UNIVERSITA' DEGLI STUDI DI VERONA

DEPARTMENT OF BIOTECHNOLOGY

GRADUATE SCHOOL OF NATURAL SCIENCES AND ENGINEERING

DOCTORAL PROGRAM IN BIOTECHNOLOGY

WITH THE FINANCIAL CONTRIBUTION OF CONSELHO NACIONAL DE DESENVOLVIMENTO CIENTÍFICO E TECNOLÓGICO (CNPq)

XXI Cycle / 2015

A NEW GENERATION OF YEASTS FOR THE DIFFERENTIATION AND IMPROVEMENT OF WINE QUALITY

S.S.D. AGR/16

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A new generation of yeasts for the differentiation and improvement of wine quality
Renato Leal Binati
PhD thesis
Verona, 18th December 2018
ISBN

Esta tese é dedicada aos meus avós.

(this thesis is dedicated to my grandparents)

There's wisdom in wine.

Jack Kerouac

SUMMARY

The optimized use of the grape environment indigenous microbiota has been proposed as an innovative tool to mimic the complexity of the spontaneous fermentation process, without taking the risks of having poor-quality wines due to the uncontrolled growth of spoilage organisms or having a "standardized" product lacking originality caused by the inoculation of a single starter culture of *Saccharomyces cerevisiae* exhaustively employed worldwide. With the aim of exploring new alternatives for the improvement of wine quality, it was proposed the screening of innovative yeasts to be used on mixed fermentations together with *S. cerevisiae*. After the mining of more than 400 yeasts isolates in samples coming from multiple Italian wine-producing regions, a collection of non-*Saccharomyces* yeasts was thoroughly described morphologically and identified. About one quarter of them belonging to three more interesting and prevalent oenological species, namely *Starmerella bacillaris*, *Lachancea thermotolerans* and *Metschnikowia* spp., were picked for an in-depth molecular and physiological characterization. After the genotyping, stress tolerance assays, enzymatic activity trials and single inoculum fermentations, important differences were acknowledged between the strains and species, allowing for the selection of a reduced number of isolates showing potentially positive oenological traits. Furthermore, the isolates of *S. bacillaris* and *L. thermotolerans* were also submitted to a safety assessment of virulence factor related to human pathogenicity and were challenged to inhibit the growth of the phytopathogen *Botrytis cinerea*, responsible for massive quantitative and qualitative losses on the wine industry, across *in vitro* and *in vivo* assays, in order to become candidates for an integrated vitivinicultural strategy of biocontrol and mixed fermentation. The chosen isolates from the preliminary selection steps were then applied in sequential inoculations with a commercial *S. cerevisiae* in natural grape must. The physicochemical analysis and the aromatic profile confirmed the great potential of all three species to a successful winemaking routine, mainly focused on the glycerol increase and reduction of acetaldehyde concentration for *S. bacillaris*, limitation of acetic acid and boosted aromatic complexity for *Metschnikowia* spp., and diminution on ethanol content and enhanced natural acidity by lactic acid production for *L. thermotolerans*. This last feature was intriguingly highly variable among the strains and thus received a more detailed attention, revealing that sequence mutations and transcriptional regulatory mechanisms could be involved in the strong variance of a very relevant metabolic pathway. Indeed, many of the distinctive features of alternative yeasts are species- or strain-dependent and the efficient selection of a new generation of starter cultures could provide satisfactory answers to multiple challenges of modern winemaking.

RESUMO

O uso otimizado da microbiota autóctone associada às uvas tem sido proposto como uma ferramenta inovadora para mimetizar a complexidade de um processo de fermentação espontânea, evitando os riscos de se obter vinhos de baixa qualidade devido ao crescimento descontrolado de microrganismos indesejados ou vinhos "padronizados" com falta de originalidade como resultado da inoculação de uma cultura pura de starter *Saccharomyces cerevisiae* exaustivamente utilizada ao redor do mundo. Com o objetivo de explorar novas alternativas para a melhoria na qualidade dos vinhos, foi proposta uma triagem de leveduras inovadoras para utilização em culturas mistas com *S. cerevisiae*. Após o isolamento de mais de 400 leveduras a partir de amostras originárias de diversas regiões vitivinícolas da Itália, uma coleção de isolados non-*Saccharomyces* foi minuciosamente descrita do ponto de vista morfológico e identificada. Cerca de um quarto dos isolados, pertencentes à três espécies de maior abundância e maior interesse enológico, a saber *Starmerella bacillaris*, *Lachancea thermotolerans* e *Metschnikowia* spp., foram escolhidos para um densa caracterização molecular e fisiológica. Após a genotipagem, ensaios de tolerância ao estresse, provas de atividade enzimática e fermentações com cultura simples, diferenças importantes entre as espécies e cepas foram observadas, permitindo a seleção de um número limitado de isolados com potenciais características enológicas positivas. Adicionalmente, os isolados de *S. bacillaris* e *L. thermotolerans* foram submetidos a uma verificação de fatores de virulência associados à patogenicidade em humanos e foram desafiados a inibir o crescimento do fitopatógeno *Botrytis cinerea*, responsável por enormes perdas qualitativas e quantitativas na indústria do vinho, em testes in vitro e in vivo, com o objetivo de obter candidatos pra uma estratégia vitivinícola integrada de biocontrole e fermentação mista. Os isolados selecionados durante as primeiras fases de triagem foram então aplicados em fermentações sequenciais com um starter comercial de *S. cerevisiae* em mosto natural de uvas. As análises físico-químicas e o perfil aromático confirmaram o enorme potencial das três espécies para uma bem-sucedida produção de vinho, focada principalmente no aumento de glicerol e redução na concentração de acetaldeído para *S. bacillaris*, diminuição no ácido acético e incremento na complexidade aromática para *Metschnikowia* spp., e limitação da quantidade de etanol com uma melhora na acidez natural pela produção de ácido lático para *L. thermotolerans*. Esta última característica se mostrou intrigantemente muito variável entre as diversas cepas e recebeu então uma atenção mais detalhada, que revelou mutações na sequência e mecanismos de regulação transcricional provavelmente envolvidos na significativa variância dessa via metabólica de alta relevância. De fato, muitas das características peculiares das leveduras alternativas são dependentes da espécie ou cepa e uma seleção eficiente de uma nova geração

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LIST OF ABBREVIATIONS

a_W – Water activity

BCA – Biological control agent

BSA – Bovine serum albumin

CFU – Colony forming units

DEPC - Diethylpyrocarbonate

DGGE - Denaturing gradient gel electrophoresis

dNTPs - Deoxynucleotide triphosphates

GC-MS - Gas chromatography-Mass spectrometry

HPLC – High performance liquid chromatography

ITS – Internal transcribed spacer

LA – L(+)-Lactic acid

LDH – Lactate dehydrogenase

OD_{600} – Optical density measured at a wavelength of 600 nm

PAN – Primary amino nitrogen

PCA – Principal component analysis

PCR - Polymerase chain reaction

qPCR – Real-time PCR

RAPD - Random amplification of polymorphic DNA

RFLP - Restriction fragment length polymorphism

ROS – Reactive oxygen species

SLAD - Synthetic low ammonium dextrose

SNP - Single-nucleotide polymorphism

SPE – Solid phase extraction

SSR – Simple sequence repeats

T_m – Melting temperature

UPGMA - Unweighted pair group method with arithmetical average

VOC - Volatile organic compound

WL - Wallerstein Laboratory Nutrient Agar

YAN – Yeast available nitrogen

YNB – Yeast nitrogen base

PREFACE

The PhD research was performed in its majority in the Laboratory of Food Microbiology, Department of Biotechnology of the University of Verona. Some chemical analysis of the wines produced in the microvinification trials were carried out in the Laboratory of Oenological Chemistry of the same university, and DNA sequencing was performed at external facilities as indicated in the chapters regarding this analysis. The thesis is structured as follows:

Chapter 1 Premise

Chapter 2 Ecology of non-*Saccharomyces* yeasts in high sugar matrices from different regions of Italy

Chapter 3 Exploring the phenotypic and genotypic diversity of a potential new generation of starter cultures for oenology

Chapter 4 Antagonistic effect of alternative yeast strains against *Botrytis cinerea* for integrated vitivinicultural biocontrol strategies

Chapter 5 Unraveling molecular and physiological divergence among strains of *Lachancea thermotolerans* in the production of lactic acid

Chapter 6 Improvement in the quality of wines elaborated with mixed fermentations of non-*Saccharomyces* yeasts and *Saccharomyces cerevisiae*

Chapter 7 Concluding remarks

Parts of the following chapters were already presented in scientific conferences, namely: International Conference in Wine Sciences “Macrowine 2018”, organized in Zaragoza, Spain, where it was presented the poster “Isolation and physiological characterization of non-*Saccharomyces* yeast strains with potential oenological role from different regions of Italy”; XII Congress

Biodiversity 2018, at Teramo, Italy, one oral presentation entitled "Exploring *Starmerella bacillaris* Diversity to Develop New Integrated Vitivinicultural Strategies" and two posters, "Phenotypic and Genotypic Diversity of *Lachancea thermotolerans* Wine Strains in the Production of Lactic Acid" and "Molecular and Phenotypic Characterization of *Metschnikowia* spp. Wine Strains from Different Regions of Italy".

In the period between February and August 2017, the student developed a research internship in the Institute for Wine Biotechnology (IWBT) of the University of Stellenbosch, South Africa. The project entitled "Transcriptome analysis of wine-related lactic acid bacteria under different conditions of oxygen and nitrogen availability" was carried out under the coordination of Prof. Maret du Toit. The results were presented as a poster in the conference Enoforum 2019, at Vicenza, Italy, entitled "Metabolic response of wine-related lactic acid bacteria to different conditions of aeration and nitrogen availability".

The main results of this PhD thesis will be organized for publication in scientific journals. Data of Chapter 2 and Chapter 3 were already published:

Renato L. Binati, Giada Innocente, Veronica Gatto, Alessandro Celebrin, Maurizio Polo, Giovanna E. Felis, Sandra Torriani. "Exploring the diversity of a collection of native non-*Saccharomyces* yeasts to develop co-starter cultures for winemaking", Food Research International, Volume 122, 2019, Pages 432-442, ISSN 0963-9969, https://doi.org/10.1016/j.foodres.2019.04.043.

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Chapter 1

Premise

CHAPTER 1. PREMISE

1.1. GENERAL INTRODUCTION

Never before in the history of mankind there were available so many different options of wine as we have in the market today, with a huge diversity regarding the style and quality. The globalization made possible for consumers all over the world to have easy access to wine bottles coming from all different regions, making the international competition become an important factor pushing towards higher quality, but also these consumers are getting more confident about their wine preferences and chasing for new styles. Climate change and the issues regarding the health of both consumers and environment can be added to the list of challenges to the wine industry worldwide, calling for technological innovations (Bisson *et al*., 2002; Pretorius and Hoj, 2005).

Among the factors that contribute to the wine quality, there is usually a consensus about the grape variety, environmental aspects, the health and ripeness state of the grapes and the human factor (viticultural and winemaking practices) (Van Leeuwen and Seguin, 2006; Bokulich *et al.*, 2016). Nevertheless, it is widely recognized that the microbial ecology in a food matrix has a crucial and complex influence on the global quality and uniqueness of the product (Capozzi and Spano, 2011). More specifically, it is also very well-defined the role played by microbial interactions in the grapevine health and during the fermentation of sugars and the maturation of the wine (Nykanen, 1986; Lambrechts and Pretorius, 2000; Barata *et al*., 2012).

Winemaking is a very complex process from the microbiological point of view. The spontaneous fermentation of grape must is carried out by a succession of metabolic active yeast and bacteria species, each one better adapted to different stages of this biochemical transformation. Yeast genera frequently found on grapes and in must include *Hanseniaspora*, *Candida*, *Metschnikowia*, *Kluyveromyces*, *Schizosaccharomyces*, *Torulaspora*, *Rhodotorula*, *Zygosaccharomyces*, and *Cryptococcus* (Heard and Fleet, 1985). It is well-known that the species *Saccharomyces cerevisiae* usually overtake the others and is the

main responsible for the alcoholic fermentation, due to its better tolerance to ethanol and SO_2 (Henick-Kling *et al.*, 1998).

The inoculation of starter cultures of *S. cerevisiae* is a very well established approach followed by many winemakers around the world, that can provide predicted results in a more controlled process, ensuring consistency, safety and quality of the final product, but on the other hand it suppresses the autochthonous microbiota responsible for the complexity and some distinctive and "unique" regional characteristics of wine. However, it has to be considered the high risk of relying on natural wild fermentations, that could result on stuck or sluggish process and/or the raise of undesired metabolites associated with depreciation and human toxicity (Pretorius, 2000; Romano *et al.*, 2003; Capozzi and Spano, 2011). Hence, a reliable biotechnological alternative to mimic the spontaneous uncontrolled fermentation, without taking the risks of spoilage, and that could at the same time provide complexity and improved organoleptic, is the formulation of mixed starter cultures (Capozzi and Spano, 2011).

In the past years, there has been an increasing number of studies regarding the role of a new generation of non-*Saccharomyces* yeasts during the fermentation of wine, their impact on the sensorial properties and their metabolic needs (Ciani *et al.*, 2010; Comitini *et al.*, 2011, Capozzi *et al.*, 2015). Initially neglected as having secondary importance or even considered as undesirable spoilage microorganisms, nowadays the research has shown that selected strains of non-*Saccharomyces* can be very positive resources for facing the challenges of changing consumer demands and environmental issues (Padilla *et al.*, 2016).

The non-*Saccharomyces* yeasts have been reported as possessing aroma-related enzymatic activities and other metabolic traits interesting for oenology, which are not present or less pronounced in *S. cerevisiae* (Esteve-Zarzoso *et al.*, 1998; Mateo and Maicas, 2016). Many publications have reported that beneficial (e.g. enhanced glycerol) or negative (e.g. overproduction of acetic acid) metabolic characteristics vary significantly between wine microbial species and also between strains belonging to the same species, and that such huge phenotypic biodiversity can be exploited by the screening of a new generation of starters for mixed fermentations, in order to select the best producers of interesting

metabolites and macromolecules, increasing the wine complexity, sensorial properties, stability and overall quality (Fleet, 2008; Capozzi *et al*., 2015).

It is important to notice that the non-*Saccharomyces* yeasts can be very useful tools for the application on mixed fermentations, either sequential or co-inoculated, in order to integrate their distinctive production of aromatic compounds and other important wine properties with the unique ability of *S. cerevisiae* to complete the fermentation process until the consumption of all residual sugar (Ciani, 2006; Fleet, 2008; Suárez-Lepe and Morata, 2012; Rossouw and Bauer, 2016).

Currently, there are already some commercial starters of non-*Saccharomyces* yeasts strains available for application in the wine industry, being used with the goal of giving the wines the above mentioned increase of complexity and improvement of sensorial properties (Petruzzi *et al*., 2017; Barbosa *et al*., 2018). Supplementary Table S.1 shows a list of these alternative starters available on the market up to date, adapted from Petruzzi *et al.* (2017).

1.2. AIMS OF THE STUDY

The main goal of this PhD thesis was to explore the diversity of non-*Saccharomyces* yeasts to be employed in winemaking processes for the elaboration of distinctive wines on innovative mixed fermentations with *Saccharomyces cerevisiae*.

The specific aims were the isolation and identification of yeasts from high-sugar matrices representative of different regions across Italy, the set-up of a vast yeast culture collection, the typing of isolates belonging to the same species, the phenotypic characterization of some interesting isolates for different oenological features involved in the overall quality of wine, the inoculation of grape must in single and sequential fermentations of new potential starter cultures belonging to the species *Starmerella bacillaris* and *Lachancea thermotolerans* and to the genus *Metschnikowia*, the comparison from a physic-chemical and aromatic point of view of the wines produced, and a genetic characterization of the different metabolism of lactic acid observed among the isolates of *L. thermotolerans*.

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Chapter 2

Ecology of non-*Saccharomyces* yeasts in high sugar matrices from different regions of Italy

CHAPTER 2. ECOLOGY OF NON-*Saccharomyces* YEASTS IN HIGH SUGAR MATRICES FROM DIFFERENT REGIONS OF ITALY

2.1. INTRODUCTION

The history of winemaking can be traced back to some tens of centuries ago. However, for most of this time the existence of yeasts has been ignored. It was only in the 19th century, in 1854, that Louis Pasteur started to describe the microbiological basis of the fermentation (from the Latin *fervere*, "to boil"). He demonstrated that yeasts are the responsible for the conversion of sugars present in the grape must to ethanol and carbon dioxide during the fermentation process (Padilla *et al.*, 2016). The scientist precursor of oenology noticed the existence of a wide variety of microorganisms in fermenting musts, and his drawings based on microscopic observations showed two types of yeasts. The first, more abundant during the early stages, had an apiculate shape (probably corresponding to what we know today as *Hanseniaspora uvarum*); the second, which became dominant during the alcoholic fermentation, was round shaped and larger (most likely the current *Saccharomyces cerevisiae*) (Barnett, 2000).

The great variability of yeasts that can be present in grape musts are already found colonizing vineyards and grapes, but are also recurrent on the winery equipment (Pretorius *et al.*, 1999). Among this complex microbiota, the most important wine yeast is undoubtedly *S. cerevisiae*, due to its excellence on the fermentation performance, its tolerance to the harsh conditions found in wine and the metabolism related to the aroma profile (Reed and Peppler, 1973; Bely *et al.*, 1990; Fleet, 1993; Dubourdieu, 1996; Úbeda and Briones, 2000; Ugliano *et al.*, 2006). The dominance of *S. cerevisiae* during the course of the fermentation is regularly observed for all kinds of wine, either on spontaneous or inoculated process (Heard and Fleet, 1985; Padilla *et al.*, 2016).

The inoculation of selected starter cultures of *S. cerevisiae* to carry out the alcoholic fermentation in grape must was first reported by Müller-Thurgau in 1890 (Pretorius, 2000; Barnett and Lichtenthaler, 2001). It is now one of the most common practices used in the wineries worldwide, with the aims of having better

predicted results in a more controlled process, avoiding the risks of contamination and unpredictable outcomes due to vintage variation. There are a lot of different active dry yeasts in the market, each one better adapted to specific conditions and showing different features according to the winemaker's needs and preferences (Beltran *et al*., 2002; Santamaría *et al.*, 2005).

However, despite all these advantages of using selected starters of *S. cerevisiae*, in more recent years there has been a strong debate among winemakers and scientists about the 'standardization' of the wines due to the use of the same pure cultures in many different regions, since the starter cultures inoculated at high concentrations rapidly dominate over the indigenous population and limit their involvement in the process (Fleet *et al.* 1984; Heard and Fleet, 1985; Henick-Kling *et al*. 1998). The homogeneity of fermentations is one of the goals of the inoculation, but at the same time the critics of the extensive use of this approach claim that the wines will lack on complexity, typicity and distinction, which could presumably be achieved with the indigenous microbiota associated with spontaneous fermentations (Lambrechts and Pretorius, 2000; Romano *et al*., 2003).

Usually, before being considered an opportunity to increase the quality of wine, the abundance of other yeast species was considered as spoilage organisms, causing stuck or sluggish fermentations or producing off-flavors (du Toit and Pretorius, 2000; Padilla *et al*., 2016). There is a wide variety within these 'supporting' yeasts, and the term "non-*Saccharomyces*" is used to designate more than 20 genera, for example *Hanseniaspora*/*Kloeckera, Candida, Metschnikowia, Pichia, Brettanomyce*s, *Kluyveromyces*, *Schizosaccharomyces*, *Torulaspora*, *Rhodotorula*, *Zygosaccharomyces,* and *Cryptococcus*, among others (Heard and Fleet, 1985; Padilla *et al*., 2016). They are actually the protagonists during the initial phases of spontaneous fermentation, reaching concentrations around 10^3 to 10^6 CFU/g in the grape berries surface and in the must (Barata *et al*., 2008). Many environmental parameters can affect the number and ratio of representatives from those species, such as temperature, nutrient availability or supplementation, antimicrobial agents, clarification practices (Fleet and Heard, 1993).

