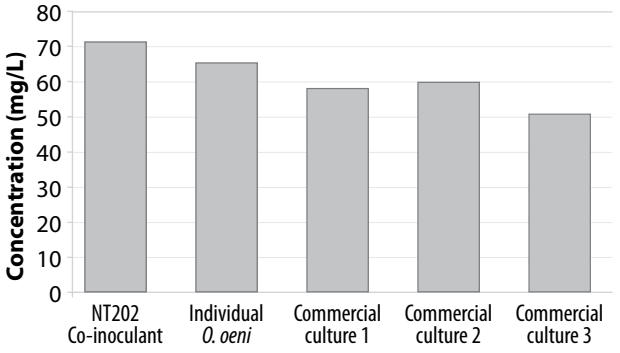


Figure 15. Kinetics of malic acid degradation in a 2010 Merlot (South Africa); Inoculation post-alcoholic fermentation [pH 3.5 / 22.9˚Balling / 13.6% (v/v)] (20)

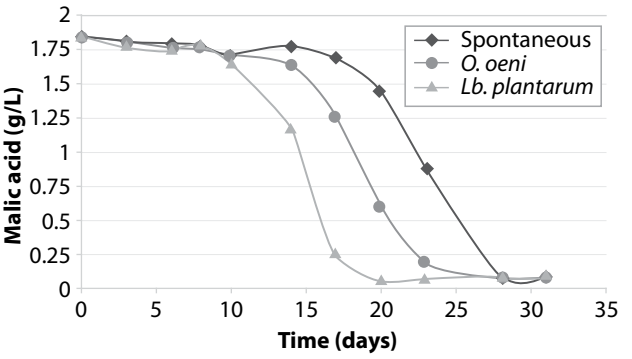


Figure 14. Fermentation-derived volatile compounds of Shiraz wines following different timing regimes of malolactic fermentation bacteria inoculation. Alcoholic fermentation (no MLF). Medium grey vectors indicate acetate esters; light grey vectors indicate ethyl esters; black vectors indicate higher alcohols (3)

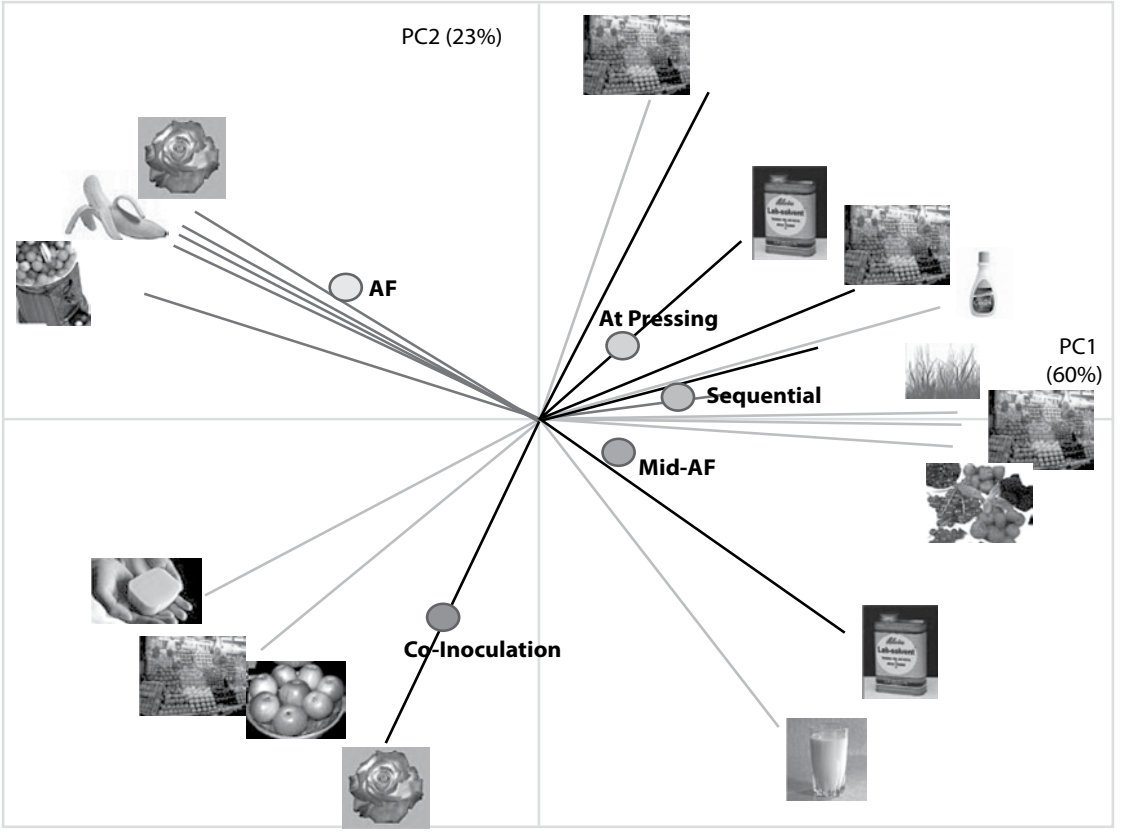
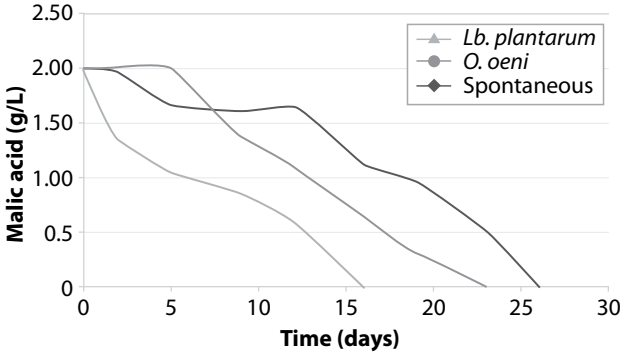


Figure 16. Kinetics of malic acid degradation in a 2009 Sangiovese (Tuscany, Italy). Co-inoculation 24 hours after yeast [pH 3.6 / 25.8 Brix / 14.3% (v/v)] (20)



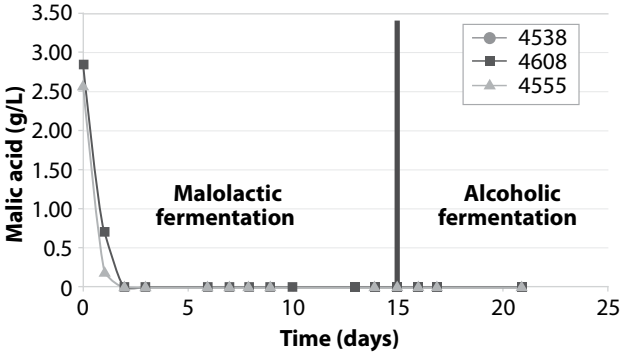
Co-inoculation with yeasts and bacteria is another possibility gaining more supporters, as climate change is creating wines with higher ethanol content. Indeed, after AF wine bacteria sometimes have to deal with ethanol levels above 16° (v/v) (20, 60). Inoculating the must with two yeasts simultaneously (or later) is an interesting alternative, as low ethanol is still synthesized. In figure 16, the kinetics of malic acid degradation during a spontaneous MLF in a Sangiovese wine can be observed, and the same wine inoculated with *O. oeni* or *L. plantarum*, both 24 hours after yeast inoculation (20). One advantage of using *L. plantarum* as a starter for MLF is that this species is homofermentative for hexoses, thus only lactic acid is formed as a final product. But *O. oeni* can produce acetic acid when degrading hexoses. In our lab, we have used this advantage to select strains of *L. plantarum* able to perform MLF in grape must (inoculation of bacteria can even precede that of yeasts), with a rapid and complete degradation of malic acid even only two or three days after the grape crushing (figure 17). With these methodologies, rapid, safe and complete fermentation (both alcoholic and malolactic) can be achieved: grape must to final wine in only a few days then rapidly stabilized.

Table 3. Examples of immobilization techniques or alternative methods to induce malolactic fermentation in wine. Figures in parentheses represent working volumes (CSTR = continuous stirred tank reactor, CCR = cell recycle continuous stirred reactor, FBR = fluidized bed reactor, nr = no reported data) (47).

Bacterium or enzyme	Immobilization supporta	Reactor (V)	Initial L-malicacid (g L^{-1})	Bioconversion rateb (%)	Operation period (h)	Reference
Lactobacillus sp.	–	CSTR (350 hL)	4.0	62.0-75.0	24	Caillet and Vayssier (1984)
Lactobacillus sp.	Calcium alginate	FBR (1.4 L)	0.9	45.0	72	Naouri et al. (1991)
Lactobacillus sp.	κ-Carrageenan	CSTR (0.5 L)	9.0	64.0	200	Crapisi et al. (1987a)
Lb. brevis	κ-Carrageenan	Shake flash (5 mL)	5.00	71.4	1	McCord and Ryu (1985)
Lb casei	Polyacrylamide	Airflow (nr)	48.00	80.0-100.0	360	Clementi (1990)
Oenococcus oeni	–	350 hL	4.0	75.0-100.0	24	Caillet and Vayssier (1984)
Oenococcus oeni	–	Screw tubes (5 mL)	1.0-6.0	94.0-99.4	6	Gao and Fleet (1994)
Oenococcus oeni	–	CCR (0.3 L)	0.8-4.6	25.0-100.0	125	Gao and Fleet (1995)
Oenococcus oeni	–	Screw tubes (10 mL)	5.0	77.0	5-48	Lafon-Lafourcade (1970)
Oenococcus oeni	–	CSTR (0.3 L)	4.2	92.0-95.0	500	Maicas et al. (1999b)
Oenococcus oeni	–	Screw tubes (10 mL)	3.5-7.0	41.0-98.0	24-500	Maicas et al. (2000)
Oenococcus oeni	Calcium alginate	CSTR (2.7 L)	8.0	97.0-100.0	17	Cuenat et Villetaz (1984)
Oenococcus oeni	Calcium alginate	FBR (1.4 L)	0.9	82.0	72	Naouri et al. (1991)
Oenococcus oeni	Calcium alginate	CSTR (30 mL)	0.3	100.0	12	Shieh and Tsay (1990)
Oenococcus oeni	Calcium alginate	CSTR (60 mL)	1.5	60.0	864	Spettoli et al. (1982)
Oenococcus oeni	Calcium alginate	CSTR (nr)d	4.5	98.7	360	Spettoli et al. (1987)
Oenococcus oeni	Cellulose sponge	Shake flask (0.1 L)	3.5	50.0	96	Maicas et al. (2001)
Oenococcus oeni	κ-Carrageenan	CSTR (0.5 L)	9.0	36.3	48	Crapisi et al. (1987b)
Oenococcus oeni	κ-Carrageenan	Shake flash (5 mL)	5.0	100.0	1	McCord and Ryu (1985)
Oenococcus oeni	Oak chips	CSTR (0.1 L)	8.0	20.0-58.0	264	Janssen et al. (1993)
Oenococcus oeni	Polyacrylamide	Shake flask (5 mL)	2.3-4.5	71.0	1	Rossi and Clementi (1984)
Malolactic enzyme	–	Membrane reactor	18.6-23.9	62.0-75.0	168	Formisyn et al. (1997)
(O. oeni)	–	(5 mL)				

[a] Matrix used when immobilizing cells or enzymes
[b] Amount of L-malic acid consumed/initial amount of L-malic acid

Figure 17. Degradation of malic acid in the early stages of winemaking when inoculating with three different strains of *Lactobacillus plantarum* (4538, 4608 and 4555)



Within three days, all the malic acid (2.8 g/L) is depleated.