S. cerevisiae can be barely found in the first 3-4 days of fermentation and some non-*Saccharomyces* species can reach up to 10^7 CFU/g, but, as said before, the first progressively becomes the dominant when the process of conversion of

the sugar into ethanol goes ahead and the other species cannot survive as a consequence of their weak tolerance to this compound (Heard and Fleet, 1985; Fleet, 1999), even though some studies already showed the detection of non-*Saccharomyces* throughout the fermentation process (Jolly *et al.*, 2006).

In this context, one suggested approach that is ever gaining popularity is the inoculation of selected species of non-*Saccharomyces* on mixed fermentations alongside with *S. cerevisiae*, in order to mimic the process of spontaneous fermentation without taking the risks of contamination by undesired species and stuck or sluggish fermentations (Jolly *et al.*, 2003; Rojas *et al.*, 2003; Romano *et al.*, 2003; Ciani *et al.*, 2006). The first non-*Saccharomyces* strain to be used intentionally in wine fermentation was in the 1960s, a selected *Torulaspora delbrueckii* to reduce the volatile acidity (Cantarelli, 1955; Belda *et al.*, 2016). Nowadays, there are many other commercial strains of non-*Saccharomyces* available, but this practice is still a challenge for researchers and oenologists because many aspects of the non-*Saccharomyces* behavior on wine fermentation are still unclear (Padilla *et al.*, 2016); the isolation and selection of suitable strains will still be an important topic for wine researchers worldwide for the next years.

It is of great interest to study the microbial resources present in different ecosystems, not only for ecology and biogeography reports, but also because the biodiversity can offer a wide variety of technological applications, and the non-*Saccharomyces* yeasts are a good example of this potential (Úbeda *et al.*, 2014). The isolation can be done from grape berries, but also from other raw and processed plant materials, as yeasts found those ecosystems suitable for their growth and colonization (Iacumin *et al.*, 2012; Úbeda *et al.*, 2014). Non-*Saccharomyces* yeasts have been reported to colonize many food and beverage products, besides grape must and wine, and many interesting species could be isolated from products containing high concentration of sugar or low water activity (a_W), such as honey, sweets, molasses, overripe or dried fruits (Deák, 2008; Benito *et al.*, 2013).

Many diverse differential-selective media have been already described in the literature for the isolation of non-*Saccharomyces* species, based on metabolic physiological characteristics of these microorganisms (Heard and Fleet, 1986; Benito *et al.*, 2013). One of them is Wallerstein Laboratory Nutrient Agar (WL), which is not a very selective medium, but can be very useful to distinguish

numerous yeast species found in wine fermentations by colony color and morphology, being used also for a preliminary identification of yeast present in fermenting musts (Green and Gray, 1950; Cavazza *et al*., 1992; Pallman *et al.*, 2001).

The classical methods of yeast identification, based on macroscopic and microscopic observations, sexual and biochemical characteristics of the isolates are laborious, time-consuming and not suitable for reliable assessments, but can be very useful for the identification and characterization studies, when they are conveniently linked to the more recent methods of molecular biology (Barnett *et al.*, 1990; Correll, 1991; Deák, 1993). Molecular methods allow the identification of microorganisms based on comparison with public databases, and nowadays they are very precise, straightforward and fast (Mannazzu and Budroni, 2005).

Polymerase chain reaction (PCR) based methods, such as restriction fragment length polymorphism (RFLP) and random amplification of polymorphic DNA (RAPD), are fast, reproducible and reliable methods for molecular characterization of yeasts (Messner and Prillinger, 1995; Masneuf *et al*., 1996; Mozina *et al*., 1997; Guillamón *et al*., 1998). RAPD is a whole-genome PCR that results in specific fingerprints by using short primers which bind to arbitrary regions within the sequence (Quesada and Cenis, 1995). One example of such primer is M13, which give characteristic band patterns for different wine yeast species (Lopandic *et al*., 2008).

These methods can be used for a primary typing of a vast diversity of isolates, after what a few representatives from the clusters formed are precisely identified based on sequencing of the internal transcribed spacer (ITS) zone located within the region between the 18S rRNA and 28S rRNA genes, and/or the genes encoding the D1/D2 domain of the large subunit of rRNA (26S), followed by comparison with public databases of sequences (Bruns *et al*., 1991; Henrion *et al*., 1994; Kurtzman and Robnett, 1998).

PCR-Denaturing gradient gel electrophoresis (DGGE) is another molecular analytical method applied to study microbial ecology in food and wine fermentations. In this technique, microbial DNA is amplified with specific primers and the amplicons generated are then separated on the basis of differences in nucleotide sequence (Muyzer and Smalla, 1998; Cocolin *et al*., 2000; Giraffa and Neviani, 2001; Prakitchaiwattana *et al*., 2004).

The main aim of this work was to establish a vast collection of yeasts isolated from diverse high-sugar substrates collected at multiple different Italian regions, in order to have access to a huge variability of interesting resources that could be used mainly for oenology and also other relevant technological applications.

The isolation and identification of the yeasts at species level allowed us to draw some conclusions about the presence, diversity and distribution of the different species in the samples grouped by diverse criteria, such as geographical location and type of sample.

2.2. MATERIAL AND METHODS

2.2.1. Samples

During two consecutive vintages, 2015 and 2016, samples with high sugar concentration, such as grapes, grape must, honey, overripe and dried fruits were collected from different regions throughout Italy.

Table 2.1 shows a summary of samples used during this study.

The samples of honey and grape must were brought to the laboratory in sterile flasks (about 100 mL), while grapes and other fruits were aseptically placed directly from the trees into sterile plastic bags and transported immediately to our facility under refrigerated conditions. The content of each bag (around 100 g) was crushed using a paddle blender homogenizer (BL Smart Astori Tecnica).

Table 2.1. List of samples collected for the isolation of yeasts.

Type	Varieties	Regions	Nº
Grapes	Alicante, Barbera, Bombino, Bonarda, Clinton, Corvina, Corvinone, Glera, Malvasia Candia, Malvasia Puntinata, Marzemino, Merlot, Moscato Giallo, Moscato Rosa, Pecorino, Pinot Grigio, Pinot Nero, Rondinella, Sangiovese, Solaris, Souvignier Gris, Vermentino, Viognier and Table grapes	Abruzzo, Emilia-Romagna, Lazio, Toscana, Trentino-Alto Adige, Veneto	101
Grape Musts	Corvina, Corvinone, Fiano, Glera, Marzemino, Negramaro, Pinot Grigio, Rondinella, Solaris	Puglia, Veneto, Trentino-Alto Adige	36

Honey	Honey, honeycomb, fermented honey (hydromel)	Abruzzo, Puglia, Sardegna, Sicilia, Toscana, Veneto	15
Dried Fruits	Barley, Date, Fig, Plum	Emilia-Romagna, Veneto	6
Overripe Fruits	Apple, Blackberry, Fig, Plum	Veneto	11
		TOTAL	**169**

2.2.2. Yeast isolation, purification and maintenance

For the isolation of yeast colonies, aliquots of the samples were diluted on physiological solution containing 0.9% of NaCl (Sigma-Aldrich) and then spread onto WL agar medium (Sigma-Aldrich), containing 100 mg per liter of chloramphenicol (Merck) to inhibit the growth of bacteria. After incubation at 27 ºC for 72 h, all colonies showing different morphology/color, regardless of their number on the plate, were streaked on new plates of the same medium in order to obtain pure and single cultures. Besides the morphological characterization of the colonies, the cells were observed on optical microscope with 1000x magnification. One colony of each isolate was used to inoculate a 10-mL tube containing YPD broth (yeast extract, 1.0%; bacteriological peptone, 2.0%; glucose, 2.0%; Sigma-Aldrich). After 48 h incubation at 27 ºC, an aliquot was taken for the cryopreservation at -80 ºC in a 25% glycerol solution and all the yeast isolates obtained during this thesis were included in the collection. Immediately before use for the characterization assays and microvinification trials, the yeasts were transferred from the stocks to new Petri dishes containing WL agar and/or tubes with YPD broth, being cultured for 48 h at 27 ºC.

2.2.3. DNA extraction

Total genomic DNA was isolated and purified using the commercial kit Wizard Genomic DNA Purification (Promega) following the manufacturer's protocol, from a 2-mL aliquot of cultures grown in YPD broth. The quality assessment of the DNA obtained was performed with a NanoDrop ND1000 UV-

Vis Spectrophotometer (Thermo Scientific) and dilution in DNAse-free water was carried out when necessary for the following molecular analysis.

2.2.4. RAPD-PCR

The analysis of RAPD-PCR was used for the dereplication of yeast isolates, employing the primer M13 (5'-GAGGGTGGCGGTTCT-3') and according to the protocol of Lieckfeldt *et al*. (1993). The reaction mix was prepared with the following concentrations for the final volume of 20 μL: 1× DreamTaq Green Buffer (Thermo Scientific); 4 mM $MgCl_2$; 0.1 mM dNTPs; 4 μM primer M13; 0.025 U/μL DreamTaq DNA Polymerase (Thermo Scientific) and 10 ng genomic DNA. The amplification program considers an initial denaturation at 94 ºC for 5 minutes, followed by 40 cycles of denaturation at 94 ºC for 1 minute, annealing at 45 ºC for 45 seconds and extension at 72 ºC for 2 minutes, concluding with a final extension of 5 minutes at 72 ºC. The amplification was conducted on Thermal Cycler 2720 (Applied Biosystems).

Band profiles were realized on electrophoresis gel at 1.2% agarose in 1× TAE Buffer (40 mM Tris, 20 mM Acetic acid, and 1 mM EDTA, Sigma-Aldrich) stained with EuroSafe colorant (Euroclone) on a 2 hours and 20 minutes run at 110 V. The molecular ladder used was O'Gene Ruler DNA (Thermo Scientific). The visualization and image capturing were made under UV light with UVITEC Gel Documentation System (Cleaver Scientific). The images were analyzed with the software BioNumerics (version 5.0, Applied Maths) and dendrograms were constructed using Pearson's correlation coefficient and the unweighted pair group method with arithmetical average (UPGMA) clustering.

2.2.5. Sequencing

A selected number of isolates was chosen from the RAPD analysis based on the clusters formed in the dendrograms obtained and their DNA was used for amplification of specific fragments (ITS region and D1/D2 domain) as detailed below. The PCR products were purified and sent to GATC Biotech (Konstanz, Germany) for the sequencing. The sequences obtained were compared with online

database using the BLAST alignment tool (http://blast.ncbi.nlm.nih.gov/) in order to identify based on the most similar species.

2.2.5.1. ITS region

For all the representative isolates of the clusters formed with the RAPD analysis, the ITS region was chosen to confirm their identities by sequencing. The PCR for amplification of the ITS region was performed with the pair of primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), according to White *et al.* (1990). The reaction mix was prepared for a final volume of 20 µL with the following components: 1× DreamTaq Green Buffer (Thermo Scientific); 1.5 mM $MgCl_2$; 0.1 mM dNTPs; 0.5 µM primer ITS1; 0.5 µM primer ITS4; 0.025 U/µL DreamTaq DNA Polymerase (Thermo Scientific) and 10 ng genomic DNA. The amplification program consists of an initial denaturation at 94 ºC for 5 minutes, followed by 35 cycles of denaturation at 94 ºC for 50 seconds, annealing at 55.5 ºC for 50 seconds and extension at 72 ºC for 1 minute, concluding with a final extension of 10 minutes at 72 ºC. The amplification was conducted on Thermal Cycler 2720 (Applied Biosystems).

2.2.5.2. D1/D2 domain

After a series of unsatisfactory results for the ITS sequencing of some isolates, it was decided to try another widely used fragment of yeasts DNA for the sequencing, the D1/D2 domain of the 26S rRNA gene. The primers used were NL-1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL-4 (5′GGTCCGTGTTTCAAGACGG-3′), following the protocol of Kurtzman and Robnett, 1998. A reaction mix with all components was prepared for a final volume of 20 µL as follows: 1× DreamTaq Green Buffer (Thermo Scientific); 1.5 mM $MgCl_2$; 0.2 mM dNTPs; 0.7 µM primer NL-1; 0.7 µM primer NL-4; 0.05 U/µL DreamTaq DNA Polymerase (Thermo Scientific) and 10 ng genomic DNA. The amplification program started with an initial denaturation at 95 ºC for 10 minutes, followed by 36 cycles of denaturation at 94 ºC for 30 seconds,

annealing at 55 ºC for 30 seconds and extension at 72 ºC for 45 seconds, and concluded with a final extension of 7 minutes at 72 ºC. The amplification was conducted on Thermal Cycler 2720 (Applied Biosystems).

2.2.6. DGGE

In order to verify the existence of more than one variant of the 26S rRNA gene in the isolates that had this gene sent to the sequencing, due to ambiguous results in the sequences received, a fragment of approximately 250 bp within the D1/D2 domain was amplified and submitted to DGGE analysis (Prakitchaiwattana *et al.*, 2004). The amplification was performed with primers NL-1 containing GC-clamp (5'-CGCCCGCCGCGCGGCGGGCGGGGCGGGGGC-GCATATCAATAAGCGGAGGAAAAG-3′) and LS-2 (5'-ATTCCCAAACAACTCGACTC-3'), according to Cocolin *et al.*, 2000. The conditions of the reaction for a final volume of 40 μL were as follows: 1× DreamTaq Green Buffer (Thermo Scientific); 2.0 mM $MgCl_2$; 0.2 mM dNTPs; 0.2 μM GC-clamp primer NL-1; 0.2 μM primer LS-2; 0.025 U/μL DreamTaq DNA Polymerase (Thermo Scientific) and 50 ng genomic DNA. The amplification program started with an initial denaturation at 95 ºC for 5 minutes, followed by 30 cycles of denaturation at 95 ºC for 1 minute, annealing at 52 ºC for 2 minutes and extension at 72 ºC for 2 minutes, and concluded with a final extension of 7 minutes at 72 ºC. The amplification was conducted in Thermal Cycler 2720 (Applied Biosystems).

The separation of GC-clamped amplicons was carried out in a D-Code™ Universal Mutation Detection System (Bio-Rad). The PCR samples were added with the same volume of loading buffer 2.0× and applied into an 8% polyacrylamide gel (acrylamide:bis-acrylamide ratio of 37.5:1) of 20x20x0.1 cm with a denaturing gradient from 30–60% of urea and formamide. The electrophoretic run was performed in a running buffer of 1% TAE at a constant voltage of 50 V for 16 hours at 60 ºC. After the end of the run, the gel was stained in solution containing EuroSafe colorant (Euroclone). The visualization and image capturing were made under UV light with UVITEC Gel Documentation System (Cleaver Scientific).

2.3. RESULTS AND DISCUSSION

2.3.1. Isolation and identification of non-*Saccharomyces* yeasts

A total of 169 samples were collected from 35 different locations distributed across 9 regions of Italy. Most of the samples were composed of grape bunches harvested directly from the vineyards, belonging to 23 varieties of *Vitis vinifera* and also 4 samples of table grapes. We also received grape musts just pressed, at the very beginning of fermentation, in order to find higher concentration of non-*Saccharomyces* yeasts, from 9 different grapes, both red and white. Additionally, 17 samples of overripe/dried fruits and 15 of honey were used for the isolation. All samples have in common the high sugar content, aiming to isolate strains adapted to growing on such conditions, as it was already shown that they are able to achieve better results on grape must fermentation than other natural yeasts isolated from environments less rich in sugars (Camarasa *et al*., 2011).

After the purification of at least one colony from every type of different morphology encountered at the WL plates spread with the diluted samples, a total number of 409 yeast isolates were organized into our culture collection. They were all described from the macroscopically and microscopically point of view. This approach can be very useful for a preliminary identification and selection of the isolates. As demonstrated by Cavazza *et al*. (1992) and Pallmann *et al*. (2001), many yeast species associated with wine fermentation present distinguishable aspects of the colonies grown on WL medium, and some of them also present very particular cell morphology when observed with optical microscope.

The morphological analysis was used as the first criteria of exclusion of some groups which have less interesting properties for the purposes of this research, the inoculation as starter cultures on mixed fermentations. We decided to do not keep for the next steps of characterization the isolates presenting flat and dark green colonies with apiculate cells (presumably associated with *Hanseniaspora uvarum* and other species of the *Hanseniaspora* genus) and the white/cream colonies with wrinkled opaque surface, fringed margins and elongate cells forming pseudohyphae (usually characteristic of *Candida* spp. and *Pichia* spp.), shown on Figure 2.1. They are both among the most common groups found in vineyards and early stages of fermentation.

This way, we were able to identify 55 isolates with the first characteristics described above, which will be called from here on as “apiculate”; and 47 showing the later characteristics on the previous paragraph, which will be now referred to as “pseudohyphae”. These isolates were maintained on the yeast collection at -80 ºC, but they were not evaluated on the next trials of this research. Nevertheless, some representatives of these two groups were included on the molecular identification procedures for the confirmation of the morphological preliminary identification. All remaining 307 yeast isolates had their genomic DNA purified and amplified according to the RAPD protocol.

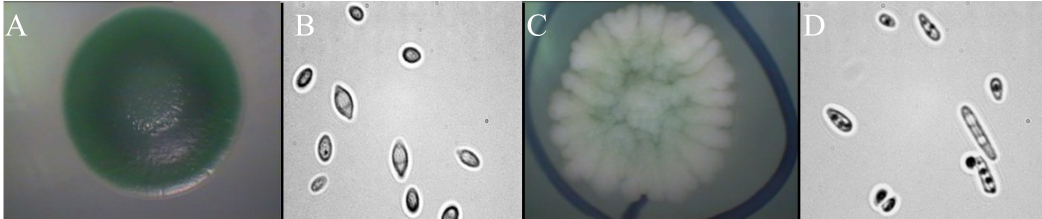


Figure 2.1. Morphology of colonies grown on WL Nutrient Agar and cells observed with 1000× magnification on optical microscope. (A) Dark green round colonies with flat elevation; (B) apiculate cells; (C) white wrinkled colonies with fringed margins; (D) elongate cells.

Figure 2.2 shows one example of the electrophoretic run on agarose gel with isolates with different band profiles.

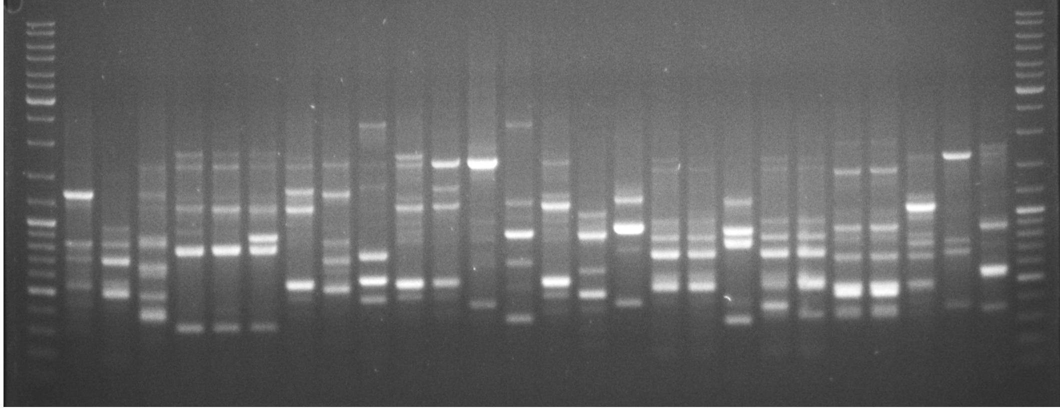


Figure 2.2. Electrophoretic run on agarose gel at 1.2% for the separation of bands amplified with primer M13 on genomic DNA of yeast isolates. First and last lanes were loaded with molecular weight marker O’Gene Ruler DNA (Thermo Scientific).

All images were grouped together for the construction of dendrograms with the software BioNumerics. A threshold of 90% was defined to group isolates belonging to the same species. At least one isolate from the clusters formed by a minimum of three isolates was chosen for the identification through ITS sequencing. Some clusters were composed by just one or two isolates and it was a choice to do not sequence their DNA, because they were not suitable for the next steps of screening, since the goal of this study was a broad characterization of diversity of isolates with major distribution and presence on the samples analyzed.

The ITS sequences obtained were compared to online databases and 24 different species could be identified among the yeast isolates, shown on Figure 2.3. Many of them are usually present on vineyards and associated with the wine environment. Not considering the groups of reduced oenological interest, as stated before, the most abundant non-*Saccharomyces* yeast was *Starmerella bacillaris*, followed by *Metschnikowia* spp., which couldn't be identified at species level as explained on the next session, *Zygosaccharomyces* spp. (*Z. bailii* and *Z. mellis*) and *Lachancea* spp. (*L. fermentati*, *L. kluyveri* and *L. thermotolerans*).

2.3.2. Divergence in the sequence analysis of *Metschnikowia* spp. isolates

The isolates of *Metschnikowia* are easily recognizable on WL plates due to the production of a pigment called pulcherrimin, which causes the formation of a red halo under the colonies (Cavazza *et al.*, 1992; Pallman *et al.*, 2001). Many isolates presented this characteristic, even if the colony morphology was not identical and slight differences on the surface color and consistency were observed, and they were grouped together on a few clusters on the dendrograms. However, they could not be identified at species level by the sequencing of ITS region. When the obtained sequence was confronted with the available databases, it presented the same level of similarity with more than one species within the genus *Metschnikowia* and could not be unambiguously associated with just one. Different isolates had their ITS region sequenced and the process was even repeated multiple times, but the results were always inconclusive and there was significant diversity among them.

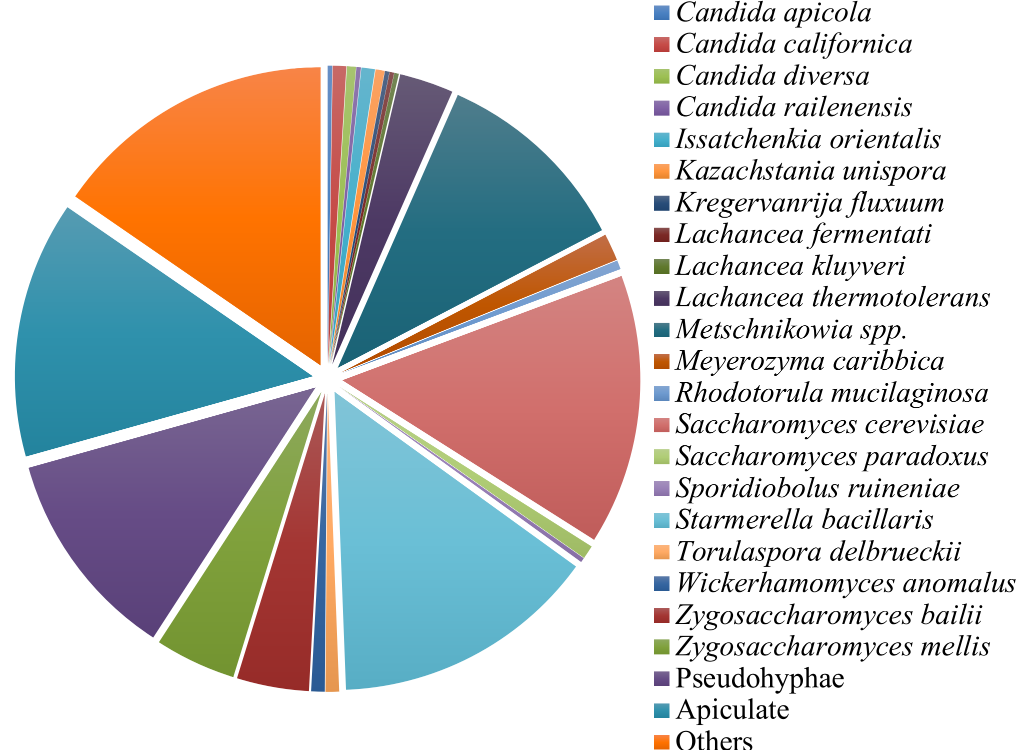


Figure 2.3. Population distribution among the 409 yeast isolates. 'Apiculate' includes *Hanseniaspora uvarum*, *H. opuntiae*, among others; 'Pseudohyphae' includes *Pichia membranifaciens*, among others.

As an alternative, the D1/D2 region of the DNA from ten different isolates of the group and three type strains of *Metschnikowia* species of oenological relevance, namely: *Metschnikowia pulcherrima* CBS5833, *Metschnikowia andauensis* CBS10809 and *Metschnikowia fructicola* CBS8853; was amplified and sent for the sequencing facility. The results were again ambiguous, as the ITS sequencing.

Figure 2.4 shows two regions on the chromatogram obtained by the Sanger sequencing of two isolates, to illustrate the presence of more than one peak with similar intensity in some positions. The nucleotides cannot be unambiguously defined in these conditions and it was still not possible to identify the isolates at species level.

Similar issues on the identification of *Metschnikowia* spp. isolates and the observation of a great diversity among isolates from this group were already reported by other authors (Pallman *et al.*, 2001; Prakitchaiwattana *et al.* 2004;

Sipiczki, 2006; Brysch-Herzberg and Seidel, 2015; Belda *et al.* 2016; Jara *et al.*, 2016).

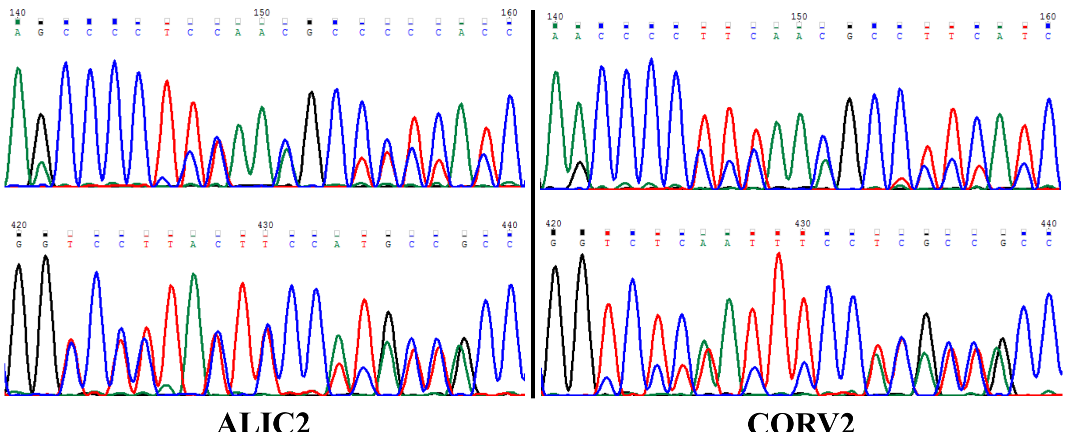


Figure 2.4. Chromatograms obtained through Sanger sequencing of D1/D2 domain of the 26S rRNA gene of the isolates ALIC2 and CORV2, highlighting two regions with elevated number of ambiguous nucleotides.

The ambiguous nucleotides on the sequence could be attributed to errors during the sequencing or to heterogeneity in the amplified DNA due to the presence of more than one fragment showing different sequences (Sipiczki *et al.*, 2013). The isolates used for the PCR reactions were restreaked multiple times on WL agar and their purity was also confirmed by microscope observations. One single colony was used for the DNA purification, making it high unlikely that the DNA from more than one strain would be present on the material sent for sequencing. Moreover, the fact that isolates from other species were sent together to the sequencing facilities and the results were satisfactory, and the ambiguous nucleotides on the *Metschnikowia* spp. were always present at around the same positions and not randomly distributed across the sequence, makes also very unlikely the possibility of sequencing errors.

To test the hypothesis of the presence of more than one fragment with different sequence, the amplified D1/D2 domain of the same ten isolates and the three type strains was then loaded to a Denaturing Gradient Gel Electrophoresis (DGGE), shown on Figure 2.5.

It can be seen from the presence of multiple bands on the DGGE run that the isolates do have repeats of non-identical sequences of this gene on their genome,

which could probably be the reason for the ambiguous nucleotides on the sequencing results. Moreover, it is interesting to note the different profiles associated with each isolate, highlighting that they could all belong to different species or this feature could be related to diverse strains of the same species. In each way, it represents a challenge for the identification of yeasts belonging to the genus *Metschnikowia*.

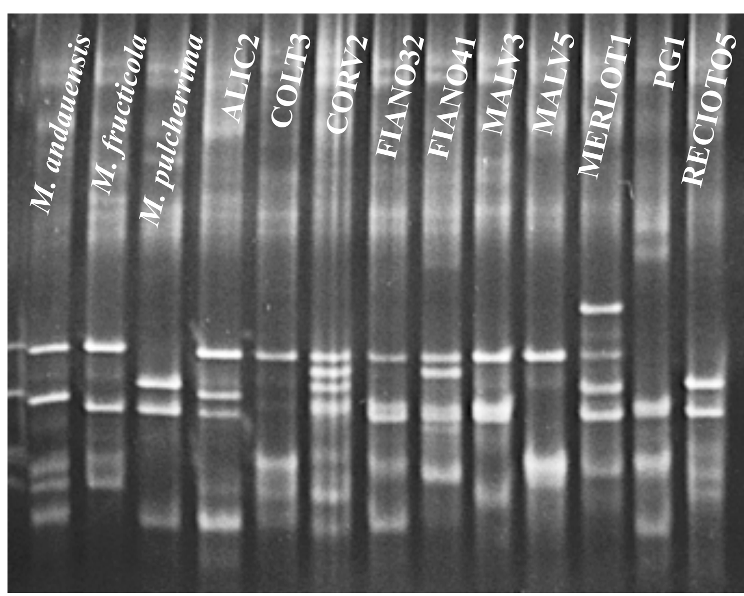


Figure 2.5. PCR-DGGE analysis (30-60% gradient) for the amplified D1/D2 domain from genomic DNA of three type strains and ten isolates of *Metschnikowia* spp.

Sipiczki *et al*. (2013) used a different approach to test the same hypothesis that *Metschnikowia* strains have diverse large subunit rRNA genes in their rDNA arrays. They cloned D1/D2 domains from single-cell cultures of type strains from *M. andauensis* and *M. fructicola* and found several different versions of D1/D2 sequence on the clone population. With this result they were clearly able to show that such ambiguous sequencing results of both species originate from divergent copies of the rDNA gene. Interestingly, their results showed that two variant regions are present within the D1 sequence, which correspond to the same positions highlighted in our study.

The D1/D2 domain sequence of the three type strains used in this study was obtained from the deposited sequences on databases and compared using the Clustal Omega 1.2.4. multiple sequence alignment tool (http://www.ebi.ac.uk/Tools/msa/clustalo/). The results shown on Figure 2.6

clearly emphasize the differences on the sequence in the same positions as the ambiguous nucleotides presented on Figure 2.4.

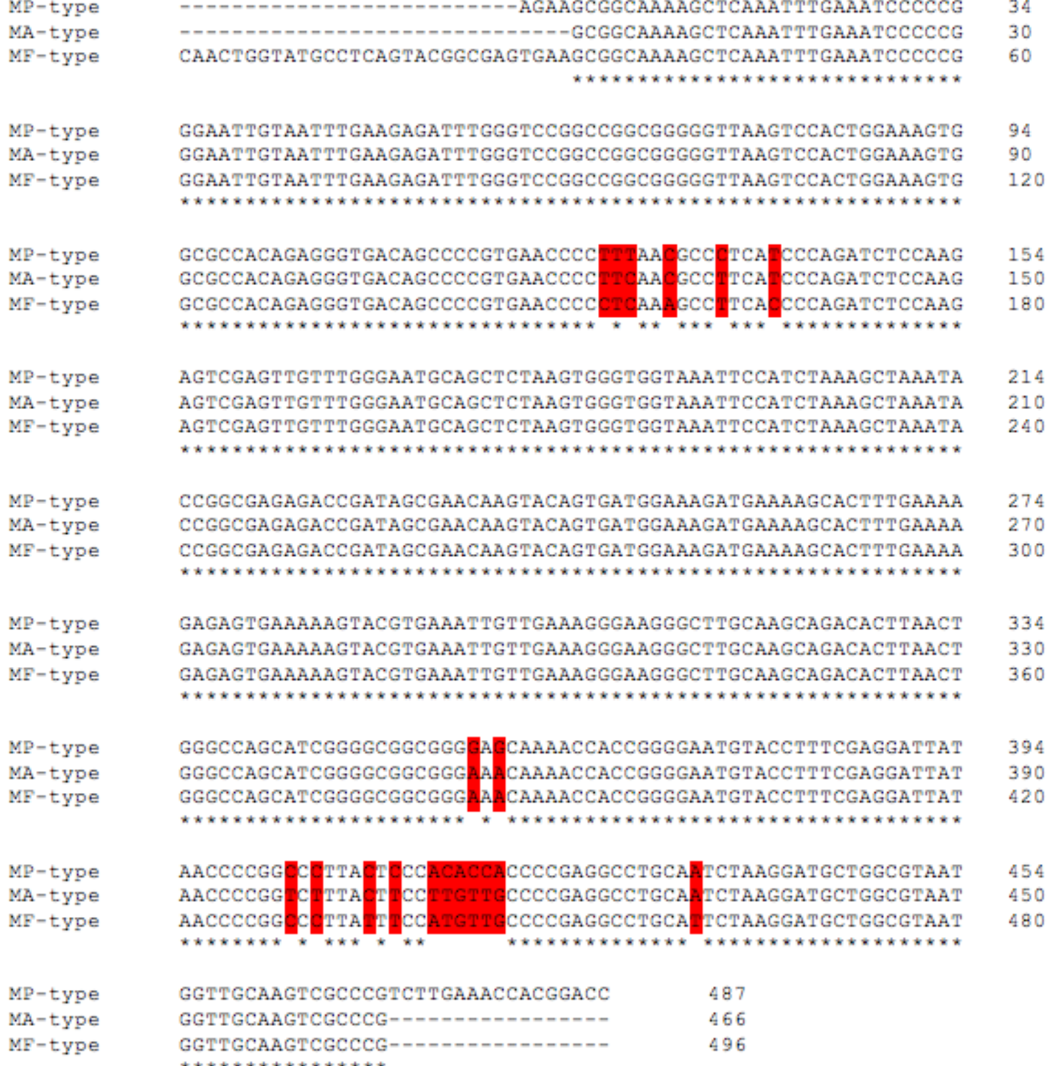


Figure 2.6. Comparison of the sequence of D1/D2 domain of 26S rRNA gene from the deposited sequences of three *Metschnikowia* type strains: *Metschnikowia pulcherrima* (MP), *Metschnikowia andauensis* (MA) and *Metschnikowia fructicola* (MF); using the Clustal Omega 1.2.4 multiple sequence alignment tool (http://www.ebi.ac.uk/Tools/msa/clustalo/).

Due to the imprecision on the identification at species level, all isolates that presented the same colony morphology and the ability to produce pulcherrimin, which moreover clustered together by the RAPD analysis, will be indicated as *Metschnikowia* spp. on the continuation of this study.

2.3.3. Distribution of non-*Saccharomyces* isolates in the different samples

From the samples of honey and honeycomb it was possible to isolate only a limited number of yeast species. *Zygosaccharomyces mellis* was present in almost every sample; one strain of *Sporidiobolus ruineniae* and one of *Rhodotorula mucilaginosa* could be isolated from the samples collected in Sicily. We analyzed also one sample of hydromel, which is an alcoholic beverage of fermented honey, and just colonies of *S. cerevisiae* were grown on the WL plates. Benito and co-workers (2013) reported the isolation of *Schizosaccharomyces pombe* from samples of unpasteurized honey and honeycombs from organic farms, using a selective differential medium. Applying the same protocol and with similar samples, we were not able to isolate any strain of this species.

In samples of dried dates there were only colonies of *Hanseniaspora opuntiae* and from the samples of barley there could only be recovered the species *Kazachstania unispora*. To sum up, these isolates from honey, dates and barley are not interesting for the aims of inoculation on grape musts and were excluded from the successive steps of characterization and microvinification. Those samples were then not considered for the observations on diversity and distribution of yeasts.

Considering the 154 samples of grapes, grape musts and overripe/dried fruits, coming from 27 different locations (in 7 Italian regions) and of 31 diverse varieties of grapes and fruits, the yeast isolates obtained were differentially distributed. It confirmed the influence that geographical origin, vineyard or fruit tree position (sunlight exposure, type of soil), climatic conditions (temperature, rainfall, wind), fruit variety (cultivar, age, history), ripeness level and health state of the fruits, human intervention (use of antimicrobial agents, training systems) can exert on microbial diversity (Fleet *et al*., 2002; Barata *et al*., 2012; Sabate *et al*., 2002). It was already demonstrated that on vineyard ecosystems this diversity is non-randomly associated with varietal, climatic and regional factors (Bokulich *et al*., 2014; Morrison-Whittle *et al*., 2017).

The following graph (Figure 2.7) shows the presence on different locations and different varieties of grapes/fruits. The isolation procedure employed during this study consisted on the purification of one colony from each slightly different morphology present on the plate with the highest dilution where the total number

of colonies was between 20 and 200. Among the colonies with identical morphology, the one to be purified was chosen randomly. The colonies present on the plates prepared with higher dilutions have a high probability to belong to the dominant species on the samples (Pulvirenti *et al.*, 2004; Solieri *et al.*, 2006; Aponte and Blaiotta, 2016). Two approaches were used to calculate the presence of the different species on the samples analyzed. Firstly, the samples were grouped by their origin, more specifically the municipality from which they were collected (even if from different fruit types), and secondly, they were grouped by the type of fruit (even if from the same location). The presence was then calculated as the number of groups with at least one positive sample for a determined species or genus divided by the total number of groups, expressed in percentage.