Figure 18. Scanning electron microscope (SEM) observation of alginate microbeads before silica coating (A), and environment scanning electron microscope (ESEM) observation of alginate microbeads after silica coating (B) (8, 30)

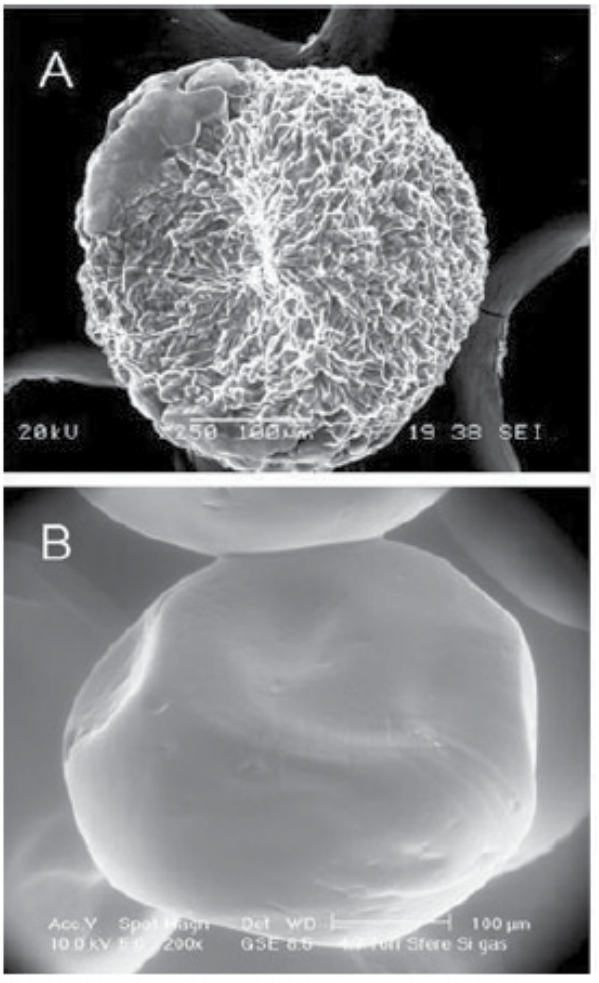
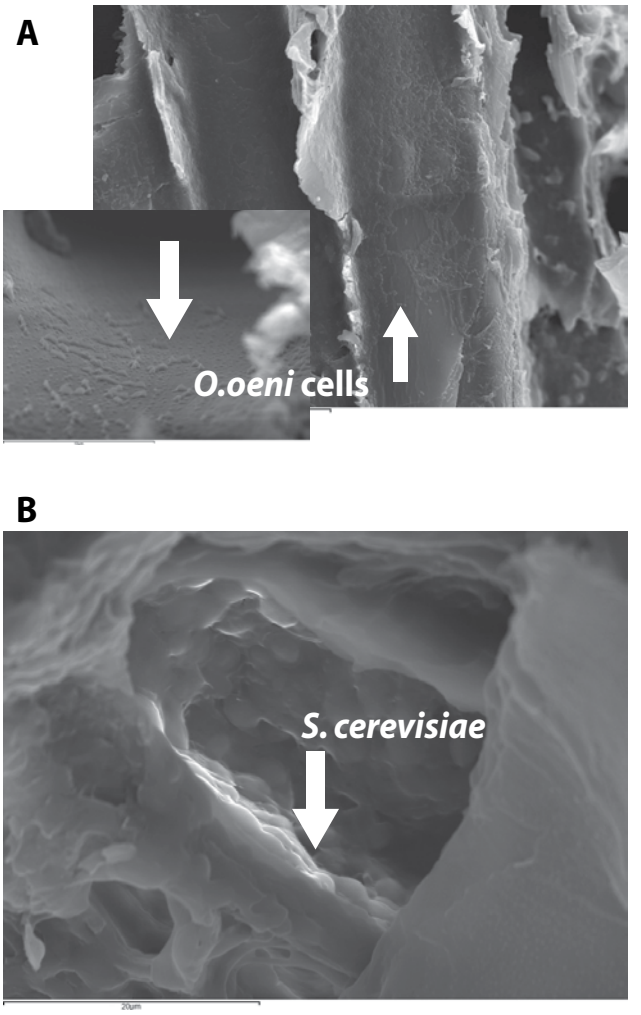


Figure 19. Cells of *Oenococcus oeni* immobilized on the surface of the natural nanotubes (A), and overlaid with yeasts placed within a gel



3. Immobilization of lactic acid bacteria

The immobilization of bacteria is an interesting field recently revisited. For years, experiments have utilized a number of immobilization techniques to perform MLF in wines. For a review see references 36 and 47 and table 3. However, these techniques have never really led to an end use in wineries, for one reason or another. Now there are two promising techniques that could change this and bring immobilization to the real world in winemaking. In the first technique, cells are entrapped in Ca-alginate microbeads, coated with an organosilica membrane obtained by two treatments: the first a sol suspension of tetraethoxysilane, the second using methyltriethoxysilane in gas phase (8, 30). The structure of these microbeads can be observed in figure 18. Their very interesting properties are based on the physicochemical features of alginate (the first layer in contact with the cells), but a coat of the or-

ganosilica membrane avoids cell leakage during fermentation and protects the cells from antimicrobial compounds (i.e., lysozyme). The second approach, improved in our lab, uses natural nanotubes to immobilize bacteria on the surface, and then overlays yeasts placed within a gel (figure 19). This technique is based on previous work on delignified cellulosic material (1, 35, 36). The advantages are clear for this new technology: (i) the use of a food-grade natural matrix to immobilize cells, (ii) an increase in ester formation and an improvement of wine aroma has been observed, (iii) it requires lower numbers of cells than conventional systems (and/or reduces fermentation times), (iv) fermentations can be conducted at low temperatures (i.e., 10°C), (v) different microorganisms (yeasts and bacteria, with combinations) can be co-immobilized, (vi) different approaches can be employed ("tea-bags," columns, spread of powder, etc.), (vii) starter removal from wine is easy, at any time during fermentation, and rapid, and (viii) after the end of both alcoholic and malolactic fermentation the wines can be rapidly stabilized.

4. Conclusion

What will the future bring? Maybe the future is already here! A new generation of bacteria is appearing before our eyes, as well as new techniques to utilize them. New challenges and dangers are coming, due to new climate conditions, along with new technologies, new foes, new friends... So let's face this exciting future with new tools!

Acknowledgements

This research has been partially supported by grants from the projects MICIN AGL2006-08495, INIA RM2007-00007-00-00 and CENIT CDTI 2008-2011.

References

1. Agouridis, N., N. Kopsahelis, S. Plessas, A. A. Koutinas, and M. Kanellaki. 2008. *Oenococcus oeni* cells immobilized on delignified cellulosic material for malolactic fermentation of wine. *Bioresource Technology.* 99:9017-9020.

2. Barre, P. 1978. Identification of thermobacteria and homofermentative, thermophilic, pentose-utilizing lactobacilli from high temperature fermenting grape musts. *J. Appl. Bacteriol.* 44:125-129.

3. Bartowsky, E., and P. Costello. 2011. Using malolactic fermentation to enhance the fruity characters of wine. *Ace Revista de Enología* http://www.acenologia.com/cienciaytecnologia/FML_vino_afrutado_cienc1211_eng.htm.

4. Bartowsky, E. J. 2005. *Oenococcus oeni* and malolactic fermentation – moving into the molecular arena. *Australian Journal of Grape and Wine Research*. 11:174-187.

5. Bernardeau, M., J. P. Vernoux, S. Henri-Dubernet, and M. Guéguen. 2008. Safety assessment of dairy microorganisms: The *Lactobacillus* genus. *International Journal of Food Microbiology*. 126:278-285.

6. Bou, M., and S. Krieger. 2006. Alcohol-tolerant malolactic strains for the maturation of wines with average or high pH. United States Patent No. 20060153822.

7. Cai, Y., H. Okada, H. Mori, Y. Benno, and T. Nakase. 1999. *Lactobacillus paralimentarius* sp. nov., isolated from sourdough. *Int J Syst Bacteriol.* 49:1451-1455.

8. Callone, E., R. Campostrini, G. Carturan, A. Cavazza, and R. Guzzon. 2008. Immobilization of yeast and bacteria cells in alginate microbeads coated with silica membranes: Procedures, physico-chemical features and bioactivity. *Journal of Materials Chemistry*. 18:4839-4848.

9. Canchaya, C., M. J. Claesson, G. F. Fitzgerald, D. van Sinderen, and P. W. O'Toole. 2006. Diversity of the genus *Lactobacillus* revealed by comparative genomics of five species. *Microbiology*. 152:3185-3196.

10. Caplice, E., and G. F. Fitzgerald. 1999. Food fermentations: Role of microorganisms in food production and preservation. *International Journal of Food Microbiology*. 50:131-149.

11. Claesson, M. J., D. van Sinderen, and P. W. O'Toole. 2007. The genus *Lactobacillus* – a genomic basis for understanding its diversity. *FEMS Microbiology Letters*. 269:22-28.

12. Collins, M., U. Rodrigues, C. Ash, M. Aguirre, J. Farrow, A. Martinez-Murcia, B. Phillips, A. Williams, and S. Wallbanks. 1991. Phylogenetic analysis of the genus *Lactobacillus* and related lactic acid bacteria as determined by reverse transcriptase sequencing of 16 S rRNA. *FEMS Microbiology Letters*. 77:5-12.

13. Costello, P. J., G. J. Morrison, T. H. Lee, and G. H. Fleet. 1983. Numbers and species of lactic acid bacteria in wines during vinification. *Food Technology in Australia*. 25:14-18.

14. Chalfan, Y., I. Goldberg, and R. I. Mateles. 1977. Isolation and characterization of malo-lactic bacteria from Israeli red wines. *Journal of Food Science*. 42:939-943.

15. Cho, G.-S., S. Krauß, M. Huch, M. du Toit, and C. M. A. P. Franz. 2011. Development of a quantitative PCR for detection of *Lactobacillus plantarum* starters during wine malolactic fermentation. *Journal of Microbiology and Biotechnology*. 21:1280-1286.

16. D'Incecco, N., E. Bartowsky, S. Kassara, A. Lante, P. Spettoli, and P. Henschke. 2004. Release of glycosidically bound flavour compounds of Chardonnay by *Oenococcus oeni* during malolactic fermentation. *Food Microbiology*. 21:257-265.

17. Davis, C. R., D. Wibowo, G. H. Fleet, and T. H. Lee. 1988. Properties of wine lactic acid bacteria: Their potential enological significance. *American Journal of Enology and Viticulture*. 39:137-142.

18. Davis, C. R., D. Wibowo, T. H. Lee, and G. H. Fleet. 1986. Growth and metabolism of lactic acid bacteria during and after malolactic fermentation of wines at different pH. *Applied Environmental Microbiology*. 51:539-545.

19. du Plessis, H. W., L. M. T. Dicks, I. S. Pretorius, M. G. Lambrechts, and M. du Toit. 2004. Identification of lactic acid bacteria isolated from South African brandy base wines. *International Journal of Food Microbiology*. 91:19-29.

20. du Toit, M., L. Engelbrecht, E. Lerm, and S. Krieger-Weber. 2011. *Lactobacillus*: The next generation of malolactic fermentation starter cultures – an overview. *Food and Bioprocess Technology*. 4:876-906.

21. Edwards, C. G., M. D. Collins, P. A. Lawson, and A. V. Rodriguez. 2000. *Lactobacillus nagelii* sp. nov., an organism isolated from a partially fermented wine. *Int J Syst Evol Microbiol.* 50:699-702.

22. Edwards, C. G., K. M. Haag, M. D. Collins, R. A. Hutson, and Y. C. Huang. 1998. *Lactobacillus kunkeei* sp. nov.: A spoilage organism associated with grape juice fermentations. *J Appl Microbiol.* 84:698-702.

23. Edwards, C. G., and K. A. Jensen. 1992. Occurrence and characterization of lactic acid bacteria from Washington state wines: *Pediococcus* spp. *Am. J. Enol. Vitic.* 43:233-238.

24. Ehrmann, M. A., M. R. A. Muller, and R. F. Vogel. 2003. Molecular analysis of sourdough reveals *Lactobacillus mindensis* sp. nov. *Int J Syst Evol Microbiol.* 53:7-13.

25. Endo, A., and S. Okada. 2005. *Lactobacillus satsumensis* sp. nov., isolated from mashes of shochu, a traditional Japanese distilled spirit made from fermented rice and other starchy materials. *Int J Syst Evol Microbiol*. 55:83-85.

26. Felis, G. E., and F. Dellaglio. 2007. Taxonomy of *Lactobacilli* and *Bifidobacteria*. *Curr. Issues Intestinal Microbiol.* 8:44-61.

27. Fumi, M. D., S. Krieger, M. Bou, A. Silva, and M. du Toit. 2010. Una nueva generación de bacterias malolácticas para vinos con pH elevado. *Enoviticultura*. 6:34-38.

28. Grimaldi, A., E. Bartowsky, and V. Jiranek. 2005. Screening of *Lactobacillus* spp. and *Pediococcus* spp. for glycosidase activities that are important in oenology. *Journal of Applied Microbiology*. 99:1061-1069.

29. Grimaldi, A., E. Bartowsky, and V. Jiranek. 2005. A survey of glycosidase activities of commercial wine strains of *Oenococcus oeni*. *International Journal of Food Microbiology*. 105:233-244.

30. Guzzon, R., A. Cavazza, and G. Carturan. 2009. Immobilization of malolactic fermentation bacteria with an innovative technique. *Bulletin O.I.V.* 82:83-92.

31. Hernández, T., I. Estrella, M. Pérez-Gordo, E. G. Alegría, C. Tenorio, F. Ruiz-Larrea, and M. V. Moreno-Arribas. 2007. Contribution of Malolactic Fermentation by *Oenococcus oeni* and *Lactobacillus plantarum* to the Changes in the Nonanthocyanin Polyphenolic Composition of Red Wine. *J. Agric. Food Chem.* 55:5260-5266.

32. Huang, Y.-C., C. G. Edwards, J. C. Peterson, and K. M. Haag. 1996. Relationship between Sluggish Fermentations and the Antagonism of Yeast by Lactic Acid Bacteria. *American Journal of Enology and Viticulture*. 47:1-10.

33. Inês, A. F. H. 2007. Abordagem polifásica na caracterização e selecção de bactérias do ácido láctico de vinhos da Região Demarcada do Douro. Ph.D. thesis. Universidade de Trás-os-Montes e Alto Douro.

34. Kandler, O. 1983. Carbohydrate metabolism in lactic acid bacteria. *Antonie van Leeuwenhoek*. 49:209-224.

35. Kandylis, P., A. Goula, and A. Koutinas. 2008. Corn starch gel for yeast cell entrapment. A view for catalysis of wine fermentation. *Journal of Agricultural and Food Chemistry*. 56:12037-12045.

36. Kourkoutas, Y., A. Bekatorou, I. M. Banat, R. Marchant, and A. A. Koutinas. 2004. Immobilization technologies and support materials suitable in alcohol beverages production: A review. *Food Microbiology*. 21:377-397.

37. Krieger-Weber, S. 2009. Application of Yeast and Bacteria as Starter Cultures. In: *Biology of Microorganisms on Grapes, in Must and in Wine*. Eds.: Helmut König, Gottfried Unden, and Jürgen Fröhlich. Springer-Verlag Berlin Heidelberg. 489-511.

38. Krockel, L., U. Schillinger, C. M. A. P. Franz, A. Bantleon, and W. Ludwig. 2003. *Lactobacillus versmoldensis* sp. nov., isolated from raw fermented sausage. *Int J Syst Evol Microbiol*. 53:513-517.

39. Kunkee, R. E. 1991. Some roles of malic acid in the malolactic fermentation in wine making. *FEMS Microbiology Letters*. 88:55-72.

40. Lafon-Lafourcade, S. 1983. Wine and brandy. In: *Biotechnology* Vol. 5. H. J. Rehm and G. Reed, eds. Verlag Chemie, Weinheim. 81-163.

41. Lafon-Lafourcade, S., E. Carre, and P. Ribéreau-Gayon. 1983. Occurrence of lactic acid bacteria during the different stages of vinification and conservation of wines. *Applied Environmental Microbiology*. 46:874-880.

42. Lerm, E., L. Engelbrecht, and M. du Toit. 2011. Selection and Characterisation of *Oenococcus oeni* and *Lactobacillus plantarum* South African Wine Isolates for Use as Malolactic Fermentation Starter Cultures. *South African Journal of Enology and Viticulture*. 32:280-295.

43. Liu, B., and X. Dong. 2002. *Lactobacillus pantheris* sp. nov., isolated from faeces of a jaguar. *Int J Syst Evol Microbiol*. 52:1745-1748.

44. Liu, S.-Q. 2002. Malolactic fermentation in wine – beyond deacidification. *Journal of Applied Microbiology*. 92:589-601.

45. Lonvaud-Funel, A. 1999. Lactic acid bacteria in the quality improvement and depreciation of wine. *Antonie van Leeuwenhoek*. 76:317-331.

46. Lonvaud-Funel, A., A. Joyeus, and O. Ledoux. 1991. Specific enumeration of lactic acid bacteria in fermenting grape must and wine by colony hybridization with non-isotopic DNA probes. *Journal of Applied Bacteriology*. 71:501-508.

47. Maicas, S. 2001. The use of alternative technologies to develop malolactic fermentation in wine. *Applied Microbiology and Biotechnology*. 56:35-39.

48. Mañes-Lázaro, R. 2010. Descripción de nuevas especies de *Lactobacillus* y desarrollo de sondas específicas para la detección de microorganismos en vinos mediante un Nanochip. Ph.D. thesis. Universitat de Valencia.

49. Mañes-Lázaro, R., S. Ferrer, A. M. Rodas, M. Urdiain, and I. Pardo. 2008. *Lactobacillus bobalius* sp. nov., a lactic acid bacterium isolated from Spanish Bobal grape must. *Int J Syst Evol Microbiol.* 58:2699-2703.

50. Mañes-Lázaro, R., S. Ferrer, R. Rosselló-Mora, and I. Pardo. 2009. *Lactobacillus oeni* sp. nov., from wine. *Int J Syst Evol Microbiol*. 59:2010-2014.

51. Mañes-Lázaro, R., S. Ferrer, R. Rosselló-Mora, and I. Pardo. 2008. *Lactobacillus uvarum* sp. nov. – A new lactic acid bacterium isolated from Spanish Bobal grape must. *Systematic and Applied Microbiology*. 31:425-433.

52. Maret, R., and T. Sozzi. 1977. Flore malolactique de moûts et de vins du Canton du Valais (Suisse). I. Lactobacilles et pédiocoques. *Annales de Technologie Agricole*. 27:255-273.

53. Maret, R., and T. Sozzi. 1979. Flore malolactique de moûts et de vins du Canton du Valais (Suisse). II. Évolution des populations de lactobacilles et de pédiocoques au cours de la vinification d'un vin blanc (un Fendant) et d'un vin rouge (une Dole). *Annales de Technologie Agricole*. 28:31-40.

54. Matthews, A., A. Grimaldi, M. Walker, E. Bartowsky, P. Grbin, and V. Jiranek. 2004. Lactic Acid Bacteria as a Potential Source of Enzymes for Use in Vinification. *Appl. Environ. Microbiol*. 70:5715-5731.

55. Michlmayr, H., C. Schümann, N. M. Barreira Braz da Silva, K. D. Kulbe, and A. M. Del Hierro. 2010. Isolation and basic characterization of a β-glucosidase from a strain of *Lactobacillus brevis* isolated from a malolactic starter culture. *Journal of Applied Microbiology*. 108:550-559.

56. Miyamoto, M., Y. Seto, D. Hai Hao, T. Teshima, Y. Bo Sun, T. Kabuki, L. Bing Yao, and H. Nakajima. 2005. *Lactobacillus harbinensis* sp. nov., consisted of strains isolated from traditional fermented vegetables "Suan cai" in Harbin, Northeastern China and *Lactobacillus perolens* DSM 12745. *Systematic and Applied Microbiology.* 28:688-694.

57. Mtshali, P. S., B. Divol, and M. du Toit. 2012. Identification and characterization of *Lactobacillus florum* strains isolated from South African grape and wine samples. *International Journal of Food Microbiology.* 153:106-113.

58. Mtshali, P. S., B. Divol, P. van Rensburg, and M. du Toit. 2010. Genetic screening of wine-related enzymes in *Lactobacillus* species isolated from South African wines. *Journal of Applied Microbiology*. 108:1389-1397.

59. Mtshali, P. S., B. Divol, P. van Rensburg, and M. du Toit. 2010. Genetic screening of wine-related enzymes in *Lactobacillus* species isolated from South African wines. Journal of Applied Microbiology 108:1389-1397.