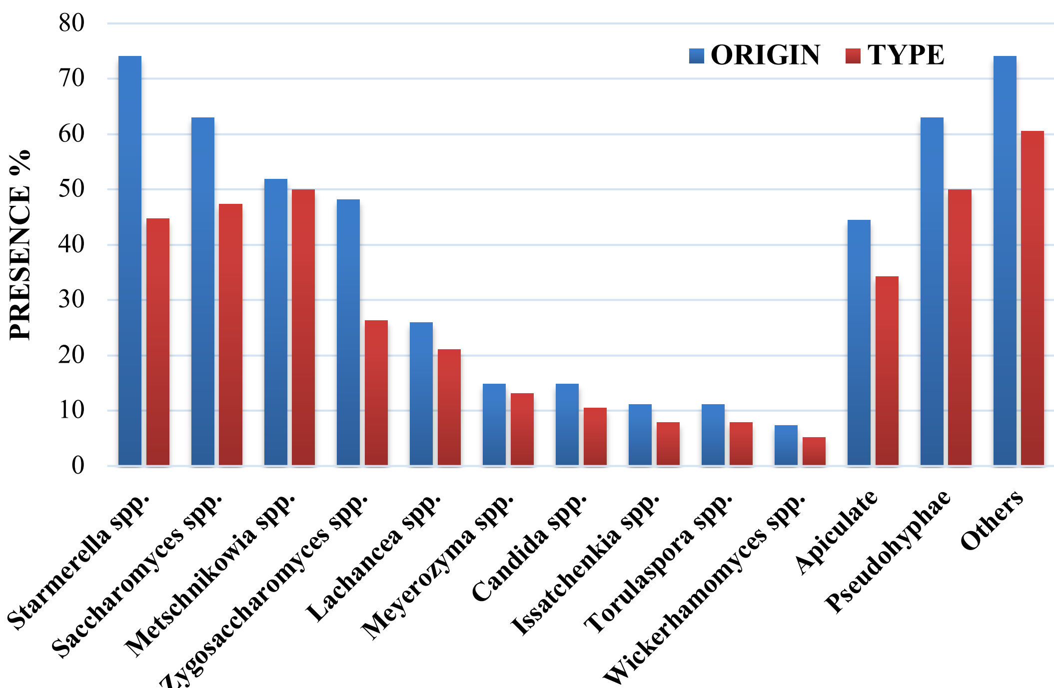


Figure 2.7. Presence of some yeast genus according to the origin and type of sample from which they were isolated.

The diversity of yeasts present was analyzed by how many times each identified species was detected in the isolation substrates, following the same approach as Solieri *et al.* (2006) and Tofalo *et al.* (2009). However, no

annotations were made neither on the total concentration of colonies grown on the Petri dishes nor the relative abundance between the different morphologies.

Starmerella bacillaris was the species most spread on the locations analyzed, being present on 74% of the territories, while isolates of the genus *Metschnikowia* were those able to colonize the highest number of different matrices, being detected on 50% of the fruits and grape varieties investigated. Moreover, *S. bacillaris* was found on 45% of types of samples and *Metschnikowia* on 52% of the locations. *S. cerevisiae* was also highly present throughout samples of different origins and type. Isolates of *Zygosaccharomyces* and *Lachancea* represented two other important groups of non-*Saccharomyces* yeast with presence above 20%.

Even if not considered of interest for the inoculation on mixed fermentation, it can be noted how apiculate and pseudohyphae-forming yeasts were highly frequent between the cultivable yeasts isolated from our samples. As said before, they are among the most common species in vineyards and beginning of fermentation, but rapidly disappear when the conversion of sugar to ethanol increases and anaerobic condition occurs. The group of other species represented on the graphic is composed by isolates that were present in less than 5% when considered alone, but when they are put together it can be seen that more than 60% of territories and varieties of samples had at least one of those minor species.

To study how and why this yeast diversity is heterogeneously distributed among the different samples, they were grouped based on diverse criteria. Firstly, it was used the geographical origin approach, meaning the samples were sorted by the territories from which they were collected and then separated according to the geopolitical division of Italy. Secondly, ecological factors were considered and the samples were divided by type of fruit, grapevine species and cultivars, and some human intervention on the grapes.

The profile for each geographical region (Figure 2.8) was calculated as the average of all samples coming from that region, without distinction of grape varieties or type of sample.

The samples coming from the Veneto region were those with the highest number of different species, showed by the higher average number of isolates obtained from each sample. Moreover, a higher variety of species in general could

be isolated from that region. Veneto have a high prevalence of strains from the species *S. bacillaris*, also present in a high number of samples from Abruzzo.

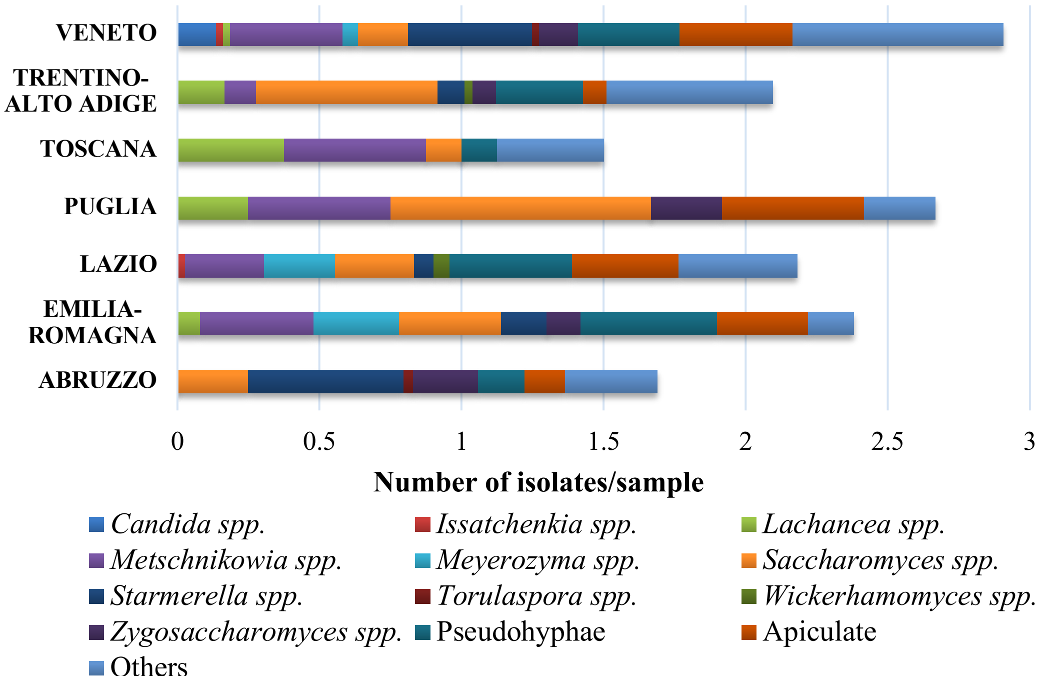


Figure 2.8. Average of the number of isolates present on the samples grouped by region of origin.

The highest similarity was found between the regions Lazio and Emilia-Romagna, due to the presence of isolates of *Metschnikowia* spp. and *Meyerozyma* spp., besides apiculate and pseudohyphae-forming yeasts. In Toscana there were also an elevated percentage of *Metschnikowia* spp. isolates and it was the region were *Lachancea* spp. was more easily isolated.

Saccharomyces spp. was the only group found in all regions, with higher probability in Trentino-Alto Adige and Puglia. It can be highlighted that while *S. cerevisiae* was found in all regions, only in Trentino-Alto Adige there were also isolates belonging to the species *S. paradoxus*.

However, these different profiles associated with each region can only indicate a few general trends on the regions, since the samples put together based on region of origin were far from being homogenous. Many factors are acting in synergy to define the microbiota present on each sample, and the territories sampled during this study have many differences on the grape varieties, climate, vineyard position and training system, human intervention.

Another way to arrange the samples is to separate the overripe and dried fruits from the grapes, and the grapes were further divided in two groups: those which were collected already as must after crushing in the winery and those which had no contact with the winery machinery and were brought directly from the tree to the laboratory. The different profiles are shown in Figure 2.9.

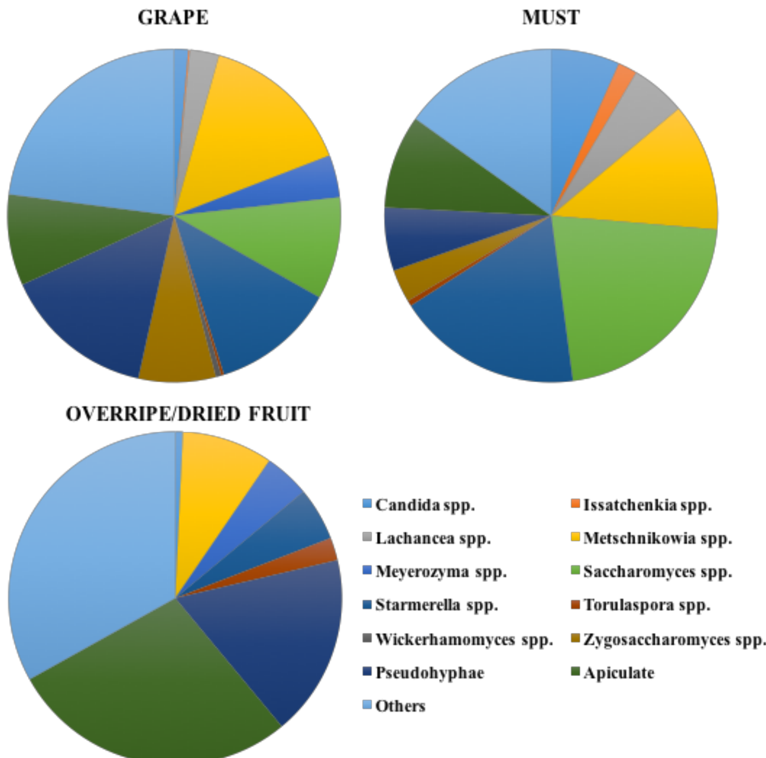


Figure 2.9. Differential distribution of main oenological species on the samples divided by the type of fruit and stage of processing.

These graphics show the influence of the types of samples that were collected on the diversity of yeast species present, regardless the region of origin. First, it can be noted how in the samples of overripe and dried fruits (apples, blackberries, figs and plums) there was a prevalence of species of no interest for this research, as apiculate, pseudohyphae and other minor non-identified species. Nevertheless, some isolates of *Metschnikowia* spp., *Meyerozyma* spp., *Starmerella* spp. and one of *Torulaspora* spp. could be recovered. Ruiz-Moyano and co-workers (2016) isolated yeasts from fig and found a prevalence of *Aureobasidium pullulans* and *Hanseniaspora uvarum*, accompanied in lower percentage of isolates identified as *Meyerozyma caribbica*, *Candida carpophila*, *Torulaspora delbrueckii*,

Metschnikowia pulcherrima and *Hanseniaspora opuntiae*, so generally speaking in accordance with our findings.

Comparing grapes and grape musts, higher diversity was found in the samples of grapes and the biggest difference that comes to attention is the increase in the presence of *S. cerevisiae* on musts, exactly as it can be expected after the grapes come in contact with the winery equipment and the fermentation slowly begins. Additionally, it is also noteworthy how the isolates of *Starmerella* spp. intensified their presence among the isolates from must in comparison with grapes.

The biodiversity tends to decrease throughout the fermentation process, as a result to the more stringent environment created by the metabolites produced. These findings were confirmed by Pinto *et al*. (2015), who also observed different microbial communities according to their appellation of origin.

In a study carried out by Belda and co-workers (2016), they also isolated a vast number of yeasts from grape musts, however they added a step before the isolation to reduce the level of *Aureobasidium pullulans* and basidiomycetes species and used Lysine Agar Medium to selectively find only non-*Saccharomyces* yeasts. They found more than 50% of the isolates to be *Hanseniaspora uvarum*, much higher than the group defined by us as “apiculate”, which also includes this species. The main similarity with our study was the concentration of *Metschnikowia* spp. and *Lachancea* spp. among the most relevant non-*Saccharomyces*, while the most notable differences were on the presence of *Saccharomyces* spp. and *Starmerella* spp. They found a significantly lower concentration of *S. cerevisiae*, most likely due to the use of lysine medium in comparison with WL medium employed in our research. However, it is very intriguing to see how they did not isolate any strain of *Starmerella* spp., which was the most prevalent group in our samples. They could also find a limited number of isolates from species such as *Torulaspora* spp., *Wickerhamomyces* spp., *Zygosaccharomyces* spp. and *Meyerozyma* spp.

In studies carried out with Xarel-lo and Garnatxa grape musts from 1995 to 2000 (Beltran *et al*., 2002), Merlot in 2006 (Zott *et al*., 2008), Tempranillo also in 2006 (Hierro *et al*., 2006), Chardonnay in 2011 (David *et al*., 2014), Cabernet Sauvignon and Malbec in 2011 (Maturano *et al*., 2015) and Grenache and Carignan in 2012 (Wang *et al*., 2015), *Candida zemplinina* (a synonym of *Starmerella bacillaris*) and *Hanseniaspora uvarum* were the most abundant yeast

species during the cold maceration process and the early stages of fermentation. Tofalo and co-workers (2009) studied a traditional Italian wine made with very high sugar most and found a restricted number of osmotolerant non-*Saccharomyces*, such as *Candida apicola*, "*Candida zemplinina"* and *Zygosaccharomyces bailii*.

Other authors also shown that after the isolation of yeast from different samples of grapes or musts at early stages of fermentation the dominant species of non-*Saccharomyces* were usually *Hanseniaspora* spp. and *Metschnikowia* spp., with important presence of *Starmerella* spp. and sometimes *Lachancea* spp. (Pallmann *et al*., 2001; Sabate *et al*., 2002; Tofalo *et al*., 2009; Brežná *et al*., 2010; Chovanová *et al*., 2011; Tristezza *et al*., 2013; Brysch-Herzberg and Seidel, 2015; Pinto *et al*., 2015; Aponte and Blaiotta, 2016; Jara *et al.*, 2016).

It was shown with the previous charts the diversity and richness of the microbiota when the geographical origin and the type of sample are considered separately. A Principal Component Analysis (PCA) with the samples divided by territory of origin and fruit variety is a possible way to visualize the impact of these factors on the diversity and how similar they are based on region of origin and type of sample (Figure 2.10).

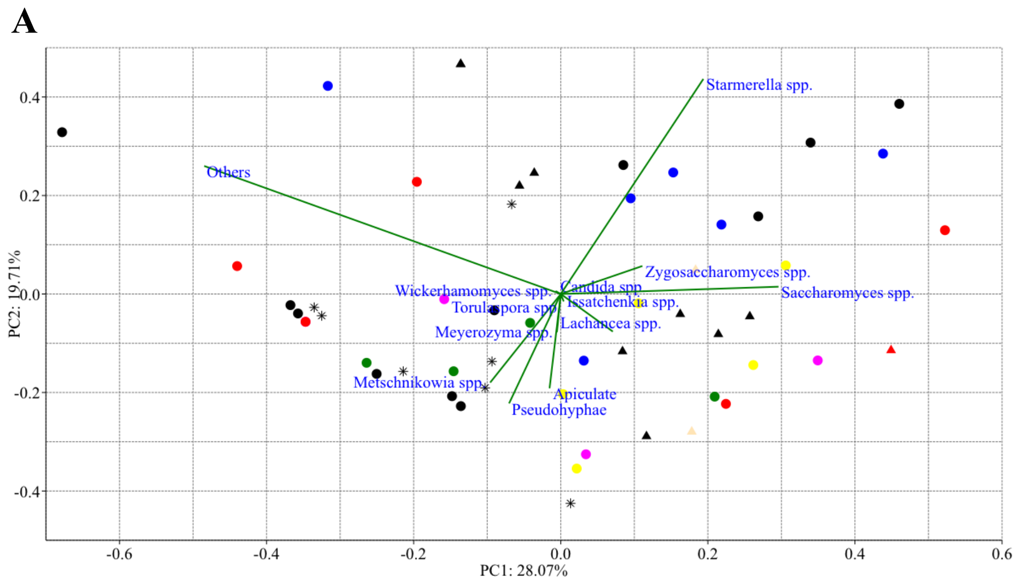
The PCA confirms the huge variability between the samples and the influence of many factors. It's possible though to see some trends that were highlighted before. The samples of grape must, represented with filled triangles, clustered relatively close due to higher presence of *Saccharomyces* spp. Overripe and dried fruits are also positioned closer and on the quadrants of the PCA with more influence of apiculate, pseudohyphae and the other minor species of yeasts.

Regarding the samples of grapes, they were well distributed over the chart and it is difficult to identify which factor has more impact on the yeast diversity. In some cases, grapes from different varieties but coming from the same region clustered together, while there were examples of the opposite behavior, samples from the same region very distant from each other on the PCA plot.

All samples from the Abruzzo region were composed of Pecorino grapes, coming from different locations within the region. It can be seen that most of them clustered together under the influence of a higher concentration of *S. bacillaris*, as already observed. In the Lazio region, a great difference can be seen among the samples from there, for example Malvasia di Candia and Malvasia

Puntinata (also known as Malvasia del Lazio) are distant from each other. The Malvasia di Candia from Emilia-Romagna is actually a bit closer to the last, and not the first from the same variety.

A



B

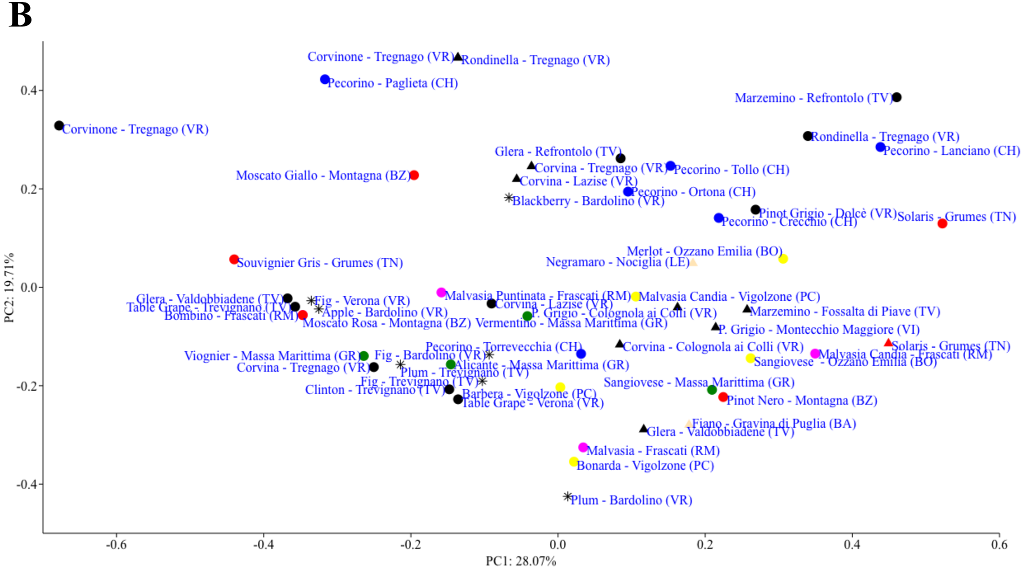


Figure 2.10. [A] Loading plot and [B] Score plot of the Principal Component Analysis of the average number of isolates present on the samples grouped by fruit variety and region of origin. Samples of grapes are represented by dots, grape musts by filled triangles and overripe/dried fruits by stars. Different colors represent different regions, as follows: Blue – Abruzzo; Yellow – Emilia-Romagna; Fuchsia – Lazio; Beige – Puglia; Green – Toscana; Black – Veneto; Red – Trentino-Alto Adige.

In general, the samples from Emilia-Romagna and from Toscana, while belonging to all different grape varieties, tended to be more closely related to those from the same region, with the exception of Sangiovese, which were sampled from these two regions and were closer to each other than to the other varieties of the same respective region.

In Trentino-Alto Adige, there were five grape varieties from two different locations, and they were very clearly separated on the graphic. Particularly, the two varieties from Grumes (TN), which were Solaris and Souvignon Gris, showed a very elevated divergence between them.