60. Nehme, N., F. Mathieu, and P. Taillandier. 2009. Impact of the co-culture of *Saccharomyces cerevisiae-Oenococcus oeni* on malolactic fermentation and partial characterization of a yeast-derived inhibitory peptidic fraction. *Food Microbiology.* 27:150-157.

61. Nielsen, J. C., C. Prahl, and A. Lonvaud-Funel. 1996. Malolactic fermentation in wine by direct inoculation with freeze-dried *Leuconostoc oenos* cultures. *American Journal of Enology and Viticulture*. 47:42-48.

62. Vandamme, P., B. Pot, M. Gillis, P. de Vos, K. Kersters, and J. Swings. 1996. Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiological Reviews*. 60:407-438.

63. Picataggio, S. K., M. Zhang, M. A. Franden, J. D. McMillan, and M. Finkelstein. 1998. Recombinant *Lactobacillus* for fermentation of xylose to lactic acid and lactate. United States Patent No. 5,798,237.

64. Pozo-Bayón, M. A., E. G-Alegría, M. C. Polo, C. Tenorio, P. J. Martín-Álvarez, M. T. Calvo de la Banda, F. Ruiz-Larrea, and M. V. Moreno-Arribas. 2005. Wine Volatile and Amino Acid Composition after Malolactic Fermentation: Effect of *Oenococcus oeni* and *Lactobacillus plantarum* Starter Cultures. *Journal of Agricultural and Food Chemistry*. 53:8729-8735.

65. Prahl, C. 1989. A method of inducing the decarboxylation of malic acid in must or fruit juice. Patent WO 89/06685.

66. Prahl, C., A. Lonvaud-Funel, E. M. Korsgaard, and A. Joyeux. 1988. Étude d'un nouveau procédé de déclenchement de la fermentation malolactique. *Connaissance de la Vigne et du Vin*. 22:197-207.

67. Ribéreau-Gayon, J., D. Dubordieu, B. Donèche, and A. Lonvaud. 2000. *Handbook of Enology. Vol I. The microbiology of wine and vinifications*.

68. Rodas, A. M., E. Chenoll, M. C. Macian, S. Ferrer, I. Pardo, and R. Aznar. 2006. *Lactobacillus vini* sp. nov., a wine lactic acid bacterium homofermentative for pentoses. *International Journal of Systematic and Evolutionary Microbiology*. 56:513-517.

69. Rodas, A. M., S. Ferrer, and I. Pardo. 2003. 16S-ARDRA, a tool for identification of lactic acid bacteria isolated from grape must and wine. *Systematic and Applied Microbiology*. 26:412-422.

70. Rodas, A. M., S. Ferrer, and I. Pardo. 2005. Polyphasic study of wine *Lactobacillus* strains: Taxonomic implications. *International Journal of Systematic and Evolutionary Microbiology*. 55:197-207.

71. Schleifer, K. H., and W. Ludwig. 1995. Phylogeny of the genus *Lactobacillus* and related genera. *Systematic and Applied Microbiology*. 18:461-467.

72. Sponholz, W. R. 1993. Wine Spoilage by Microorganisms. *Wine Microbiology and Biotechnology*. Ed.: G. H. Fleet. Hardwood Academic Publishers. Switzerland. 395-420.

73. Swiegers, J. H., E. J. Bartowsky, P. A. Henschke, and I. S. Pretorius. 2005. Yeast and bacterial modulation of wine aroma and flavour. *Australian Journal of Grape and Wine Research*. 11:139-173.

74. Vaquero, I., A. Marcobal, and R. Munoz. 2004. Tannase activity by lactic acid bacteria isolated from grape must and wine. *International Journal of Food Microbiology*. 96:199-204.

75. Wibowo, D., R. Eschenbruch, C. R. Davis, G. H. Fleet, and T. H. Lee. 1985. Occurrence and growth of lactic acid bacteria in wine: A review. *American Journal of Enology and Viticulture*. 36:302-313.

76. Yoon, J. H., S. S. Kang, T. I. Mheen, J. S. Ahn, H. J. Lee, T. K. Kim, C. S. Park, Y. H. Kho, K. H. Kang, and Y. H. Park. 2000. *Lactobacillus kimchii* sp. nov., a new species from kimchi. *Int J Syst Evol Microbiol.* 50:1789-1795.

THE USE OF NON-*SACCHAROMYCES* YEASTS IN DEVELOPING A NEW METHOD OF AGING ON LEES

José Antonio SUÁREZ-LEPE and Antonio MORATA

enotecUPM, Dept. Food Technology, ETS Ingenieros Agrónomos, Universidad Politécnica de Madrid

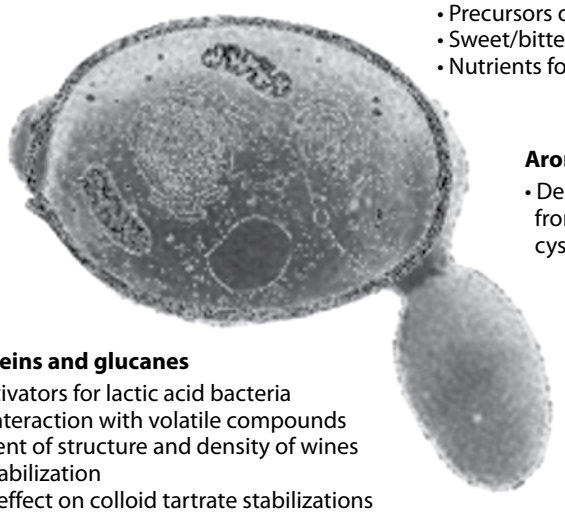
1. Introduction

Aging on lees (AOL) is a technique used to improve wine quality through a maturation process of the wine with yeast cells that can release cell constituents over time, enhancing the sensory properties of the wine. AOL has been used extensively in such white wines such as 1) those fermented in barrels (typical for Burgundy Chardonnays), 2) during the second (malolactic) fermentation, 3) during bottle-aging of natural sparkling wines, and 4) during the biological aging of Sherries.

2. Aging on Lees

During AOL, yeasts are able to release different compounds into the wine that have sensory repercussions (figure 1). Some peptides can provide sweet and/or bitter flavours and can also facilitate the development of malolactic fermentation (MLF). Volatile compounds from cell wall or cytosolic content will enrich the aromatic fraction of wines. And cell wall polysaccharides can act as nutrients for lactic acid bacteria (LAB) nutrients, favouring MLF, and can participate in the sensory profile, increasing the structure and density of wines. These nutrients can influence the colloidal stability, improving colour stability and decreasing crystalline precipitations.

Figure 1. Yeast cell components that have sensory repercussions



Peptides
- Precursors of aroma compounds
- Sweet/bitter flavourings
- Nutrients for malolactic fermentation

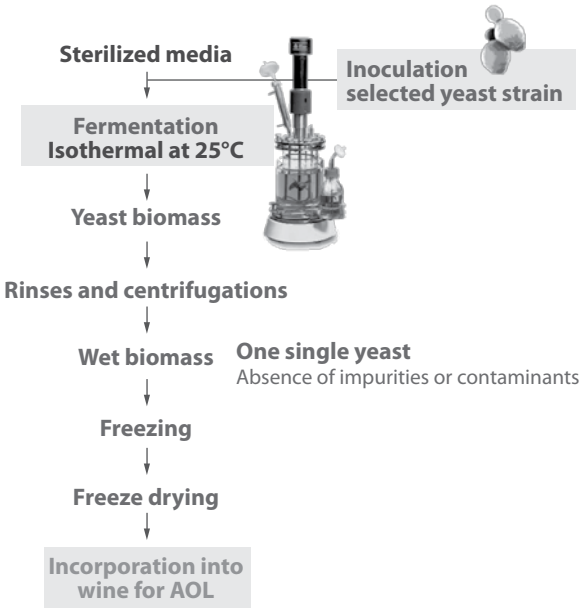
Aromas
- Derived volatile compounds from either cell wall or cystolic molecules

Mannoproteins and glucanes
- Growth activators for lactic acid bacteria
- Potential interaction with volatile compounds
- Improvement of structure and density of wines
- Pigment stabilization
- Protective effect on colloid tartrate stabilizations

The AOL process can be carried out through different techniques. The use of **gross lees** is the easiest technique and can be done by keeping the wine together with all the yeast lees after alcoholic fermentation (AF). The main drawback of this technique is the excessive input of nutrients, which facilitates undesired microbial growth and reduction off-odours (volatile sulphur) (Suárez-Lepe et al. 2005). In the **fine lees** technique after AF, the wine is racked, leaving only a fraction of the lees in suspension. The wine is aged in barrels or tanks with only this fraction of lees in suspension. The problem is the heterogeneous population of yeasts due to the fermentation. This technique is better than gross lees, and is less likely to produce organoleptic or microbiological deviations during aging.

We now use a new process where the lees are generated externally in a fermenter (figure 2) in order to utilize a selected yeast strain with the appropriate features for yeast autolysis and to eliminate the possibility of yeast alterations (Suárez-Lepe and Morata Barrado 2006).

Figure 2. Yeast cell components with sensory repercussions in exterior fermenter



3. Yeast Selection According to Autolysis Properties

The ability of yeast strains to release cell-wall polysaccharides during the autolysis process is strain dependant. Therefore, the selection of strains with fast autolysis can help us accelerate the AOL process. The autolysis process can be studied by measuring the contents of either extracellular proteins or cell-wall polysaccharides.

High-performance liquid chromatography (HPLC) coupled with a refractive index (RI) detector and size exclusion columns is a powerful chromatographic technique to separate and analyze cell-wall polysaccharides (Palomero et al. 2007). Using LC-RI analysis of cell biomass produced according to our technique (Suárez-Lepe and Morata Barrado 2006) and under the autolysis process in model media, we have measured the autolysis time/capacity of different *Saccharomyces* yeasts (figure 3), and have arrived at the following main conclusions:

- There are significant differences between yeasts in the amount of cell-wall polysaccharides released into the medium/wine
- Autolysis time is specific to each yeast
- The autolysis process is very slow (six or seven months).

We have also been using this procedure to analyze the effects of other ways to accelerate AOL, such us the use of β-glucanase enzymes (Palomero et al. 2009A).

3.1 Non-*Saccharomyces* yeasts versus aging on lees

The use of non-*Saccharomyces* yeasts is a powerful tool in the new oenological biotechnology toolbox. Non-*Saccharomyces* yeasts have special physiological and metabolic features that can help improve winemaking technology; currently they are being studied and utilized to improve the aromatic profile of wine, to make ecological interactions and to improve structure due to the production of glycerol or other polyalcohols.

The cell walls of osmophilic yeasts normally have a different composition, increasing their resistance to highly concentrated musts. The cell wall of *Schizosaccharomyces pombe* has a two-layer structure with a polysaccharide composition different from that normally found in *Saccharomyces* (figure 4).