In Veneto region, it is very interesting to note how the samples were separated based more in the grape conditions than in the variety or location. Almost all samples of grape must were clustered together, while the overripe and dried fruits concentrated in another quadrant. The samples of unknown table grapes and Clinton, which is an American hybrid variety also considered as table grape, ended up in the same cluster as the overripe/dried fruits, instead of being with the varieties of *Vitis vinifera*. It is also very clear on the PCA how almost all samples of withered grapes, which are Corvina, Corvinone and Rondinella from Tregnago (VR), and Marzemino, were placed on the top borders and separated from the other grapes, suggesting that this process of withering, i.e. drying the grapes to concentrate the sugar, is responsible for changes on the microbial ecology of the grapes. Some samples of those withered grapes were very close to samples of Pecorino from Abruzzo, due to the high presence of *S. bacillaris*.

One possible explanation for diverse profiles on different grape cultivars coming from exactly the same territory and subjected to the same climatic conditions and human intervention could be the stage of ripening of the berries, because it varies from one cultivar to the other and plays a major role on the richness and diversity of the yeast community colonizing the berry surface (Raspor *et al*., 2006). Sabate and co-workers (2002) suggested that the microbiota on unripe grapes is very similar to other plant substrates, but when the berry surface starts to become richer in sugar due to increasing weakness of the peel with the maturation, the composition changes to favor some more fermentative yeast genus, such as *Hanseniaspora*, *Candida* and *Pichia*. This trend continues when the grapes are crushed to must and the higher sugar content combined with low pH make the environmental pressure towards fermentative yeasts always

stronger versus oxidative microorganisms resident on grape surface. Afterwards, the dominance of *S. cerevisiae* is inevitable due to its resistance to ethanol and SO_2 and more efficient fermentative performance, as already exhaustively shown (Pretorius, 2000).

The grape cultivar may also influence the microbiota due to berry and cluster characteristics that are particular of each variety, such as size, skin thickness, proximity and arrangement, which finally could create differences on humidity, pH, nutrient availability, susceptibility (Lederer *et al*., 2013).

2.4. CONCLUSIONS

The main goal of the present work was the set-up of a vast culture collection composed of non-*Saccharomyces* yeasts isolated from samples with high sugar content, most of them composed by grapes and grape musts, in order to further explore the potential of a few selected species to the use in winemaking.

A total of 409 isolates were obtained by using traditional culture-dependent methods and more than 20 species were identified by combining morphological and molecular approaches, confirming the tremendous potential of such substrates as yeasts reservoir, because of their ubiquitously presence with high richness and diversity.

It was confirmed the usefulness of WL medium to the isolation and differentiation of the main non-*Saccharomyces* species associated with oenological interest. The combined use of morphological description in this medium with molecular methods such as RAPD-PCR and ITS sequencing is a powerful tool for the identification of major and also minor species present in the studied samples.

A surprising and worth deeper investigation observation during this study was the high diversity among the pulcherrimin-producing colonies, which could be associated with the genus *Metschnikowia* spp. but not identified at species level. Further research is needed to clarify the taxonomic situation of this group and establish if they could belong to different species or strains under the same epithet.

The results of the frequency and distribution of the isolates are in general concordance with other reports, highlighting the presence of *Hanseniaspora*,

Metschnikowia, *Lachancea* and *Starmerella*, and some species of important oenological interest were obtained. Major differences in yeast diversity and richness were found among the samples and many factors could be accounted for this result. Furthermore, the results suggest that the particular microbiome associated with each territory could play an important role in the typicity and distinctiveness of wines obtained from each appellation.

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Chapter 3

Exploring the phenotypic and genotypic diversity of a potential new generation of starter cultures for oenology

CHAPTER 3. EXPLORING THE PHENOTYPIC AND GENOTYPIC DIVERSITY OF A POTENTIAL NEW GENERATION OF STARTER CULTURES FOR OENOLOGY

3.1. INTRODUCTION

Many genera of yeast belonging to the group called non-*Saccharomyces* participate in the winemaking process by initiating the spontaneous alcoholic fermentation of grape juice to wine, although they are usually surpassed by *S. cerevisiae* due to their limited tolerance to ethanol and sulphite (Heard and Fleet, 1985; Henick-Kling *et al.*, 1998; Jolly *et al.*, 2003). Nevertheless, the non-*Saccharomyces* yeasts can have a great impact in the progression of the fermentation process and the outcome of wine quality, due to relevant enzymatic activities and production of metabolites of oenological significance that influence the wine organoleptic profile (Ciani and Maccarelli, 1998; Egli *et al.*, 1998; Soden *et al.*, 2000).

The non-*Saccharomyces* yeasts have some features that are less pronounced in *S. cerevisiae* or even non-expressed in the leading yeast responsible for the wine fermentation (Esteve-Zarzoso *et al.*, 1998; Mateo and Maicas, 2016). The main contribution of those alternative yeasts is on the aromatic profile, not the traditional fermentative aromas associated with *S. cerevisiae*, but more related to enhancing the varietal profile of some grape varieties, since their enzymatic pool can act by catalyzing the release of volatile aromatic compounds from non-volatile precursors already present in the grape must (Fernández-Gonzáles *et al.*, 2003; Hernandez-Orte *et al.*, 2008); some molecules are produced by their own metabolic activity and some extracellular enzymes can transform molecules derived from *S. cerevisiae* metabolism (Bisson and Kunkee, 1991; Boulton *et al.,* 1996; Clemente-Jimenez *et al.*, 2004).

The most important yeast enzymes regarding the release of primary aroma compounds are glycosidases, which hydrolyze the non-volatile odorless precursors bound to sugars as glycosides (Gunata *et al.*, 1988), and carbon-sulfur lyases, that release volatile thiols from cysteine-bound conjugates (Tominaga *et*

al., 1998). Monoterpenes liberated from terpene glycosides by glycosidases and β-glucosidases, and volatile thiols generated during fermentation from their conjugated form with cysteine can contribute to positive aromas of wines (Padilla *et al.*, 2016).

The molecules discussed on the previous paragraph are called primary aromas because they come from precursors formed during the grape ripening, which are released from the bound form by the action of yeast enzymes. Instead, the volatile fatty acids, higher alcohols, esters, aldehydes, volatile phenols and sulfur compounds are also molecules related to the aromatic profile of a wine, but are considered as secondary aromas, because they arise directly from the fermentation process (Rapp and Versini, 1991; Padilla *et al.*, 2016).

These compounds derived from yeast metabolism can be either positive or negative to the overall quality of wine depending on their concentration, and since the activity of enzymes and biosynthesis of aroma compounds is species- or strain-dependent, the selection of more suitable strains is a step of primary importance to their biotechnological application (Dubourdieu *et al.*, 2006; Padilla *et al.*, 2016).

Nevertheless, the enzymatic activity is not only aroma-related and can be held responsible for other sensorial and technological features (Belda *et al.*, 2016). For example, the pectinolytic activity of non-*Saccharomyces* yeasts can be useful to help improve clarification and filtration process and the release of more color and flavor compounds entrapped in the grape skin, as some pectinolytic enzymes (such as polygalacturonases) are already used in enology to degrade the plant cell wall polysaccharides present on the grape skin and pulp (Lang and Dörnenburg, 2000; Van Rensburg and Pretorius, 2000). Proteases can also be beneficial for winemaking to replace bentonites in solving problems of protein haze, since the last can cause negative effects on the wine aroma. Proteolytic activity was also reported to facilitate the juice extraction and clarification, wine filtration and could help the release of nitrogen for yeast growth (Heard and Fleet, 1985; Van Rensburg and Pretorius, 2000; Marangon *et al.*, 2012; Capozzi *et al.*, 2015).

On the other hand, enzymatic activities can also cause damage to the wine sensorial profile, such as sulfite reductase, which is responsible for the production of hydrogen sulfide during wine fermentation, a molecule related to the characteristic rotten-egg off-flavor (Swiegers and Pretorius, 2007). For this

reason, the lack of sulfite reductase activity is a positive characteristic to avoid the risk of wine reduction (Belda *et al*., 2016).

Additionally, the activity of these yeasts can produce glycerol, organic acids, mannoproteins, which could impact the production process and ultimately other aspects related to the wine quality (Ciani and Comitini, 2011; Jolly *et al*., 2014; Barbosa *et al*., 2018).

In this context, there is a wide variety of applications extensively proven or expected from the non-*Saccharomyces* yeasts, and since many of these relevant oenological characteristics are species- or even strain-specific, it is important to carry out selection protocols in order to highlight the most suitable natural isolates in the middle of all the diversity and metabolic heterogeneity, the same way as it has been done with *S. cerevisiae*, following the ultimate goal of developing innovative fermentation processes with improved sensorial, technological and safety aspects (Suárez-Lepe and Morata, 2012; Belda *et al*., 2016).

The workflow for the selection of interesting yeast isolates has been evolving over the years and, besides the properties that influence the outcome in the wine quality and style, which were explained on the previous paragraphs, there are two other categories that can be usually added: properties related to the performance of the fermentation process and properties affecting the commercial production of the starters in large scale (Fleet, 2008).

Regarding the performance in the grape must fermentation, it is important that the yeasts are able to survive the harsh environment found in grape juice and wine, being tolerant to the concentrations of ethanol and sulphur dioxide usually present. They are produced by *S. cerevisiae* during alcoholic fermentation, but SO_2 is also generally added in oenology for controlling the growth of unwanted microorganisms and to prevent oxidation of the must. Non-*Saccharomyces* yeasts were already reported as being sensitive to it (Henick-Kling *et al*., 1998; Barbosa *et al*., 2018).

Other compounds that could be present in the must remained from the grapes due to certain vineyard conditions. Copper is one of the oldest methods used in viticulture for the protection against fungal diseases, and its application has been intensified in the last years due to the possibility of use by organic growers (Cavazza *et al*., 2013). Even if it is used on vines, it is possible that copper will remain in the grape must and hinder yeast growth (Gava *et al*., 2016).

The presence of gluconic acid is particularly increased in wines obtained from grapes affected by gray rot, due to the metabolic activity of the mold *Botrytis cinerea*. The sensory properties of wines are considerably altered by the presence of gluconic acid, which decreases the wine microbiological stability and raises long-term storage problems that can be solved only by reducing its concentration in the wine. For this reason, it would be interesting to inoculate in musts alternative yeasts able to metabolize this acid (Peinado *et al*., 2004, 2009).

In the selection of *S. cerevisiae* strains, it is essential that they show a vigorous and complete fermentation of the grape sugars to high levels of alcohol, while it has to be considered the limitation of most non-*Saccharomyces* species to arrive until the end of the fermentation and thus be given less importance to this criterion, since the fermentation process will be concluded by a *S. cerevisiae* sequentially or co-inoculated. However, the best the fermentation performance exhibited by the non-*Saccharomyces* isolates, the higher the impact they will have on the final wine and the lower the chance to have stuck or sluggish fermentations (Bisson, 1999).

In order to become a commercial product, the yeast isolates selected for their good fermentation performance and interesting inputs to wine organoleptic profile must also be compatible to the large-scale production on preferably inexpensive substrates, resistant to the process of drying and packaging, resilient during the storage and readily active after the rehydration performed by the winemakers, without losing the important features for which they have been chosen (Soubeyrand *et al.*, 2006; Fleet, 2008).

Moreover, it is of summary importance to also evaluate the genotypic diversity among the species and strains of non-*Saccharomyces*. The oenological potential is usually evaluated by the phenotypic properties, but the genetic structuration of the species and the relationships between the strains are relevant to determine whether the phenotypic diversity is representative of a given species and to facilitate the selection of the top isolates. Some genotypic diversity could be related to the different geographical locations and conditions the isolates were recovered, since the colonization of different ecosystems impairs their adaptation to diverse environmental pressures and led to evolutionary differentiation (Banilas *et al.*, 2016; Masneuf-Pomarede *et al.*, 2016).

Several methods have been used for the discrimination between species, such as those used for the identification of isolates in the previous chapter, but they do not give a discrimination that is powerful enough for the definition of genetic relationships within a given species and therefore exists an increasing need for developing molecular marker-assisted genotyping methods. Such techniques should be ideally highly discriminatory, simple and affordable. Different molecular approaches have been developed so far for the typing of wine yeasts, as for example microsatellites or simple sequence repeats (SSRs), most of them already successfully applied to *S. cerevisiae* and to a lesser extent to non-*Saccharomyces* (Banilas *et al.*, 2016; Masneuf-Pomarede *et al.*, 2016). SAU-PCR is a PCR-based method which starts with the genomic DNA digestion by the restriction endonuclease Sau3AI and then selective amplification with primers whose core sequence is based on the enzyme's recognition site, such as SAG1 and SCA, and was already applied for the strain differentiation within the species *S. bacillaris* (Corich *et al.*, 2005; Rantsiou *et al.*, 2012; Englezos *et al.*, 2015; Lemos Junior *et al.*, 2016).

Among the different species of non-*Saccharomyces* identified in the first steps of this research, three genera were chosen for the characterization assays due to interesting oenological properties already reported, as shown below, and to the broad range of isolates obtained for these groups, what enabled us to perform a more representative and promising screening.

Starmerella bacillaris (synonym *Candida zemplinina*) is one of the most studied species in wine microbiology and due to its osmo- and psychrotolerant behavior is commonly found in grapes with high sugar content, like overripe and botrytized grapes, and musts at low temperatures, for example during cold maceration process (Sipiczki, 2003; Csoma and Sipiczki, 2008; Duarte *et al.*, 2012; Pfliegler *et al.*, 2014; Maturano *et al.*, 2015; Wang *et al.*, 2015). Strains from this species have already been described and tested on single and mixed fermentations aiming to explore its fructophilic character, reduced acetic acid and increased glycerol production, low ethanol yield (Magyar and Tóth, 2011; Rantsiou *et al.*, 2012; Tofalo *et al.*, 2012; Bely *et al.*, 2013; Wang *et al.*, 2014, Englezos *et al.*, 2015). It has been reported to have high tolerance to the ethanol present in wine and so it can survive until the end of the alcoholic fermentation, differing from other non-*Saccharomyces* yeasts (Rantsiou *et al.*, 2012).

The low production of volatile acidity is also a characteristic of *Lachancea thermotolerans*, another remarkable non-*Saccharomyces* yeast which biotechnological potential is mostly considered by the high production of L-lactic acid (Kapsopoulou *et al*., 2005; Hranilovic *et al*., 2017). Formerly known as *Kluyveromyces thermotolerans*, it is the type species of the ascomycetous genus *Lachancea* (Kurtzman, 2003) and occupies diverse natural habitats, such as soil, insects, plants, fruits, and in particular grapes and must (Naumova *et al*., 2007; Lachance and Kurtzman, 2011; Freel *et al*., 2014). The ability to act as acidifying agent is of increasing interest to compensate the insufficient acidity of specific grape cultivars, as *L. thermotolerans* can provide an effective acidification through its elevated production of L-lactic acid (Mora *et al*., 1990; Kapsopoulou *et al*., 2007). Other interesting oenological traits already reported for mixed fermentations with *L. thermotolerans* are the reduction of ethanol content, increasing of glycerol and improved organoleptic profile (Gobbi *et al*., 2013; Benito *et al*., 2016).

The genus *Metschnikowia* gathers around 40-50 species and represents one of the most divergent ascomycetous genera known (Kurtzman and Robnett, 1998; Liu *et al*., 2018). Its members can be found on sea-water, freshwater crustaceans, and in terrestrial habitats are usually associated with flower, fruits and transmitted to new niches by insects, within which were already isolated in the gut lumen or on eggs (Lachance, 2011; Guzmán *et al*., 2013; Molnár and Prillinger, 2005). *M. pulcherrima* strains may inhibit the growth of some spoilage yeasts (*Brettanomyces*/*Dekkera*, *Hanseniaspora*, and *Pichia*) by the formation of a red pigment, pulcherrimin, which depletes the free iron in the medium thus generating an environment unsuitable for microorganisms requiring such element for the growth (Oro *et al.*, 2014; Sipiczki, 2006). *M. pulcherrima* and close relatives (*M. andauensis*, *M. chrysoperlae*, *M. fructicola*, *M. shanxiensis*, *M. sinensis*, *M. zizyphicola*) are regularly found in nutrient-rich plant materials that serve as breeding and feeding sites for insects; and especially *M. pulcherrima* and *M. fructicola* were already found in grapes and musts at early fermentation stages (Fleet *et al*., 2002; Lachance, 2011; Guzmán *et al*., 2013). Some recent studies showed that *M. pulcherrima* can be successfully used in mixed fermentations to reduce the ethanol content in the wine and produce good quality sensory profile

with beneficial volatile composition (Contreras *et al*., 2014, 2015; Varela *et al*., 2016, 2017).

The genotypic and phenotypic characterization of a large number of yeast isolates had the main objective of giving a clearer understanding on the limitations and potential that each species could offer for a successive technological application, especially in oenology. The links between the genetic and physiological profiles were an attempt to clarify the inter and intraspecific relations and the factors that could be exerting an influence on them. Based on the integration of the characterization data obtained it was possible to choose a limited number of selected isolates to be further analyzed with new criteria aiming to the use in winemaking.

3.2. MATERIAL AND METHODS

3.2.1. Yeasts strains and culture conditions

From the yeast collection that was organized with 409 isolates recovered from grapes, grape must, honey, overripe and dried fruits, 104 isolates from the genera *Lachancea*, *Starmerella* and *Metschnikowia* were chosen for the genotypic and phenotypic studies (Supplementary Table S.2). It has also been added the type strains of the species *Lachancea thermotolerans* (DBVPG 6232^T), *Starmerella bacillaris* (NCAIM Y016667^T), *Metschnikowia andauensis* (CBS10809^T), *Metschnikowia fructicola* (CBS8853^T) and *Metschnikowia pulcherrima* (CBS5833^T); and a commercial starter of *L. thermotolerans* (Viniflora® Concerto™).

Starting from the frozen cultures kept at -80 ºC, the isolates were reactivated on WL (Sigma-Aldrich) agar plates incubated at 27 ºC for 72 hours. One single colony was then inoculated in YPD broth (yeast extract, 1.0%; bacteriological peptone, 2.0%; glucose, 2.0%; Sigma-Aldrich) and incubated under static conditions at 27 ºC for 48 hours. A fresh YPD-containing tube was then inoculated at 1% with the previous grown culture and put in agitation overnight at 27 ºC to reach the early stationary phase. The culture was then centrifuged at 3,000×*g* for 5 minutes, washed twice and resuspended in physiological solution

0.9% (w/v) NaCl (Sigma-Aldrich). The inocula were standardized for all yeast cultures with an optical density at 600 nm (OD_{600}) of 1.6, corresponding to approximately 1×10^7 cells/mL. This inoculum was then used for the stress tolerance trials on microtiter plates, enzymatic assays on Petri dishes and microvinifications.

3.2.2. DNA extraction

Total genomic DNA was isolated and purified using the commercial kit Wizard Genomic DNA Purification (Promega) following the manufacturer's protocol, from a 2-mL aliquot of cultures grown in YPD broth. The quality assessment of the DNA obtained was performed with a NanoDrop ND1000 UV-Vis Spectrophotometer (Thermo Scientific) and dilution in DNAse-free water was carried out when necessary for the following molecular analysis.

3.2.3. Rep-PCR

The analysis of Rep-PCR was used for the typing of yeast isolates belonging to the genus *Metschnikowia*, employing the microsatellite oligonucleotide sequence $(GTG)_5$ (5'-GTGGTGGTGGTGGTG-3') and according to the protocol of Lieckfeldt *et al*. (1993), modified by Pfliegler *et al*. (2014). The reaction mix was prepared with the following concentrations for the final volume of 25 µL: 1× DreamTaq Green Buffer (Thermo Scientific); 2.5 mM $MgCl_2$; 0.2 mM dNTPs; 1 µM primer $(GTG)_5$; 0.04 U/µL DreamTaq DNA Polymerase (Thermo Scientific) and 20 ng genomic DNA. The amplification program considers an initial denaturation at 94 ºC for 5 minutes, followed by 30 cycles of denaturation at 94 ºC for 50 seconds, annealing at 50 ºC for 50 seconds and extension at 72 ºC for 50 seconds, concluding with a final extension of 5 minutes at 72 ºC. The amplification was conducted in Thermal Cycler 2720 (Applied Biosystems).

Band profiles were realized in electrophoresis gel at 1.5% agarose in 1× TAE Buffer (40 mM Tris, 20 mM Acetic acid, and 1 mM EDTA, Sigma-Aldrich) stained with EuroSafe colorant (Euroclone) in a 2 hours and 20 minutes run at 110 V. The molecular ladder used was O'Gene Ruler DNA (Thermo Scientific).

The visualization and image capturing were made under UV light with UVITEC Gel Documentation System (Cleaver Scientific). The images were analyzed with the software BioNumerics (version 5.0, Applied Maths) and dendrograms were constructed using Pearson's correlation coefficient and the unweighted pair group method with arithmetical average (UPGMA) clustering.

3.2.4. SAU-PCR

For the molecular typing of *Starmerella bacillaris* and *Lachancea* spp. isolates, the SAU-PCR technique was chosen, following the protocol described by Corich *et al*., 2005. Ten microliters of the extracted genomic DNA diluted at 20 ng/µL were digested at 37 ºC overnight with 10 units of the restriction endonuclease *Sau*3AI (Promega), in a final volume of 20 µL with the corresponding restriction buffer solution. Afterwards, the amplification was performed with the following composition for the final volume of 25 µL in the reaction mix: 1× DreamTaq Green Buffer (Thermo Scientific); 1.5 mM $MgCl_2$; 0.2 mM dNTPs; 2 µM primer SAG1 (5′-CCGCCGCGATCAG-3′); 0.02 U/µL DreamTaq DNA Polymerase (Thermo Scientific) and 2 µL of the digested DNA (10 ng/µL). The amplification program considers a preliminary step to fill-in the protruding *Sau*3AI ends, starting at 25 ºC for 5 seconds, then gradually raising the temperature to 60 ºC (0.1 ºC/s) and maintaining for 30 seconds. In the first amplification cycle, there is a low-stringency phase composed by a denaturation at 94 ºC for 60 seconds, subsequently the temperature is brought to 50 ºC and after 15 seconds is gradually lowered to 25 ºC (0.1 ºC/s), then it is raised again to 50 ºC (0.1 ºC/s) and maintained for 30 seconds. This low stringency phase is repeated twice. The program continues with the high-stringency amplification phase, which is carried out by 35 repetitions of a denaturation at 94 ºC for 15 seconds, annealing at 48 ºC for 60 seconds and extension at 65 ºC for 2 minutes, concluding with a final extension of 5 minutes at 65 ºC. The amplification was conducted on Mastercycler Nexus Thermal Cycler (Eppendorf).

The separation of the amplification products, image acquisition and band profiles comparison were made following the same protocol as described for Rep-PCR, except that the agarose gel was prepared with a concentration of 2%.

3.2.5. Stress tolerance assays

The growth tests to evaluate the tolerance of the isolates to some stress conditions usually encountered on the grape and must environments were performed in 96-well microtiter plates. The wells were filled in with 198 μL of medium according to the different tests described below, and inoculated with 2 μL of the cell suspension prepared as explained above (par. 3.2.1.). All yeast isolates tested were inoculated in quadruplicate and the commercial strain of *S. cerevisiae* EC 1118 was used as control.

The microplates were then incubated at 20 ºC for 72 hours with constant agitation on orbital shaker at 150 rpm (IKA KS 260 basic). The optical density at 600 nm was measured every 24 hours with a microtiter plate reader (BIO-RAD Model 680). The relative cell growth (%) was calculated by the ratio between the OD_{600} in the medium with and without the stress factor added, at the specific incubation times. The results were then used to construct dendrograms using Pearson's correlation coefficient and the unweighted pair group method with arithmetical average (UPGMA) by the software for statistical analysis PAST (Hammer *et al*., 2001).

3.2.5.1. Ethanol

The ethanol tolerance was tested in YPD broth (yeast extract, 1%; bacteriological peptone, 2%; glucose, 2%; Sigma-Aldrich) supplemented with 4, 8 and 12% (v/v) absolute ethanol (Sigma-Aldrich), added after the sterilization of the base medium in autoclave. The same medium without the addition of ethanol was used as the control.

3.2.5.2. High sugar content

The growth in the presence of a high sugar concentration was evaluated in YPD broth with the addition of glucose to reach concentrations of 220, 270 and 320 g/L of this sugar. The glucose was autoclaved separately and added

afterwards to the sterile medium containing the peptone and yeast extract, in order to avoid the caramelization of sugars by the reaction of Maillard. The control growth was carried out in YPD with the standard concentration of 20 g/L glucose.

3.2.5.3. Sulphur dioxide

The tolerance to SO_2 was verified in YPD medium with pH adjusted to 3.30 and added potassium metabisulfite ($K_2S_2O_5$; Sigma-Aldrich), to reach a SO_2 concentration of 150 and 200 mg/L. Control was normal YPD broth without SO_2.

3.2.5.4. Copper

To verify the tolerance of the isolates to copper, this element was added to YPD broth in the form of copper sulfate ($CuSO_4$; Sigma-Aldrich) in order to reach final concentrations of 2.5, 5 and 10 mM. YPD without $CuSO_4$ was the control.

3.2.5.5. Gluconic acid

The ability to metabolize gluconic acid was evaluated after growth in medium containing this acid as the sole carbon source (Peinado *et al.*, 2004). The cells were inoculated in YM broth (yeast extract, 0.3%; malt extract, 0.3%; peptone, 0.5%; Sigma-Aldrich) supplemented with 3% gluconic acid (Sigma-Aldrich) as carbon source and pH adjusted to 6.5.

3.2.6. Enzymatic activities

Some enzymatic activities relevant for the wine quality were tested, with the protocols described in the following paragraphs. All tests were carried out with spot inoculation on Petri dishes filled with the specific media. From the cell suspension prepared as described in section 3.2.1., a droplet of 10 μL was deposited on the agar surface and dried under biosafety cabinet. Ten spots were inoculated on each plate and all isolates were inoculated in quadruplicate, with the commercial strain *S. cerevisiae* EC 1118 as a control.

The plates were incubated at 27 ºC for a specific time according to each test. The results were verified by changes on the aspect of the colony and/or the surrounding medium.

3.2.6.1. Sulfite reductase

To verify the ability of the isolates to produce hydrogen sulfide (H_2S), they were spot-inoculated on Biggy agar (Oxoid) plates and incubated at 27 ºC. After 48 hours, the colonies were classified for their H_2S production in a scale from 1 to 5 based on their color: white colonies have no or very low production and were classified as 1, while black colonies are high producers of H_2S and represent 5 in the scale, colonies showing different intensities of brown were classified from 2 to 4 and are medium producers (Comitini *et al.*, 2011).

3.2.6.2. β-Glucosidase

The medium used to check the β-glucosidase activity was prepared with 5 g/L of arbutin (hydroquinone β-D-glucopyranoside), 6.7 g/L of Yeast Nitrogen Base (YNB) with amino acids and 20 g/L of agar (all reagents Sigma-Aldrich). The pH was adjusted to 5.0 and the medium autoclaved. After the sterilization, 20 mL/L of a filtered ferric ammonium citrate solution (1% w/v) was added. The plates were incubated at 27 ºC and after 72 hours a positive result was indicated as the discoloration of the medium to brown color and also the colonies became brown, while negative colonies remained white (Rosi *et al.*, 1994).

3.2.6.3. Glycosidase

The glycosidase activity was evaluated on medium containing 6.7 g/L of YNB with amino acids, 1 g/L of glucose, 2 g/L of rutin (quercetin-3-rutinoside; Sigma-Aldrich) and 20 g/L of agar, mixed and sterilized in autoclave. The plates incubated at 27 ºC were observed after 72 hours and a positive result was indicated by a clear zone around the colony (Hildebrand and Caesar, 1989).

3.2.6.4. Esterase

The presence of esterase activity in the yeast isolates was verified in medium composed by 10 g/L of bacteriological peptone, 5 g/L of NaCl, 0.1 g/L of $CaCl_2$ and 15 g/L of agar. After the adjustment of the pH to 6.8, the medium was autoclaved and 5 mL/L of sterile Tween 80 were added subsequently. Incubation of plates was made at 27 ºC and an opaque halo around the colonies after 72 hours represented a positive result (Slifkin, 2000; Buzzini and Martini, 2002).

3.2.6.5. Pectinase

The medium for the pectinase activity assay was prepared with 12.5 g/L of polygalacturonic acid, 6.8 g/L of potassium phosphate pH 3.5, 6.7 g/L of YNB with amino acids, 10 g/L of glucose and 20 g/L of agar. The pH was adjusted to 5.0 and the medium sterilized in autoclave. After the inoculation, the plates were incubated at 27 ºC for 72 hours. The results were obtained after rinsing the colonies off the plate with physiological solution, then staining the surface of the plate with a ruthenium red solution 0.1% (w/v) and a positive result was characterized by a purple halo around the area where the colonies were grown (Charoenchai *et al*., 1997; Strauss *et al*., 2001).

3.2.6.6. Protease

For the verification of proteolytic activity in the yeast isolates, they were inoculated in YPD agar plates modified by the addition of skim milk powder (2% w/v; Sigma-Aldrich). After 5 days of incubation at 27 ºC, the positive colonies were identified by a clear halo around them (Strauss *et al*., 2001; Belda *et al*., 2016).

3.2.7. Single culture fermentation

To check the fermentative behavior and other relevant oenological parameters, the isolates of *S. bacillaris* and *Lachancea* spp. were tested through laboratory scale fermentations in natural grape must, inoculated as single starter cultures.

3.2.7.1. Fermentation conditions

Natural must from Trebbiano grapes (103 g/L glucose, 107 g/L fructose, 150 mg/L yeast assimilable nitrogen, pH 3.3) was transferred to 500-mL flasks tapped with silicon stoppers. They were inoculated in duplicate with a concentration of 1×10^6 cells/mL of each isolate of *Lachancea* spp. and *Starmerella bacillaris* listed on Table 3.1, and *S. cerevisiae* EC 1118 as a control. Fermentations were carried out at 22 ºC under static conditions for 10 days.

3.2.7.2. Analytical determinations

The parameters analyzed after 10 days of fermentation were: concentration of malic, lactic, succinic and citric acids, glucose, fructose, glycerol, ethanol, acetic acid, total acidity and pH. They were evaluated with infrared spectrophotometry (WineScan FOSS Analytical) and high performance liquid chromatography (HPLC; Thermo Scientific). The fermentation kinetics was estimated following the production of CO_2 during the time. A Principal Component Analysis (PCA) was performed with the results of the chemical determinations using the software for statistical analysis PAST (Hammer *et al*., 2001).

3.3. RESULTS AND DISCUSSION

3.3.1. Molecular characterization

To test the diversity of *Lachancea* spp. isolates, the band patterns obtained with the SAU-PCR technique were compared and used to construct the similarity dendrograms. Twelve isolates identified as *L. thermotolerans*, as described in the previous chapter, were submitted to this molecular characterization, alongside with one type strain and one commercial strain from the same species, one isolate identified as *L. kluyveri* (FIANO22) and one identified as *L. fermentati* (LS16).

The cluster analysis of the 16 profiles shown in Figure 3.1 allowed us to see the clear differentiation of the three species, whereas there was less than 30% similarity between them. When the coefficient of similarity was 80%, it can be

distinguished the presence of two clusters and three single strains, among the *L. thermotolerans* isolates. Interestingly, the type strain and the commercial strain CONCERTO are two of those separated single strains, showing 80% and 50% similarity with the natural isolates, respectively.

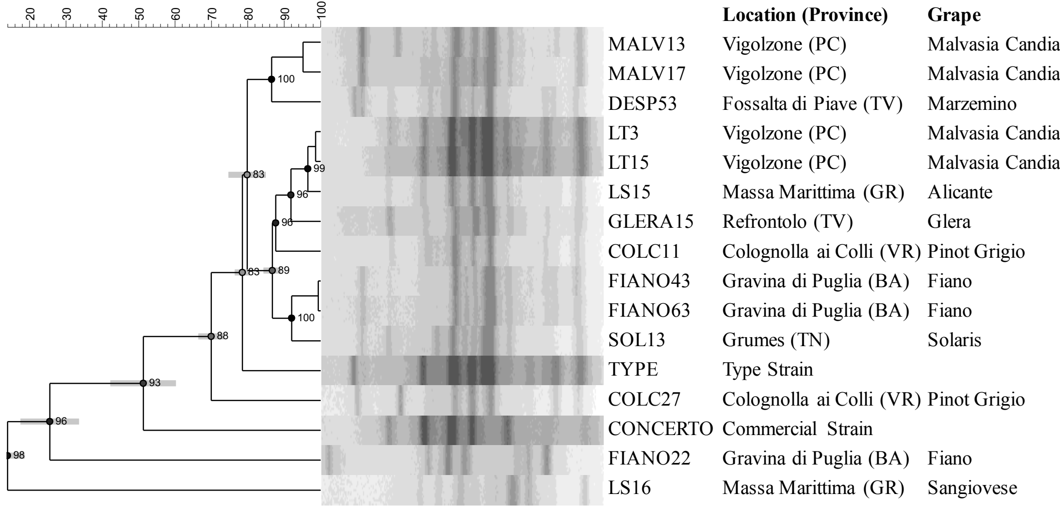

Figure 3.1. Molecular characterization of *Lachancea* spp. isolates by means of SAU-PCR.

The molecular characterization revealed a high level of similarity between isolates of *L. thermotolerans* coming from different sources. In particular, isolates coming from the same grape variety tended to be closer in the dendrogram. This is the case for the isolates from Malvasia Candia and Fiano, however, the two isolates from Pinot Grigio were not clustered together, as one of them (COLC27) was placed in a single strain cluster with 70% similarity in comparison with the others. Two previous studies with microsatellites (Banilas *et al.*, 2016; Hranilovic *et al.*, 2017) and one with sequencing of mitochondrial genome (Freel *et al.*, 2014) for the genotyping of *L. thermotolerans* isolates have shown that geography played an important role in the differentiation within this species.

For the characterization of 46 isolates of *S. bacillaris*, the protocol of SAU-PCR was used as well. The dendrogram created with the similarity of the band patterns is shown in Figure 3.2. It can be seen from the dendrogram that the isolates of *S. bacillaris* sourced from different locations and grape varieties had a rather high level of similarity. At a similarity of 60%, the isolates form two major clusters and one single strain cluster (COLC34). When the coefficient of

similarity was 80%, eight major clusters and four single strain clusters could be separated. All clusters, but one, were composed by isolates coming from different locations and grape varieties. This only exception was the cluster formed by PG21 and PG24, both isolated from Pinot Grigio musts.

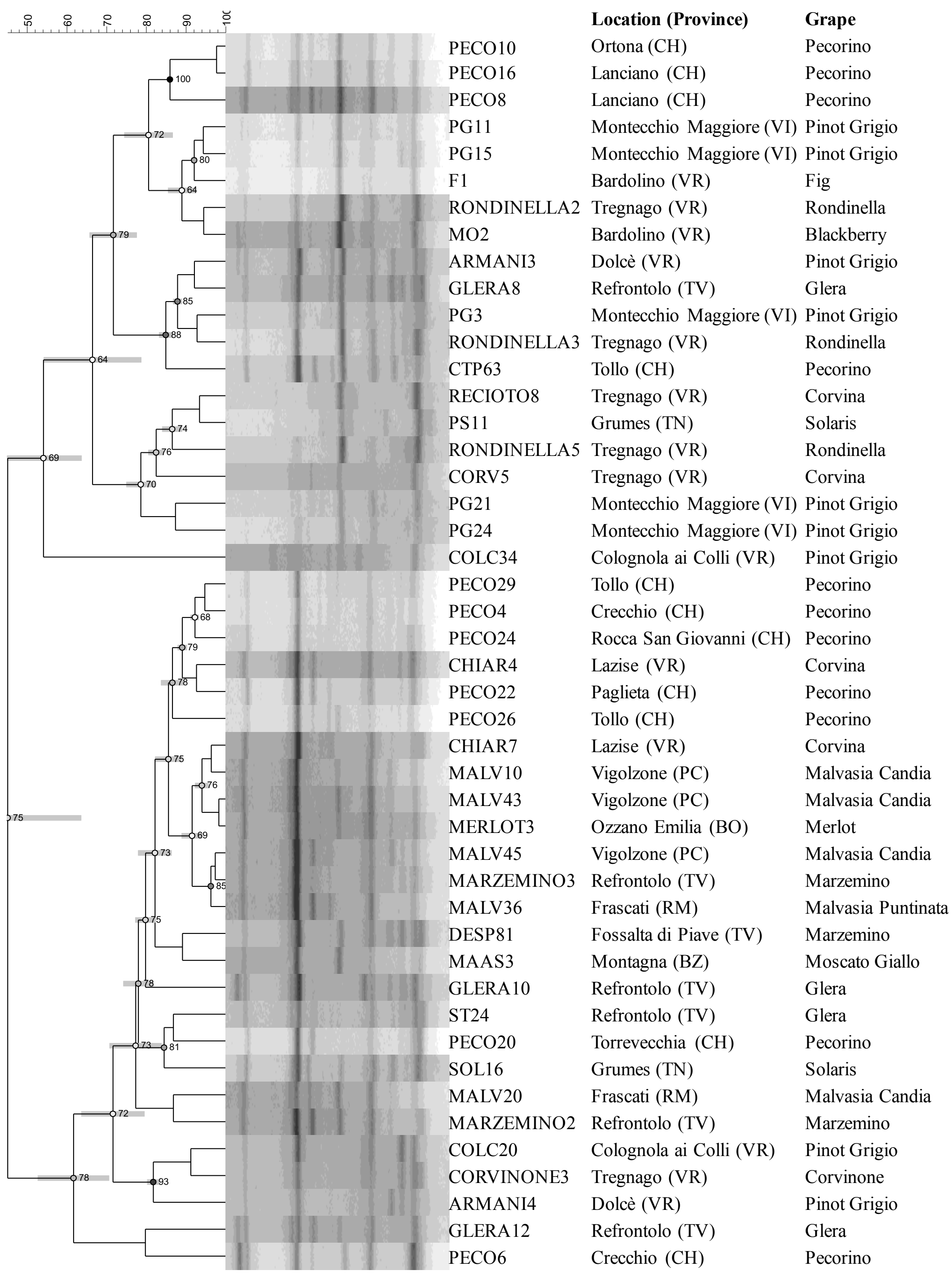

Figure 3.2. Molecular characterization of *Starmerella bacillaris* isolates by means of SAU-PCR.

All isolates from Emilia-Romagna clustered together, while those from other regions were distributed along the different groups. The most isolates were obtained from Veneto, and in the dendrogram it can be noted how those coming from Verona and Vicenza provinces are separated from Treviso isolates.

The high level of similarity between isolates of *S. bacillaris* was already seen by other authors. Pfliegler and his colleagues (2014) tested five different RAPD and microsatellite primers in order to assess the biodiversity within 35 strains from different countries, and concluded that the diversity was relatively low. Englezos and co-workers (2015) found six clusters and three single strains at 70% similarity on the dendrogram obtained by the combined analysis of SAU-PCR and Rep-PCR. They were working with 63 isolates from two different Italian regions and four grape varieties. In a study with 36 isolates from two wineries in the same Italian region, Lemos Junior and colleagues (2016) could individuate 14 groups in the dendrogram obtained with SAU-PCR, at 70% similarity. In our study, considering the level of similarity also at 70% in order to compare, we were able to see four major clusters and one single strain, within a total of 46 isolates coming from five different Italian regions and 12 grape varieties (plus one strain isolated from blackberry and one from fig).