The use of such non-*Saccharomyces* yeasts as *Schizosaccharomyces pombe* or *Saccharomycodes ludwigii* increases the release of polysaccharides during autolysis for 28 days. Furthermore, the molecular size of the polysaccharide fragments is larger than in *Saccharomyces cerevisiae*. Figure 5 shows LC-RI chromatograms of the polysaccharides released during autolysis for *Schizosaccharomyces pombe*, *Saccharomycodes ludwigii* and *Saccharomyces cerevisiae* (Palomero et al. 2009B).

Figure 3. LC-RI chromatograms of two yeast strains after 6 and 7 months of autolysis

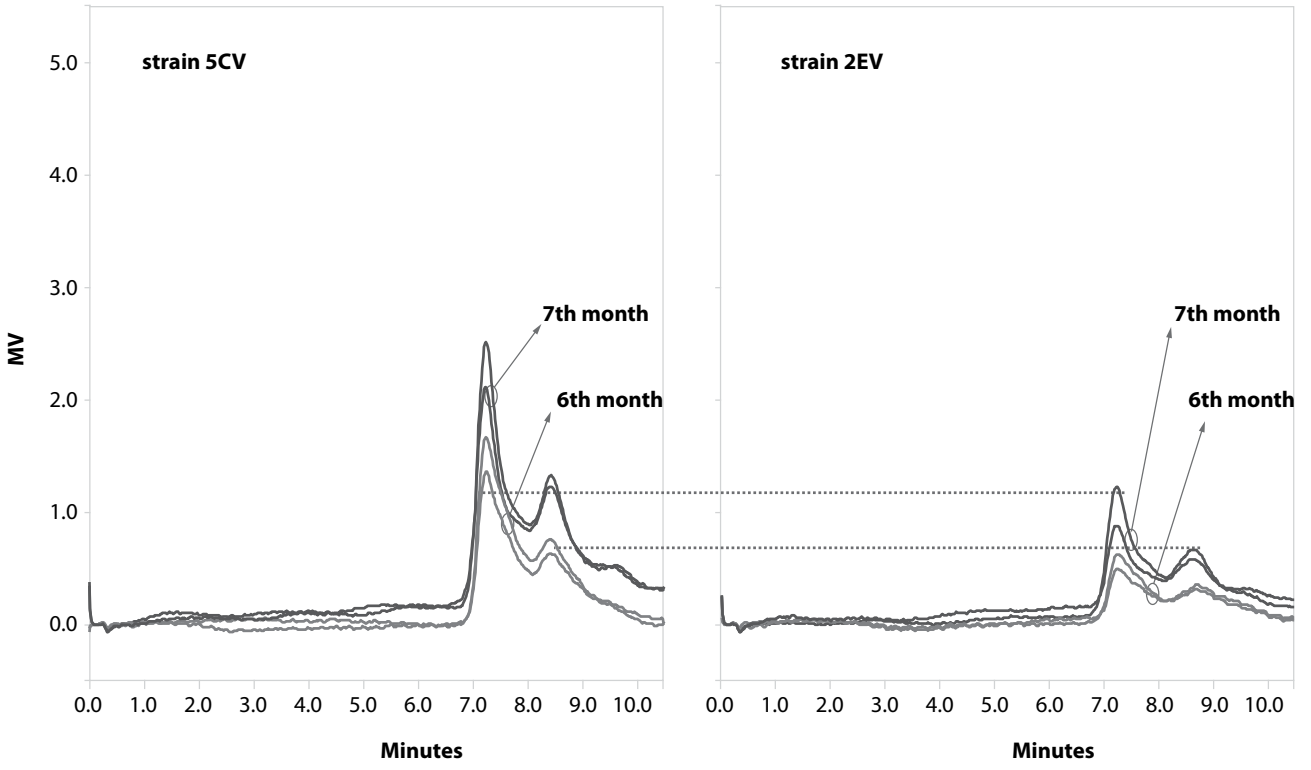


Figure 4. Cell wall structure of *Schizosaccharomyces pombe* (upper image) and *Saccharomyces cerevisiae* (lower image) (Palomero et al. 2009B)

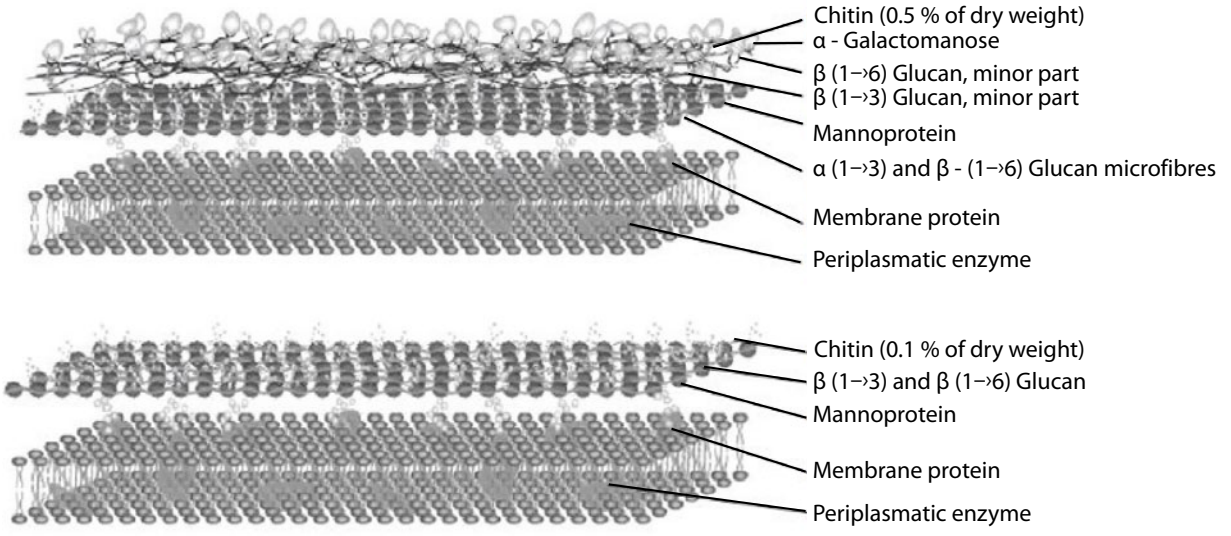
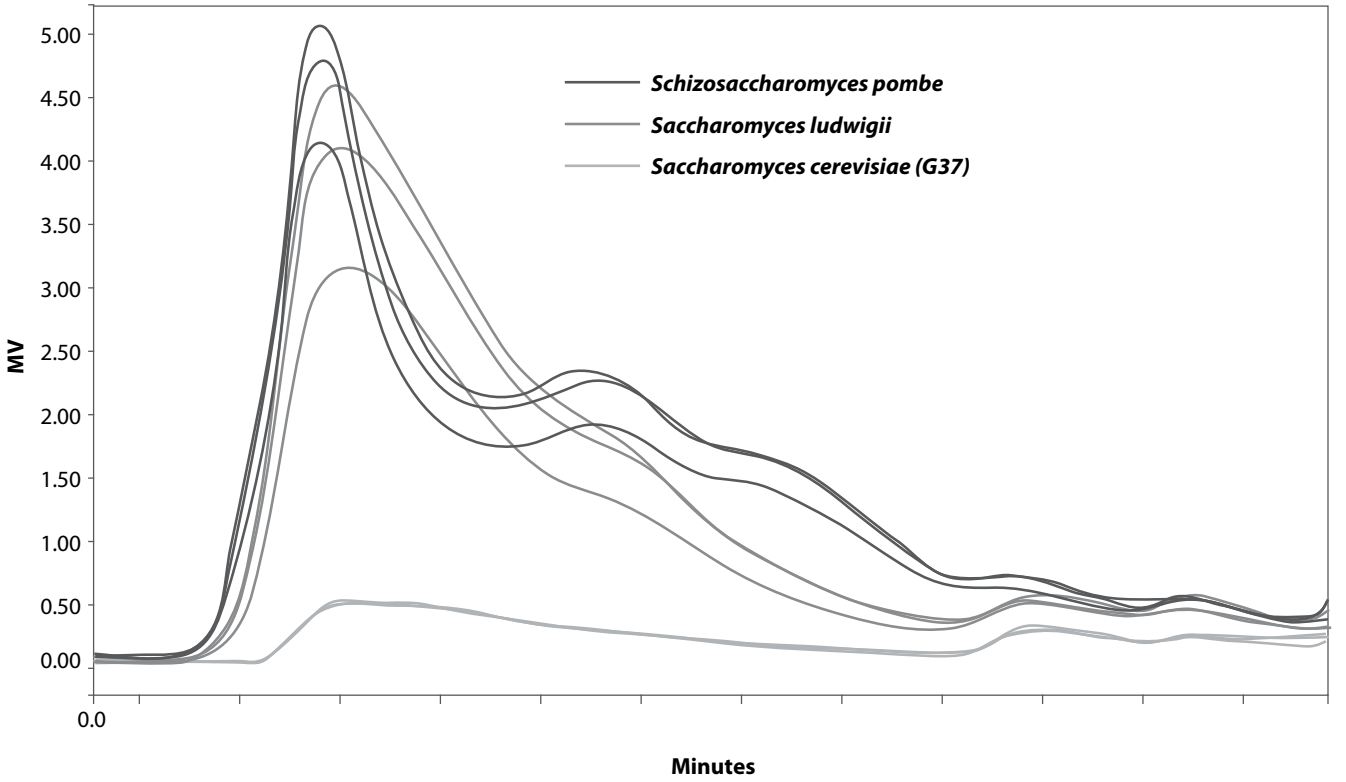


Figure 5. LC-RI chromatograms of the polysaccharides released during autolysis for *Schizosaccharomyces pombe*, *Saccharomycodes ludwigii* and *Saccharomyces cerevisiae*



During AOL, non-*Saccharomyces* yeasts can be useful to accelerate the aging process, increase the amount of polysaccharides of higher molecular size fragments. When these species are used in wines during AOL, the sensorial evaluation of the wines compared to wines aged with *Saccharomyces cerevisiae* is positive for *Schizosaccharomyces pombe*, but *Saccharomyces ludwigii* can develop some off-flavours.

4. Conclusions

According to the new technique for aging on lees, the use of pure cultures of selected strains of *Saccharomyces cerevisiae* yeast can improve the microbiological security of AOL. Moreover, certain selected strains can accelerate the process, thereby reducing costs, and can increase the amount of polysaccharides, improving the sensory perception of the wines. When this technique is associated with the use of certain non-*Saccharomyces* yeasts, such as *Schizosaccharomyces pombe*, we can produce wines even faster than with *Saccharomyces* and increase the amount of large-size polysaccharides.

References

Palomero, F., A. Morata, S. Benito, M. C. González, and J. A. Suárez-Lepe. 2007. Conventional and enzyme-assisted autolysis ageing over lees in red wines: Influence on the release of polysaccharides from yeast cell walls and on wine monomeric anthocyanin content. *Food Chemistry*. 105:838-846.

Palomero, F., S. Benito, A. Morata, W. Tesfaye, C. González, and J. A. Suárez-Lepe. 2009A. Effect on the autolysis process and the colouring matter of several commercial preparations with β-glucanase action in red winemaking. *Eur. Food Res. Technol*. 229:585-592.

Palomero, F., A. Morata, S. Benito, F. Calderón, and J. A. Suárez-Lepe. 2009B. New genera of yeasts for over-lees aging of red wine. *Food Chemistry*. 112:432-441.

Suárez-Lepe, J. A., A. Morata, F. Calderón, S. Somolinos, M. C. González, and B. Colomo. 2005. Utilización de levaduras seleccionadas en la crianza sobre lías de vinos tintos. Nuevo método de crianza sobre lías. *Alimentación, Equipos y Tecnología*. 207:39-43.

Suárez-Lepe, J. A., and A. Morata Barrado. Nuevo método de crianza sobre lías en vinos tintos. Spanish Patent: P200602423. 25/09/2006. Universidad Politécnica de Madrid.

MICROBIAL INTERACTIONS IN WINEMAKING: ANALYSIS AND PRACTICAL CONSEQUENCES

Pierre STREHAIANO
Université de Toulouse, INP-ENSIACET, Laboratoire de Génie Chimique, UMR 5503,
Département Bioprocédés et Systèmes Microbiens, Toulouse, France

1. Introduction

In winemaking, we can observe an enormous difference in the microbial population between the grape and the wine (as discussed by Aline Lonvaud-Funel). On the grapes, the microbial population is low and very heterogeneous, with moulds, yeasts and bacteria found. The major portion of this population remains unknown and the diversity of the species encountered varies according to the year, the climate and the agricultural practices. But in the wines, the microbial population is high, composed of only a few strains, species and genera of yeasts and bacteria.

2. Different Kinds of Microbial Interactions

The equilibrium of the microbial community is due to the interactions among the different kinds of microorganisms. These interactions are governed by such winemaking practices as adding sulphur dioxide or utilizing selected microflora, and by the metabolic activities of microbes providing growth metabolites, removing or producing toxic end-products, consuming oxygen and producing carbon dioxide. Clearly, the major changes occur during alcoholic fermentation (AF) and malolactic fermentation (MLF).

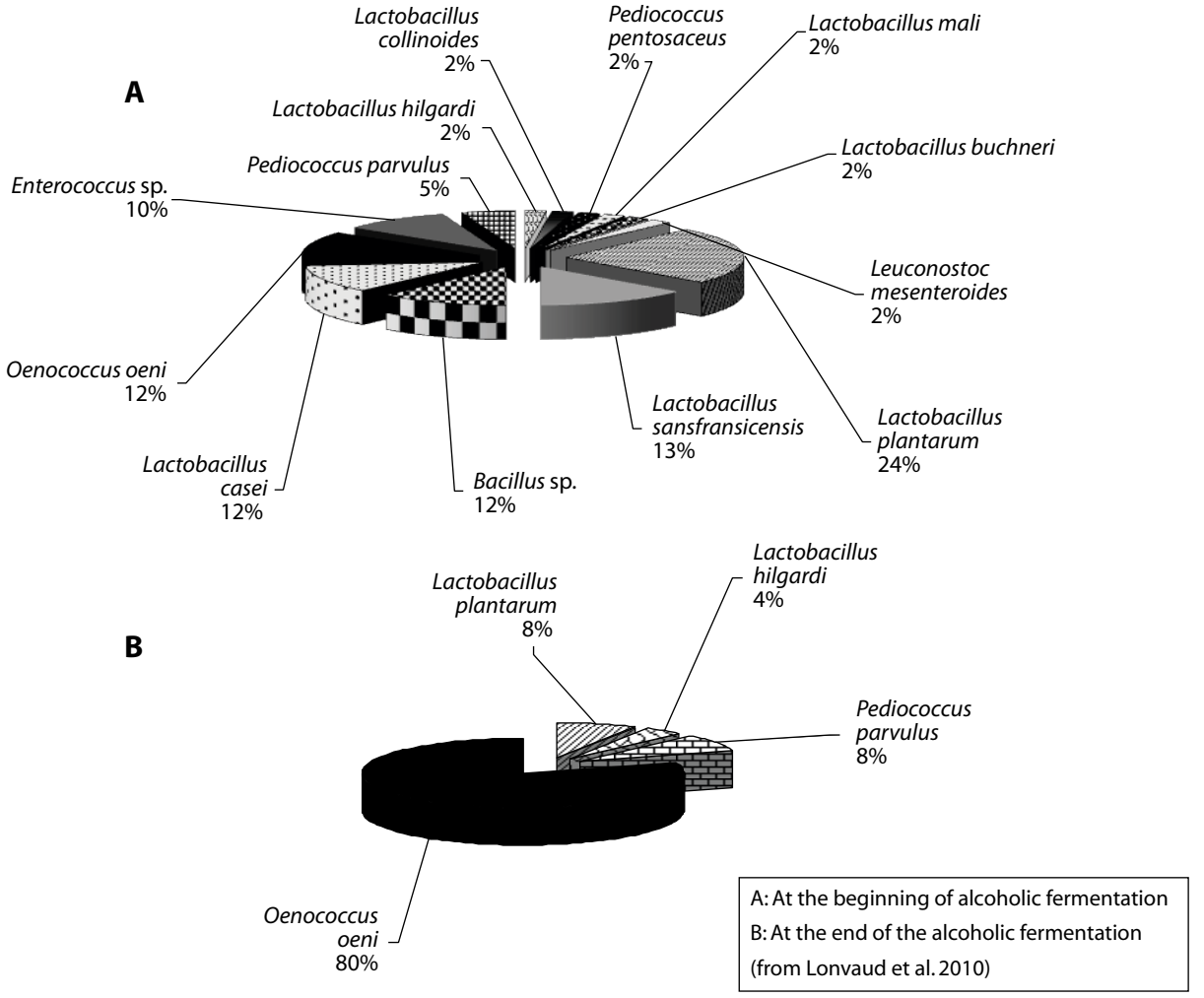
An illustration of this behaviour is found in the work of Dubourdieu and Frezier (1990). Their three-year study on the yeast populations in different cellars showed that at the beginning of AF many species of *Saccharomyces* are present in the must. But at the end of AF only one or two remain.

Recently (Lonvaud et al. 2010), a similar situation was observed with lactic acid bacteria (LAB). *Oenococcus oeni*, which made up only 12% of the total population at the beginning of AF, reached 80% by the end (figure 1).

Since the 1980s, selected *Saccharomyces* yeast strains have been commonly used as the starter in order to obtain good fermentation activity and sensory quality. More recently, the same has been done with LAB. And very recently non-*Saccharomyces* yeasts for AF are being analyzed. In all cases the same questions are asked: As the medium (i.e., the must or wine) is not sterile, is the preferred microorganism the one really responsible for the fermentation? Does it implant efficiently? What kind of relationships does it have with other microorganisms? The answers are complicated and we need to know as precisely as possible what happens when different microorganisms are growing at the same time. What kinds of interactions are there, and how do we analyze them?

The replacement of a microbial population by a less diverse one, and the ability of one species to overcome the others present are the result of interactions among the or-

Figure 1. Summary of fermentation conditions related to the production of rosé wines, and the theoretical curves for density and yeast population during alcoholic fermentation



ganisms. When first analyzed by Frederickson (1977), he distinguished direct interactions, where physical contact between the microorganisms is needed, and indirect interactions, with no contact between them.

The three kinds of direct interactions described by this author are predation, parasitism and symbiosis. None of them occur in winemaking. Recently, a new mode of direct interaction was described – "cell-to-cell" contact – which supposes that the contact between two cells can induce the death of one of them. Some authors think this can occur in winemaking.

As for indirect interaction, the action of one microorganism on another may be due to:

- Competition for a common substrate. In this case, the microorganism whose growth was promoted is the one with the higher growth rate
- A molecule produced by one of the microbes acting on the other.

Different possibilities are defined:

- Amensalism means organism A acts negatively on the growth of organism B
- Commensalism means A acts positively on the growth of B
- Mutualism is when A and B act positively on each other
- Quite recently, a new mode has been defined: "quorum sensing." This supposes cells are able to excrete specific molecules that act on the producing cells themselves and then stop the growth at a given level.

In winemaking, cases of amensalism are usual and quorum sensing may explain why the growth stops while substrates are still available (Bisson 1999). Table 1 gives an overview.

Table 1. Different kinds of microbial interactions (from Frederickson 1977). New mechanisms are in bold typeface.

No interaction

• Neutralism

Direct interactions

• Physical contact between interacting organisms (predation, parasitism and symbiosis) – no examples in winemaking

• **Cell-to-cell contact** (Aoki et al. 2005)

Indirect interactions

No contact between the organisms. Action is due to:

• Competition for a common substrate

• A chemical compound is excreted by A and acts on B:

- Amensalism: A acts negatively on the growth of B
- Commensalism: A acts positively on the growth of B.
- Mutualism: A and B act positively on each other

Quorum sensing

3. Methods of Interaction Analysis

As interactions are complex systems, the first step to studying them is to use the appropriate method: The growth of each of the different microorganisms growing together needs to be studied separately. Currently, four different approaches are available, each having its advantages and its limits: 1) The agar plate method, 2) The method based on sequential cultures, 3) The use of mixed cultures, and 4) The use of a specific apparatus in which the organisms are growing in the same medium but are separated by a membrane. Note that, in most cases, only one pair of microorganisms is studied at a time.

For the agar plate method, microorganism A is poured onto the agar gel while microorganism B is placed in specific spots then their interaction is evaluated by the clear zone surrounding the inhibiting microorganism. Quite similar to the well-known antibiogram method, this method is easy to manage and frequently used, giving qualitative results, and is often utilized in the first step of the analysis. However, the disadvantages are numerous: The diffusion of the active molecule in the agar is unknown, it is impossible to quantify the growth of the microorganisms, and it is impossible to know the changes in the composition of the medium. We proved several years ago that it is impossible to correlate with precision the results obtained this way with the results obtained in liquid cultures or observed at the industrial scale. Thus, in our mind, this method must be used only in the first step of study.