The isolates of *Metschnikowia* spp. were molecularly characterized by both techniques of RAPD-PCR with primer M13 (protocol in the previous chapter) and Rep-PCR with primer $(GTG)_5$. Figure 3.3 shows the combined dendrogram obtained by the average of these two analysis. A total of 44 isolates were used for the characterization and the type strains of three species were added, namely *M. andauensis*, *M. fructicola* and *M. pulcherrima*. Interestingly, the three type strains were clustered together with a similarity of 92%, while the overall similarity between the isolates was 68%. As deeply discussed in the previous chapter, there are some issues in the identification and taxonomical organization of *Metschnikowia* spp. strains due to a high level of heterogeneity in the sequences and presence of multiple divergent copies of the same genes within the genome.

At a similarity level of 80%, they could be divided into six different major clusters and one single strain (MALV3). Five out of six clusters were composed by isolates coming from different regions and grapes. The exception was the cluster formed by ALIC2 and ALIC3, both isolated from Alicante grapes in Toscana.

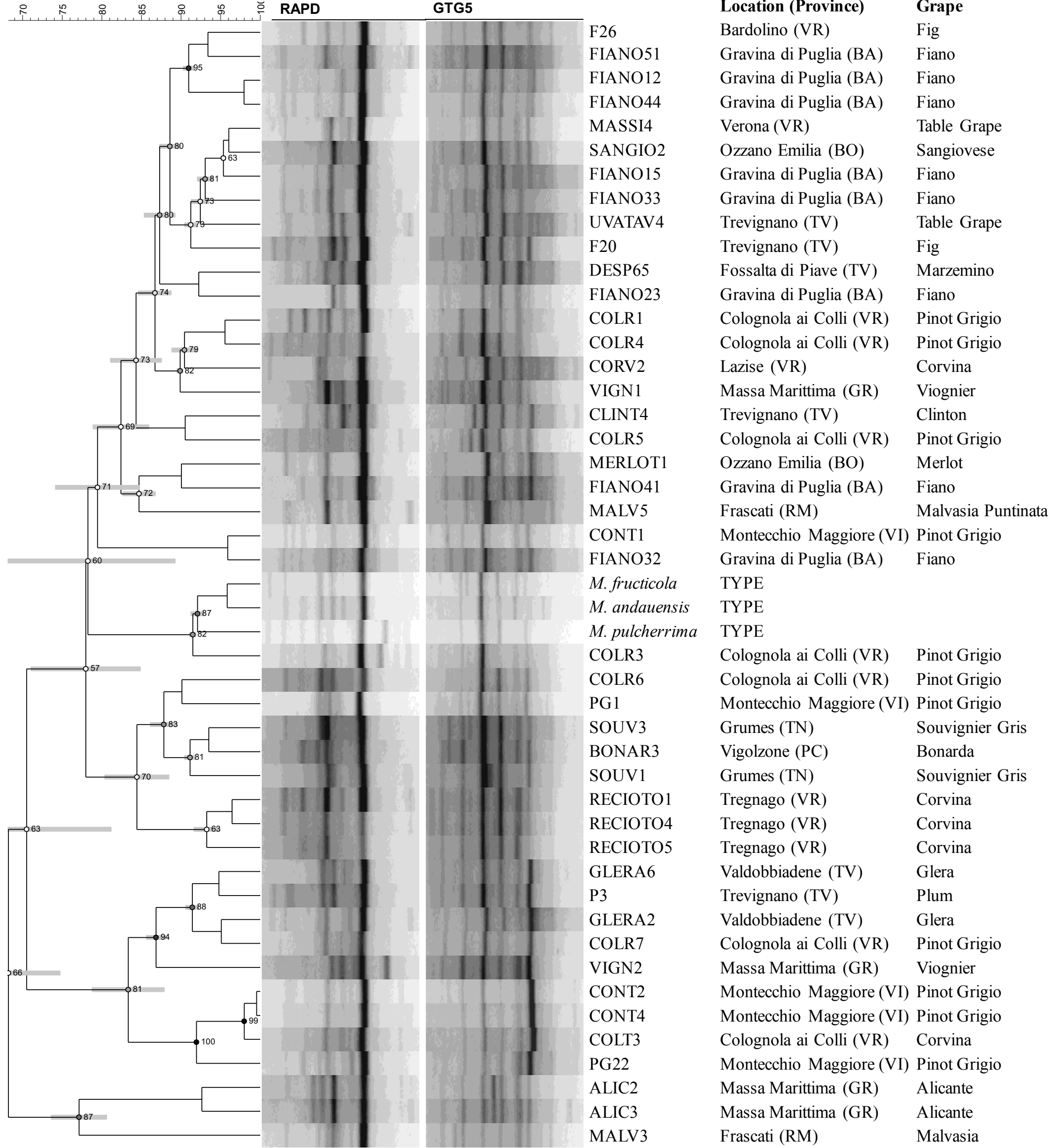

Figure 3.3. Molecular characterization of *Metschnikowia* spp. isolates by means of RAPD-PCR with primer M13 and Rep-PCR with primer $(GTG)_5$.

When considered separately, the fingerprinting with Rep-PCR gave a higher discrimination power for the isolates and type strains of *Metschnikowia* spp. When considering a similarity level of 70%, the analysis with Rep-PCR allowed the separation into five different clusters, while with RAPD-PCR all isolates were grouped into a single cluster. This result disagrees with the one reported by Barbosa and co-workers (2018), who concluded that RAPD-PCR allowed a better strain discrimination than Rep-PCR, using 65 strains of *M. pulcherrima* and the same primers as we did.

3.3.2. Physiological characterization

The results of the relative growth of the isolates in each stress tolerance assay (high sugar level, ethanol, sulfite, copper and gluconic acid) were put together to calculate the similarity based on this phenotypic characterization. The clusters for *Lachancea* spp., *S. bacillaris* and *Metschnikowia* spp. are shown in Figures 3.4, 3.5 and 3.6, respectively. The response profiles are represented by heat-maps and the results of the sulfite reductase activity are also shown next to them.

It is possible to observe that some inter- and intraspecies differences were present. In general, about the different behaviors at species level, *Lachancea* spp. and *S. bacillaris* were more resistant to high concentrations of ethanol than *Metschnikowia* spp. Most of the isolates from this last group could not grow at 8% ethanol and all of them were severely inhibited at 12% ethanol, while the isolates from the two other groups found some inhibition only at 12%, and specially isolates of *S. bacillaris* proved to be the more resistant to this condition. These results are in agreement with Barbosa and co-workers (2018), who carried out a characterization of 65 *M. pulcherrima* isolates and found only a few of them being tolerant to 9% ethanol and all sensitive to 12%. Aponte and Blaiotta (2016) performed a characterization with different species of non-*Saccharomyces*, finding isolates of *M. pulcherrima* to tolerate a maximum of 4-5% ethanol, while *L. thermotolerans* and *S. bacillaris* could grow to a concentration of 10%. Working with 25 isolates of *L. thermotolerans*, Banilas and co-workers (2016) described most of them being resistant to 6% ethanol and different behaviors at 8%, from low to high tolerance. Englezos and colleagues (2015) found similar results to ours working with *S. bacillaris*, where 90% of the isolates were able to grow at all concentrations tested, from 8 to 14%. Also Tofalo and co-workers (2009) found "*Candida zemplinina*" to be tolerant to 8% ethanol and half of the strains could grow at 14%. Ethanol stress represents toxicity through intracellular ROS generation in addition to deleterious damage to cell membrane, by altering its organization and permeability, and functional proteins (D'Amore *et al.*, 1990; Alexandre *et al.*, 1994; Costa *et al.*, 1997).

Regarding the high content of glucose in the growth medium, the results were very similar between the different species. All isolates were able to grow in the three concentrations tested (22, 27 and 32%), but some limitation of growth was

observed as being directly proportional to the increase of sugar concentration, even though some intraspecific variability was present. Tofalo and co-workers (2009) tested some strains of "*Candida zemplinina*" and all of them grew in media containing 20 and 40% glucose, and most of them grew even faster on 20% compared to the control with 2% glucose. Glucose at 60% inhibited most of the isolates and only one was able to grow. Pfliegler and colleagues (2014) described a high variability among strains of "*Candida zemplinina*" in increasing concentrations of glucose in the growth medium. The effect of the higher sugar concentrations in the growth could be attributed to a slower proliferation of yeast cells due to the osmotic pressure caused by high glucose content (Thomas and Ingledew, 1990; Zhao and Lin, 2003).

When looking at the tolerance to copper, all isolates of *S. bacillaris* and *Metschnikowia* spp. were tolerant to all concentrations tested (2.5, 5 and 10 mM), while divergent responses can be seen for *Lachancea* spp., a few isolates were fairly tolerant and others were completely inhibited. Barbosa and co-workers (2018) also reported tolerance to copper, but the maximum concentration used by them was 2 mM.

All isolates tested were dramatically sensitive to the SO_2 concentrations tested, even though a few isolates of *S. bacillaris* showed slight growth. Some diverging reports were found in literature. As regarding *Metschnikowia* spp., Barbosa *et al*. (2018) found all tested isolates of *M. pulcherrima* able to grow in 128 ppm of SO_2 and two of them were even resistant to 256 ppm. With isolates of *L. thermotolerans*, Banilas and colleagues (2016) found some of them with high growth in 100 ppm of SO_2 and some could not grow at all. *S. bacillaris* isolates were reported to be sensitive to concentrations of 100 and 150 ppm of SO_2, and only about 50% of them could grow in 50 ppm (Englezos *et al*., 2015). In a study conducted with strains from many different non-*Saccharomyces* species, all of them were described as resistant to 200 mg/L of potassium metabisulfite, including *M. pulcherrima*, *L. thermotolerans* and *S. bacillaris* (Aponte and Blaiotta, 2016).

The comparison of the growth in medium containing gluconic acid as the sole carbon source showed that isolates of *Metschnikowia* spp. have a much higher potential to metabolize this compound than the other species analyzed. Peinado and colleagues (2004, 2009) used isolates from the species *Schizosaccharomyces*

pombe to remove the gluconic acid from must obtained with rotten grapes and wine before the aging process.

The level of hydrogen sulfide production was not used for the construction of the dendrograms, but its results are shown right next to the heat maps of the stress tolerance assays. All isolates of *S. bacillaris* showed the same level of production, positioned in the medium to high spectrum of the scale. Among the isolates of *Metschnikowia* spp. and *Lachancea* spp., some variability was observed, ranging from low-medium to high production. Our results differ from those reported by Belda *et al*. (2016), who described a very low or even absent sulfite reductase activity in *Metschnikowia* spp. isolates, but agreed with them regarding the high variability among *L. thermotolerans* isolates. Aponte and Blaiotta (2016) concluded that isolates of *M. pulcherrima* were fair producers and of *S. bacillaris* were high producers, while again isolates of *L. thermotolerans* showed a high diversity among them. Most of the *L. thermotolerans* isolates assayed by Banilas and colleagues (2016) produced medium to high amounts of H_2S, even though also for them some variability among isolates was present. For *S. bacillaris*, the very low variability among the isolates was shown also by Englezos and co-workers (2015), where 4% of them produced low levels of H_2S and all the others produced a medium amount; and Pfliegler and colleagues (2014), who observed almost all 35 isolates tested producing high amounts of H_2S.

The most discriminant feature among the isolates of *Lachancea* spp. was the resistance to 10 mM of copper. It can be seen how two major clusters were formed, one of them containing the isolates sensitive to 10 mM of copper and another cluster with four isolates tolerant to all concentrations of this element. Interestingly, the isolates in the first cluster showed a general higher production of H_2S than those in the second cluster. The isolate FIANO43 was then differentiated from the others within the first cluster due to its higher tolerance to ethanol, especially at 12%. Comparing the three species of the genus *Lachancea* tested in the trials, *L. fermentati* LS16 and *L. kluyveri* FIANO22 had a slightly lower tolerance to the osmotic pressure caused by high sugar concentration and higher resistance to SO_2, compared to *L. thermotolerans*.

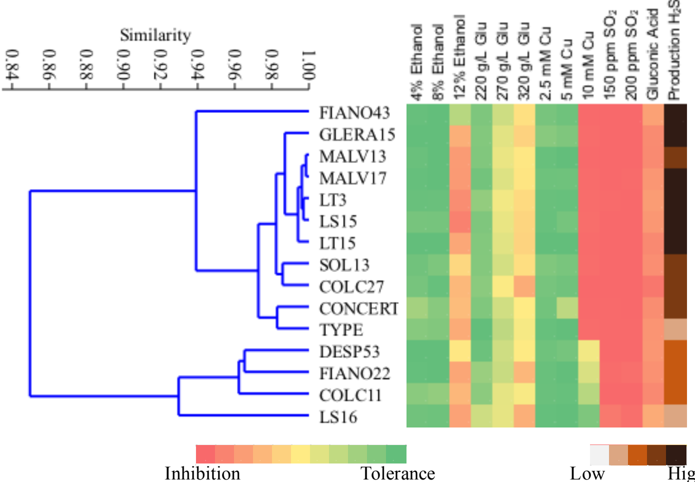

Figure 3.4. Physiological characterization of *Lachancea* spp. strains in stress tolerance assays and sulfite reductase activity.

The four isolates obtained from Malvasia Candia grapes were clustered together with a very high similarity, while in the genotypic characterization they were positioned in two different pairs (MALV13/MALV17; LT3/LT15) with around 80% similarity between them. The two isolates from Pinot Grigio were again distant from each other, as in the genotyping.

For the isolates of *S. bacillaris*, the feature that resulted in the higher differentiation was the tolerance to 12% ethanol, which resulted in the formation of two major clusters, one with low and the other with medium to high tolerance. The second most discriminant feature was the tolerance to the highest concentrations of sugar. The isolates CTP63 and CORV5 were separated from the others in their respective clusters due to the lower tolerance to copper.

The molecular characterization did not correspond with the physiological characterization, whereas the clusters formed were very different. The isolates from Emilia-Romagna, which were close to each other in the genotyping, were placed separately after the physiological characterization. On the other hand, some pairs of isolates coming from the same location and grape variety, such as ARMANI3/ARMANI4, GLERA8/GLERA12, CHIAR4/CHIAR7, which were distant from each other in the SAU-PCR analysis, were placed much closer after the stress tolerance assays.

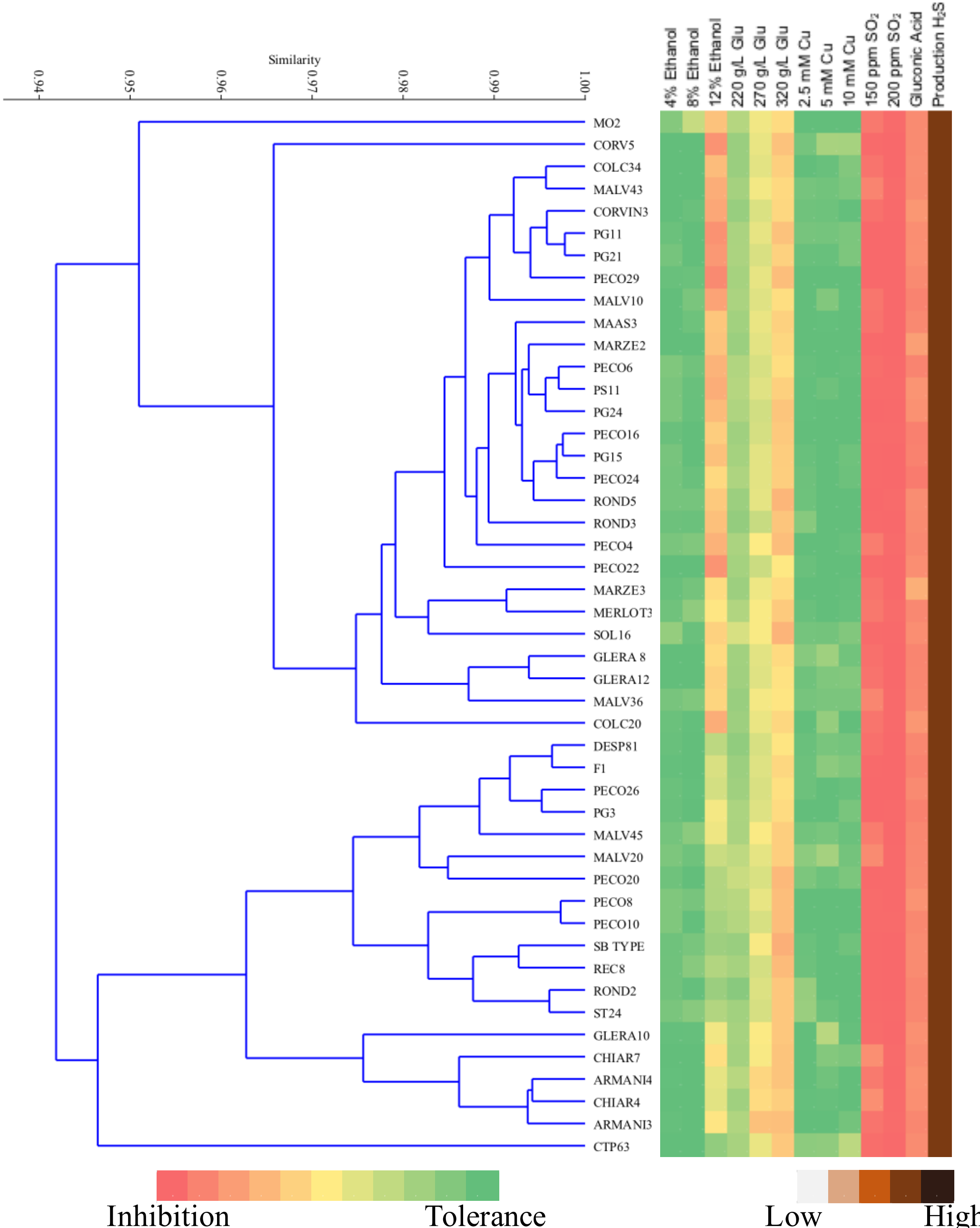

Figure 3.5. Physiological characterization of *Starmerella bacillaris* strains in stress tolerance assays and sulfite reductase activity.

Among the isolates of *Metschnikowia* spp., the ability to grow in the medium with gluconic acid was the most discriminant characteristic. One cluster was immediately separated from the others formed with the isolates with very low capacity to metabolize this acid. The isolates with lower growth in gluconate generally showed also lower tolerance to ethanol, sugar and copper, and the

highest production of H_2S. The second most discriminant feature was the tolerance to high sugar concentrations.