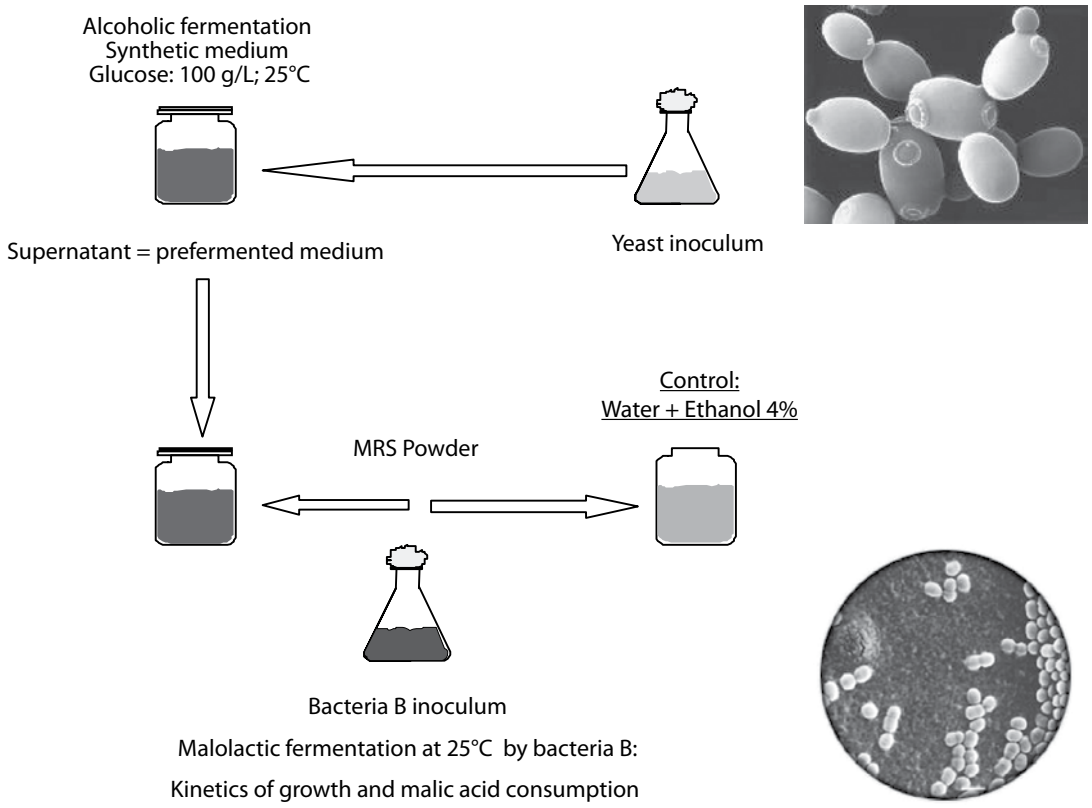
The second method is based on sequential cultures of the mircoorganisms and the interaction between *Saccharomyces cerevisiae* and *Oenococcus oeni* is illustrated in figure 2.

First, the yeast A (*S. cerevisiae* in this example), is cultivated in a liquid medium. At the end of growth, cells are eliminated from the medium and new nutrients are poured in to avoid nutritional deficiencies. Then the LAB B (*O. oeni* in the example) is inoculated into the medium. Comparing the growth of B in these conditions to its growth in a control medium allows us to observe if there is any relationship between the microorganisms.

The procedure is easy to carry out and provides interesting and practical data, especially quantitative data. Nevertheless, things are not exactly as they are in real life, and, for example, a competition mechanism may be held back.

The third method is based on mixed cultures of the tested microorganisms. The microorganisms are inoculated together into the culture medium, and the growth of each is checked separately by sampling and spreading cells on agar plates. Colonies are then identified. As more than two microorganisms may be utilized at the same time, this approach is clearly the best way to analyze the relationships between them. But the disadvantages are numerous. First, each of the colonies to be identified must be very large for statistical reasons. Second, the nature of the agar medium may promote the growth of some microorganisms (or the opposite). Third, the identification method may be

Figure 2. Sequential culture method (example with *Saccharomyces cerevisiae* and *Oenococcus oeni*)



difficult to manage, time consuming and very expensive. Nevertheless, this method is the only one to be used for ecological studies. Recent studies (see figure1) show that the use of quantitative polymerase chain reaction (qPCR) gives excellent and reliable results when the population is made up of different species of microorganisms. Unfortunately, qPCR does not work when studying mixed cultures of microorganisms belonging to the same species.

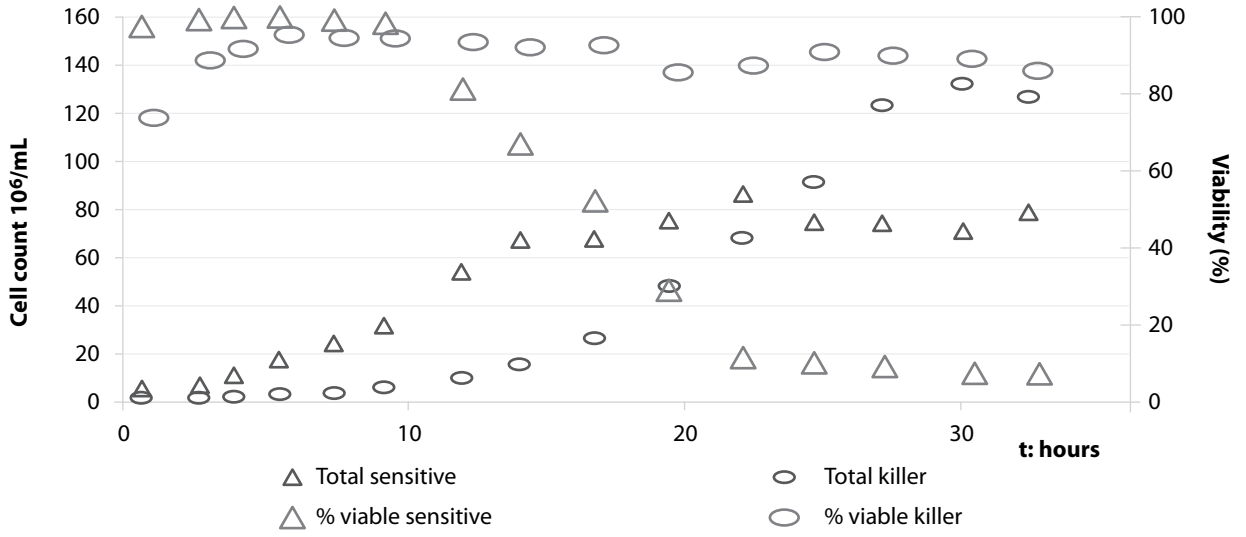
For many years, researchers have sought a method able to give accurate quantitative results. Different devices based on the physical separation of microorganisms have been elaborated, such as the Corning Transwell® system and membrane bioreactors (MBRs). As the two microorganisms to be analyzed are growing separately but share the same medium, it is possible to use simple methods, such as Thoma cell-counting chambers, absorbance, etc., for estimating the growth of each microorganism. The MBR (Salgado et al. 2000) utilized in our study is made of two jars connected by a hollow fibre membrane module immersed into one of them. The membrane's pores let metabolites and permeates pass through, while blocking microbial cells. The most important point is to have exactly the same medium in each jar. In this type of reactor, medium flow and mixing are obtained by applying pressure alternatively in the head space of each vessel. Even if the apparatus is quite heavy to manage, it provides sound quantitative data on the growth of the microbes. There are some disadvantages, however. Only two strains can be analyzed at a time, and the nature of the membrane and the size of the pores must be carefully selected to avoid adsorption or retention of active molecules or cells.

4. Interactions in Winemaking: Some Cases and Methods Utilized

4.1 Killer mechanism analyzed using the membrane bioreactor

A classic type of interaction is amensalism, called the "killer" phenomenon – one of the most famous examples of the strong amensalism type of interaction. It occurs between yeast strains of the same species sharing the same culture medium. One yeast, called killer (K), has the property to excrete a toxin that affects another yeast, called sensitive (S), by damaging the cell membrane and, in the end, killing it. In winemaking, the grape must is not sterile and the efficiency of a selected yeast depends on its ability to overcome the indigenous population. Using the MBR

Figure 3. Behaviour of killer cells and sensitive cells in a membrane bioreactor (from Pommier et al. 2002)



made it possible to distinguish the K population from the S one, and the viable cells from the dead cells within each population. Figure 3 illustrates the behaviour of these four groups during the culture.

The initial K/S ratio was 10%. The efficiency of the killer factor appears clearly: Within as little as 10 hours, the growth of the K strain overcame the growth of the S strain. At the end of the culture, sensitive cells are dead while the viability of the killer strain is nearly 100%.

4.2 Interactions between *Saccharomyces cerevisiae* and *Oenococcus oeni*

For the first qualitative approach, the agar plating method was utilized. A total of 12 yeast strains and 24 *O. oeni* LAB strains were tested, which means 288 pairings. Table 2 gives an overview of the results: In 98% of cases, *O. oeni* was more or less inhibited by *S. cerevisiae*.

Table 2. Interactions between *Oenococcus oeni* and *Saccharomyces cerevisiae* analyzed by the agar plating method; 288 pairings (12 yeasts strains and 24 bacteria strains) (from Tataridis 2001)

	Kind of interaction			
	Neutrality		Inhibition	
	Slight	Medium	Strong	
% of pairings by kind of interaction	3%	57%	31%	9%

In a second step, 10 pairings representing the different kinds of interaction were studied using the sequential culture method. Both growth and malolactic activity were analyzed. Neutrality was not observed. The inhibition of the growth of *O. oeni* varies from 40% to 80%, while the inhibition of malolactic activity varies from 20% to 80% (from Taillandier et al. 2002, and Nehme 2008). It was also noted that growth was dependent on the environmental conditions (Gilis et al. 1996). This method of sequential cultures was proposed by Costello et al. (2002) as a standardized method for testing LAB and yeast compatibility.

4.3 Yeast-yeast interactions

Saccharomyces/Saccharomyces

This method of sequential cultures was also utilized to analyze possible interaction between different yeasts. The objective was to explain the difficulty sometimes encountered to inoculate a wine in a state of stuck fermentation. The medium was inoculated with different *Saccharomyces*. At the end of the growth period, cells were eliminated and nutrients poured into the medium. Then the medium was inoculated with the same microorganisms and the sugar consumption rate was utilized to compare the activity of the cells. A culture on a fresh medium (with the same alcohol content as the prefermented medium) was used as a control. The results are reported in table 3. Three points are noticeable: First, the growth of all microorganisms in the prefermented medium was more or less inhibited; second, in every case a phenomenon of autoinhibition was observed; third, the sensitivity of the different microorganisms varies greatly from one to another.

Table 3. Substrate consumption rates (g/L/h) by different *Saccharomyces* yeasts growing on a medium prefermented by one of these species (from Strehaiano et al. 1985)

Inoculated strain				
Growth medium	*S. c. A*	*S. c. B*	*S. c. sake*	*S. uvarum*
Control (fresh medium)	1.55	1.82	1.54	0.95
Medium prefermented with *S. c.* A	0.80	1	0.80	0.42
Medium prefermented with *S. c.* B	0.80	1	0.83	0.42
Medium prefermented with *S. c. sake*	0.73	1	0.80	0.42
Medium prefermented with *S. uvarum*	0.49	1	0.50	0.53

Saccharomyces* and *Torulaspora

For this pairing, the results obtained utilizing the mixed culture method were compared with those from cultures in the MBR. Using the MBR, Lai (2010) observed that the growth of *Torulaspora delbrueckii* was reduced in mixed cultures while that of *Saccharomyces* remained unchanged. Renault (2010), using another kind of MBR and microrganisms different from those of Lai, also observed an inhibition of *Torulaspora* growth by *Saccharomyces*. But when studying the mixed cultures of these yeasts, they showed that the inhibition was higher in mixed cultures than in the MBR apparatus. Therefore they suggest a mechanism of cell-to-cell contact as did Nissen et al. (2003), utilizing the same yeasts.

5. Interactions and Winemaking Practices

Although the precise mechanisms of interactions are not yet well known, some rules may be considered in winemaking.

Our first example is inoculating the must with a selected yeast. Because of the competition between the selected yeast and the indigenous ones, the dominance of the selected strain depends on the initial ratio of this strain and the indigenous population: A 5 to 10 ratio leads to a 90% success rate; if the selected strain is a killer, a 4 to 5 ratio leads to the same success rate. But to reach a 100% success rate, the ratio must be more than 20. The main conclusion is: Beware of the level of the indigenous microbial population in the must!

Our second example addresses the problem of stuck fermentations. How can the winemaker ensure the success of a re-inoculation when the must is polluted by the metabolites produced by a "bad health" population?

Some rules may be given: The wine composition must be checked and corrected, if needed. A treatment by yeast ghosts (i.e., yeast hulls), which remove the inhibitors, may be useful. The must will be inoculated with a different yeast from the initial one, and this inoculum must be progressively adapted to the wine conditions by several pre-cultures.

The third example deals with the use *O. oeni* starters. The question is: Co-inoculation or sequential inoculation?

The most common way is to inoculate the must with *O. oeni* LAB at the end of AF, and in most cases that leads to good results. But problems may occur due to the strong inhibition of the LAB by *Saccharomyces*, as shown above. Co-inoculation is another way (i.e., yeast and LAB at the same time). *O. oeni* is expected to gradually adapt to the changes in its environment during AF. Good results have been reported with certain co-inoculation kits available on the market.

The issue of using non-*Saccharomyces* yeast has recently been studied. *Torulaspora* is currently the non-*Saccharomyces* yeast most utilized, but the same question arises: Co-inoculation or a two-step process? In co-inoculation, the two yeasts are put together and the light development of *Torulaspora* is expected to lead to the desired benefits. Then *Torulaspora* disappears and *Saccharomyces* takes over. The question is: Are we sure that *Torulaspora* works? If the interaction is to strong, *Torulaspora* could disappear from the medium too soon.

The alternative is a two-step process – a sequential culture. First, the must is inoculated with *Torulaspora* yeast and then two or three days later the must is inoculated with *Saccharomyces*, ensuring most of the AF. Good results have been reported by Renault (2010) and, most recently, Salmon (2012).

6. Conclusion

Clearly, it appears that microbial interactions involve many very complex mechanisms and that their study requires analytical devices that are very onerous to manage. Some of these mechanisms are now quite well understood, but many remain to be discovered or explained. We can expect exciting advances thanks to new tools of molecular biology, such as the quantitative polymerase chain reaction. Although current knowledge is not yet all-encompassing, some rules and processes are available to winemakers now, and recent research into new strains, hybrids and techniques in the use of microorganisms, such as immobilized cells, may soon give winemakers excellent new tools.

References

Aoki, S. K., R. Pamma, A. D. Hernday, J. E. Bickham, B. A. Braaten, and D. A. Low. 2005. Contact-dependent inhibition in *Escherichia coli*. *Science*. 309:1245-1248.

Bisson, L. F. 1999. Stuck and sluggish fermentations. *Am. J. Enol. and Viti*. 50:107-119.

Costello, P. J., P. A. Henschke, and A. J. Markides. 2002. Developing a standardized method for testing malolactic bacteria and wine yeast compatibility. Proceedings of *Lallemand Technical Meetings*. 10:5-14.

Dubourdieu, D., and V. Frezier. 1990. Application de l'électrophorèse en champ pulsé à l'étude de l'écologie des levures en fermentation spontanée. *Rev. Fr. Oenol.* 125:37-40.

Frederickson, A. G.1977. Behavior of mixed cultures. *Ann. Rev. Microbiol.* 31:63-87.

Gilis, J. F., M. L. Delia, and P. Strehaiano. 1996. Qualitative and quantitative study of interactions between *Saccharomyces cerevisiae* and *Leuconostoc oenos*. *J. Int. Sc. Vigne Vin*. 30:151-157.

Lai, Q. P. 2010. Utilisation de levures non-*Saccharomyces* en œnologie : Étude des interactions entre *Torulaspora delbrueckii* et *Saccharomyces cerevisiae* en cultures mixtes. Ph.D. thesis. Institut National Polytechnique de Toulouse.

Lonvaud-Funel, A., V. Renouf, and P. Strehaiano. 2010. *Microbiologie du Vin. Bases fondamentales et applications*. Lavoisier Ed. Paris.

Nehme, N. 2008. Étude des interactions entre *Saccharomyces cerevisiae* et *Oenococcus oeni* : Impact sur la réalisation de la fermentation malolactique en cultures séquentielles et mixtes. Ph.D. thesis. Institut National Polytechnique de Toulouse.

Nissen, P., D. Nielsen, and N. Arnborg. 2003. Viable *Saccharomyces* cells at high concentration causes early growth arrest of non-*Saccharomyces* yeasts in mixed cultures by a cell-cell contact-mediated mechanism. *Yeast*. 20:331-341.

Pommier, S., C. Albasi, P. Strehaiano, and M. L. Delia. 2002. Modelisation of killer yeast systems: Quantification of mixed culture dynamics with a specific membrane bioreactor. Proceedings of *Foodsim 2002* (2nd International Conference on Simulation in Food and Bio Industries). 197-201.

Renault, P. E. 2010. Caractérisation phénotypique de l'espèce *Torulaspora delbrueckii* en conditions œnologiques. Application de la co-inoculation avec l'espèce *Saccharomyces cerevisiae*. Ph.D. thesis. Université de Bordeaux 2.

Salgado Manjarrez, E., C. Albasi, and J. P. Riba. 2000. A two reservoir hollow fibre reactor for the study of mixed population dynamics: Design and validation of the approach. *Biotechnol. Bioeng*. 69:401-408.

Strehaiano, P., M. Mota, and G. Goma. 1985. Inhibitions non-conventionnelles des croissances levuriennes : Effets interspécifiques et essais de levée d'inhibition. *Conn. Vigne Vin*. 19(2):97-107.

Taillandier, P., P. Tataridis. and P. Strehaiano. 2002. A quantitative study of antagonism between *Saccharomyces cerevisiae* and *Oenococcus oeni*. Proceedings of *Lallemand Technical Meetings*. 10:21-26.

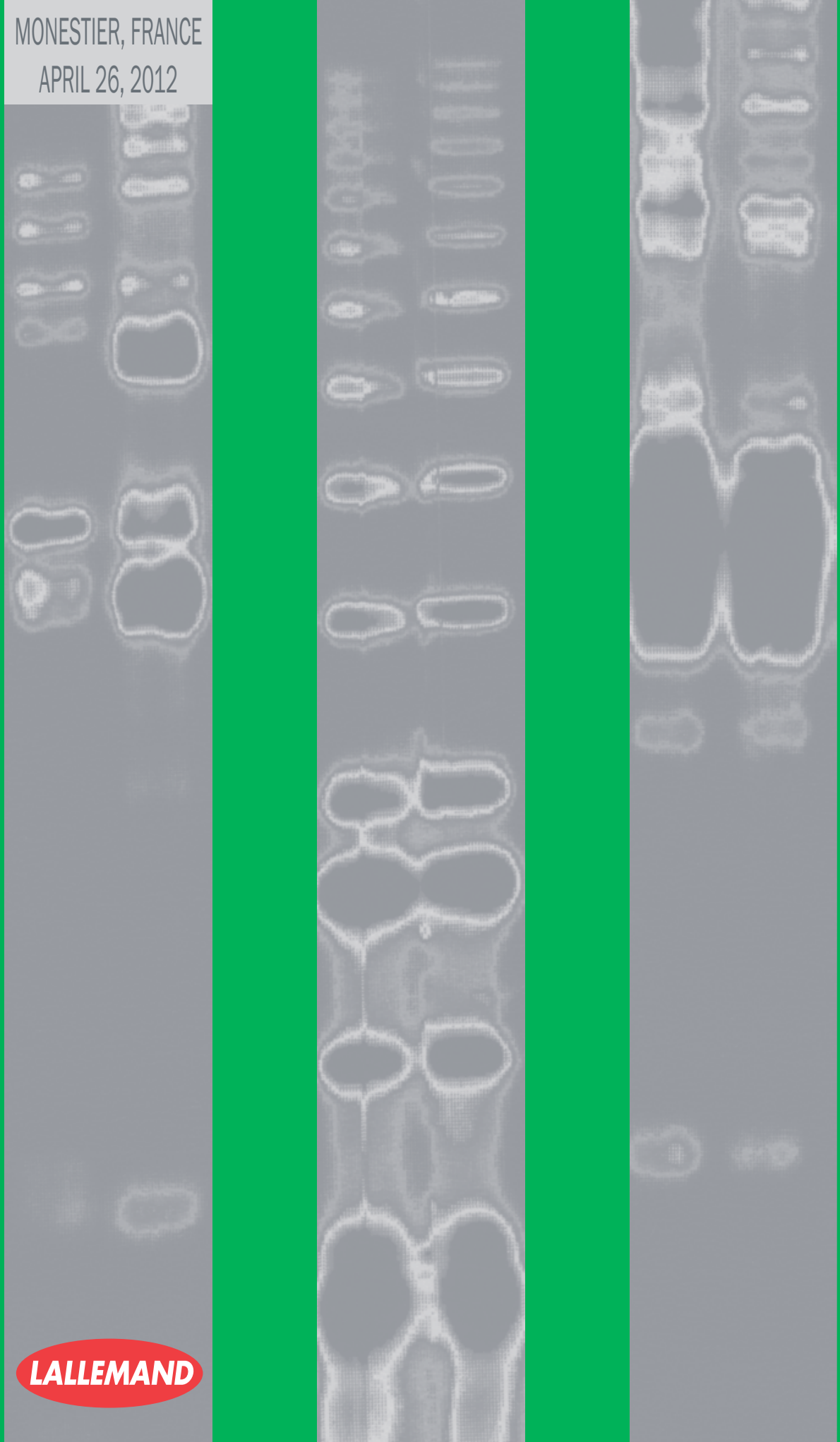
Tataridis, P. 2001. Étude des interactions entre microorganismes du vin: du qualitatif au quantitatif. Ph.D. thesis. Institut National Polytechnique de Toulouse.

Original Cover Design: Bruno Loste – Layout and Printing: MODULI INC.

LALLEMAND S.A.S. – 19, rue des Briquetiers - B.P. 59 - 31702 Blagnac CEDEX – Tel.: +33 (0)5 62 74 55 55 – Fax: +33 (0)5 62 74 55 00
www.lallemandwine.com

MONESTIER, FRANCE
APRIL 26, 2012

19



LALLEMAND