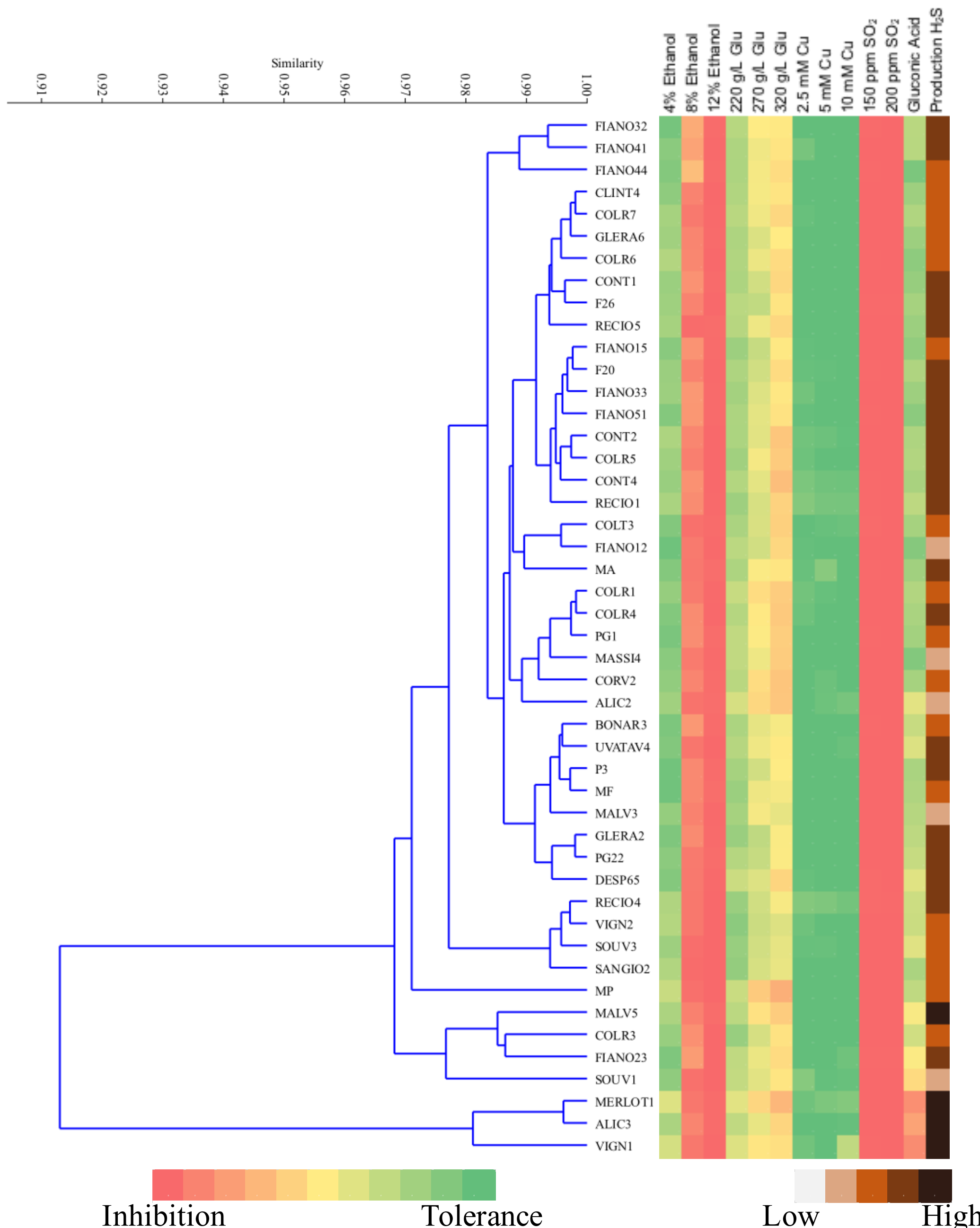

Figure 3.6. Physiological characterization of *Metschnikowia* spp. strains in stress tolerance assays and sulfite reductase activity.

The physiological characterization of *Metschnikowia* spp. isolates also did not reflect the genotyping. Most of the isolates grouped together after the RAPD- and Rep-PCR analysis did not show the same similarity on the stress tolerance assays.

The exceptions were the pairs CONT2/CONT4 and COLR1/COLR4, which had a high similarity in both analysis and moreover were isolated from the same type of grape must and location, most likely representing two isolates of the same strain.

As regarding the enzymatic activities, the isolates of *S. bacillaris* and *L. thermotolerans* did not show positive result for any of the tests. *L. fermentati* was positive for β-glucosidase and *L. kluyveri* had only pectinase activity. Some studies have already shown that most yeast species are unable to produce pectic enzymes, even though polygalacturonase activity has been described in a few wine isolates (Strauss *et al*., 2001; Merín *et al*., 2011; Belda *et al*., 2016). On the other hand, β-glucosidase activity is widespread in non-*Saccharomyces* yeasts according to several screenings (Padilla *et al*., 2016).

The isolates of *Metschnikowia* spp. presented some positive activities of esterase, protease and β-glucosidase, as shown in Table 3.1, organized according to the clusters formed through the stress tolerance assays. The test of glycosidase in medium containing rutin did not result positive for any of the isolates assayed.

The proteolytic activity in skim milk was present in almost all isolates, only about 10% of them did not show any activity, while the others ranged from low to high activity. The activity of β-glucosidase was also positive for most of the tested isolates, 85% of them. The seven isolates that could not grow and/or change the color of the medium containing arbutin were also negative for the esterase activity. Around 28% of the isolates gave a positive result in the medium containing Tween 80. Only the strain ALIC3 was negative for all enzymatic activities assayed in this study. On the other hand, the isolates BONARDA3, CLINT4, COLR7, COLT3, CONT2, CONT4, FIANO12, MERLOT1, P3, PG1, PG22 and RECIOTO1, gave positive results for all three tests shown in Table 3.1, which represents around one quarter of the *Metschnikowia* spp. isolates used.

The results reported by Barbosa and colleagues (2018) agreed with ours regarding the β-glucosidase activity of *Metschnikowia* spp. isolates, when almost all isolates showed positive activity, and they also found a high diversity in the activity of sulfite reductase. However, their reports on the protease activity was the opposite, whereas most of our isolates were positive and almost all of theirs were negative.

Table 3.1. Enzymatic activities of *Metschnikowia* spp. isolates. Esterase and β-Glucosidase were described as negative or positive, while Protease was evaluated with different intensities for the positive result.

Isolate	Esterase	β-Glucosidase	Protease
FIANO32	-	+	++
FIANO41	-	+	+++
FIANO44	-	+	++
CLINT4	+	+	++
COLR7	+	+	+++
GLERA6	-	+	+
COLR6	-	+	++
CONT1	-	+	+
F26	-	+	++
RECIOTO5	-	+	-
FIANO15	-	+	++
F20	-	-	++
FIANO33	-	-	++
FIANO51	-	-	++
CONT2	+	+	++
COLR5	-	-	+++
CONT4	+	+	+++
RECIOTO1	+	+	+++
COLT3	+	+	++
FIANO12	+	+	++
MA	-	+	+++
COLR1	-	+	+
COLR4	-	+	++
PG1	+	+	+
MASSI4	-	+	++
CORV2	-	+	+
ALIC2	-	-	++
BONARDA3	+	+	+
UVATAV4	-	+	+++
P3	+	+	+
MF	-	+	++
MALV3	-	+	++

Isolate	Esterase	β-Glucosidase	Protease
GLERA2	-	+	+
PG22	+	+	+
DESP65	-	+	+++
RECIOTO4	-	+	+++
VIGN2	-	+	-
SOUV3	-	+	+
SANGIO2	-	-	+++
MP	-	+	++
MALV5	-	+	+
COLR3	-	+	-
FIANO23	-	+	++
SOUV1	-	+	++
MERLOT1	+	+	+
ALIC3	-	-	-
VIGN1	+	+	-

For *S. bacillaris*, the work by Lemos Junior and colleagues (2016) reported only two strains as protease producers and all others were negative for the same enzymatic activities that we assayed as well. Englezos and co-workers (2015) were able to find 5% of the isolates being positive for β-glucosidase, 77% for protease and 11% for esterase, while pectinase and glycosidase activities corresponded with our findings and any isolate was positive.

In a very vast study with 770 yeast isolates belonging to 15 different species, many enzymatic activities were tested by Belda *et al*. (2016). Protease and β-glucosidase were the two most present enzymes, while polygalacturonase (pectinase) was very rare. The isolates of *L. thermotolerans* did not show any of those three activities, and most of *Metschnikowia* spp. had positive results for β-glucosidase and protease, in agreement with our findings, with the only exception that they found also the pectinolytic activity to be widespread among *Metschnikowia* spp. (Belda *et al*., 2016).

Another enzymatic characterization with different yeast species was carried out by Aponte and Blaiotta (2016). Regarding the same groups evaluated in the present study, only *M. pulcherrima* was able to produce β-glucosidase in medium

containing arbutin, and any isolate of *M. pulcherrima*, *L. thermotolerans* or *S. bacillaris* could give a positive result for esterase test in medium with Tween 80.

3.3.3. Single culture fermentation

The isolates from the species *L. thermotolerans*, *L. fermentati*, *L. kluyveri* and *S. bacillaris* were further characterized by their fermentation performance in natural grape must. A commercial strain of *S. cerevisiae* (EC 1118) was used as control. The most important oenological parameters calculated for each species are shown in Table 3.2, represented as the mean value of all isolates for the species *L. thermotolerans* and *S. bacillaris*.

After 10 days of incubation, none of the non-*Saccharomyces* isolates used in this fermentation trials was able to complete the alcoholic fermentation, as it can be seen by the high concentration of residual sugars and low production of ethanol, considering that the grape must initially contained around 210 g/L of sugars. On the other hand, the commercial strain of *S. cerevisiae* fermented almost to dryness and produced 12.21 %v/v ethanol. There was some variation among the isolates of *S. bacillaris* and *L. thermotolerans*, but in general *L. fermentati* and *L. thermotolerans* were best fermenters than *L. kluyveri* and *S. bacillaris*.

It is noteworthy to point out how all isolates of *Lachancea* spp. consumed more glucose than fructose, while all isolates of *S. bacillaris* consumed more fructose, confirming the fructophilic character of this species.

S. cerevisiae also consumed more malic acid than the non-*Saccharomyces* isolates. As regarding succinic acid, *S. bacillaris* produced much less than the other species, with *L. kluyveri* being the highest producer. At the same time, this species did not produce detectable amounts of lactic acid, a very well-known feature of *L. thermotolerans* and *L. fermentati*. The quantity produced by this last species was exceptionally high and for *L. thermotolerans* the metabolism of lactic acid was very strain-dependent. The concentrations varied from 0.26 to 10.54 g/L, and such huge diversity was further explored by different molecular and physiological tests in order to clarify the mechanisms of this very relevant metabolic trait, which are explained in detail in the next chapter.

The higher production of volatile acidity (g/L of acetic acid) by *S. cerevisiae* can be explained by the complete consumption of sugars. Nevertheless, even

though this character varied among the different isolates of *S. bacillaris* and *L. thermotolerans*, it can be seen how the first presented a mean value much higher than the second. Usually, values between 0.20 and 0.70 g/L are considered optimal, while above its threshold of 0.70-1.10 g/L the acetic acid can becomes unpleasant to the flavor (Lambrechts and Pretorius, 2000). *L. thermotolerans* and *S. bacillaris* are usually reported as low producers of volatile acidity, when compared to *S. cerevisiae* (Padilla *et al*., 2016).

The different values for the total acidity and the pH can be related to the diverging profiles on the production of lactic acid and acetic acid by each species.

The production of glycerol was also very strain-dependent for *L. thermotolerans* and *S. bacillaris*, although it is possible to highlight that most of the isolates of both species produced more than *L. kluyveri* and *L. fermentati* and some isolates of *S. bacillaris* resulted in more glycerol than *S. cerevisiae*, keeping in mind that this last was the only one to complete the fermentation of sugars. The higher production of glycerol could be considered positive, as it contributes to smoothness, sweetness and complexity in wines (Ciani and Maccarelli, 1998). In a study performed in 2016, Rossouw and Bauer also found high glycerol yields for isolates of *L. thermotolerans* and *S. bacillaris*.

Aponte and Blaiotta (2016) performed microvinification trials in Aglianico grape must with single cultures of different non-*Saccharomyces* yeasts, including *L. thermotolerans* and *S. bacillaris*. They showed a similar fermentation power, leaving almost the same quantity of residual sugars at the end, which was lower than the value found in the present study. Similarly to what we observed, *S. bacillaris* consumed much more fructose, while *L. thermotolerans* preferred glucose. *S. bacillaris* produced more glycerol while *L. thermotolerans* more succinic acid, moreover all values were higher than ours. They also found a significant diversity in the production of lactic acid by *L. thermotolerans*. The production of acetic acid was much lower for *L. thermotolerans* and similar to our findings, while *S. bacillaris* produced quantities slightly above the threshold described before.

Table 3.2. Chemical analysis of the wines obtained after single inoculation, represented as the mean values and standard deviation of all isolates belonging to each species. N/D = not determined.

Species (No of strains)	Residual Glucose (g/L)	Residual Fructose (g/L)	Citric Acid (g/L)	Malic Acid (g/L)	Succinic Acid (g/L)	Lactic Acid (g/L)	Glycerol (g/L)	Acetic Acid (g/L)	Ethanol (%v/v)	Total Acidity (g/L)	pH
L. fermentati (1)	51.04	66.20	0.21	3.24	0.38	13.03	3.63	0.20	5.33	15.39	2.39
L. kluyveri (1)	59.30	84.37	0.19	3.43	0.93	0	4.27	0.21	3.8	6.79	3.35
L. thermotolerans (13)	54.00 ± 8.23	65.98 ± 8.19	0.19 ± 0.016	3.17 ± 0.25	0.50 ± 0.10	3.68 ± 2.99	4.48 ± 0.51	0.13 ± 0.095	5.17 ± 0.93	9.11 ± 1.96	3.14 ± 0.23
S. bacillaris (47)	93.12 ± 6.18	54.72 ± 8.14	0.22 ± 0.024	3.08 ± 0.21	0.095 ± 0.043	N/D	5.46 ± 0.82	0.56 ± 0.10	3.86 ± 0.53	6.56 ± 0.28	3.56 ± 0.026
S. cerevisiae (1)	2.22	1.46	0.20	2.63	N/D	N/D	5.21	0.59	12.21	7.09	3.62

Microvinifications of pasteurized grape must were conducted by Banilas and co-workers (2016) with single cultures of *L. thermotolerans*. The fermentations were only stopped when the weight loss became constant, around 20 days. Some variability was observed among strains, but they were able to consume more sugars, produce more ethanol and more volatile acidity than our isolates. The production of lactic acid was highly variable among the strains, ranging from 1.0 to 16.6 g/L, consequently also the total acidity was variable.

In 2016, Lemos Junior and colleagues carried out single fermentations with *S. bacillaris* isolates in synthetic grape must and stopped the fermentation after 26 days, but they found similar results to ours: high residual sugar and limited ethanol production, preference for fructose consumption, high glycerol yield, but they found lower values for the volatile acidity. In 2015, Englezos and colleagues performed the fermentations in Barbera grape must during 14 days, confirming also the high fructophilic character and slower fermentation, compared to *S. cerevisiae*. The acetic acid and glycerol production were higher than those observed in the present study.

In order to compare and better visualize the differences among the isolates of *L. thermotolerans* and *S. bacillaris*, a Principal Component Analysis was carried out with the most important parameters for all isolates of each species. The results are shown in Figures 3.7 and 3.8.

The first two components were able to explain 72% of the variability (Figure 3.7). On the top-left quarter, the isolates showed higher fermentation performance, but produced excessive amounts of lactic acid and volatile acidity. On the top-right side the isolates had a good ability to increase the glycerol content and reduce the volatile acidity, however had the slowest growth and probably faced more trouble adapting to the grape must. Other isolates were placed more intermediary and could be interesting in the balance between having a good growth rate and producing adequate quantities of the important metabolites. As it could be expected, a higher production of lactic acid was associated with the higher reduction of pH. The production of succinic acid and glycerol was positively related to a higher consumption of fructose instead of glucose, even though all isolates preferred glucose.

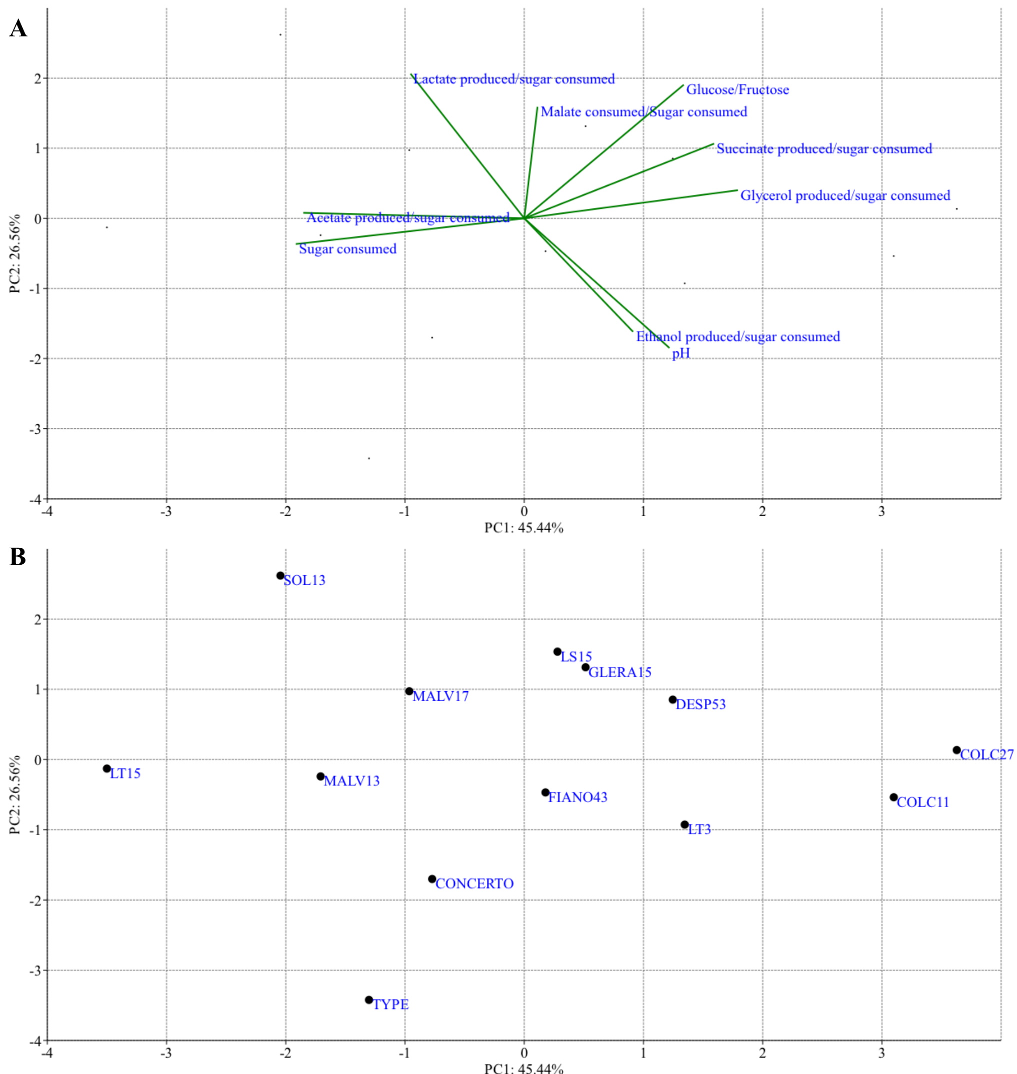

Figure 3.7. [A] Loading plot and [B] Score plot of the Principal Component Analysis of some important oenological parameters obtained after the single inoculation of Trebbiano grape must with isolates of *Lachancea* spp.

For the isolates of *S. bacillaris*, the first two components of the PCA explained 64% of the variability (Figure 3.8). The isolates placed on the left side had a faster fermentation performance and showed a higher fructophilic character, even though all isolates consumed more fructose than glucose. On the right side there were the isolates that presented higher yields in the production of glycerol and acetate. Most of the isolates were distributed close to the center of the PCA, showing intermediary features.

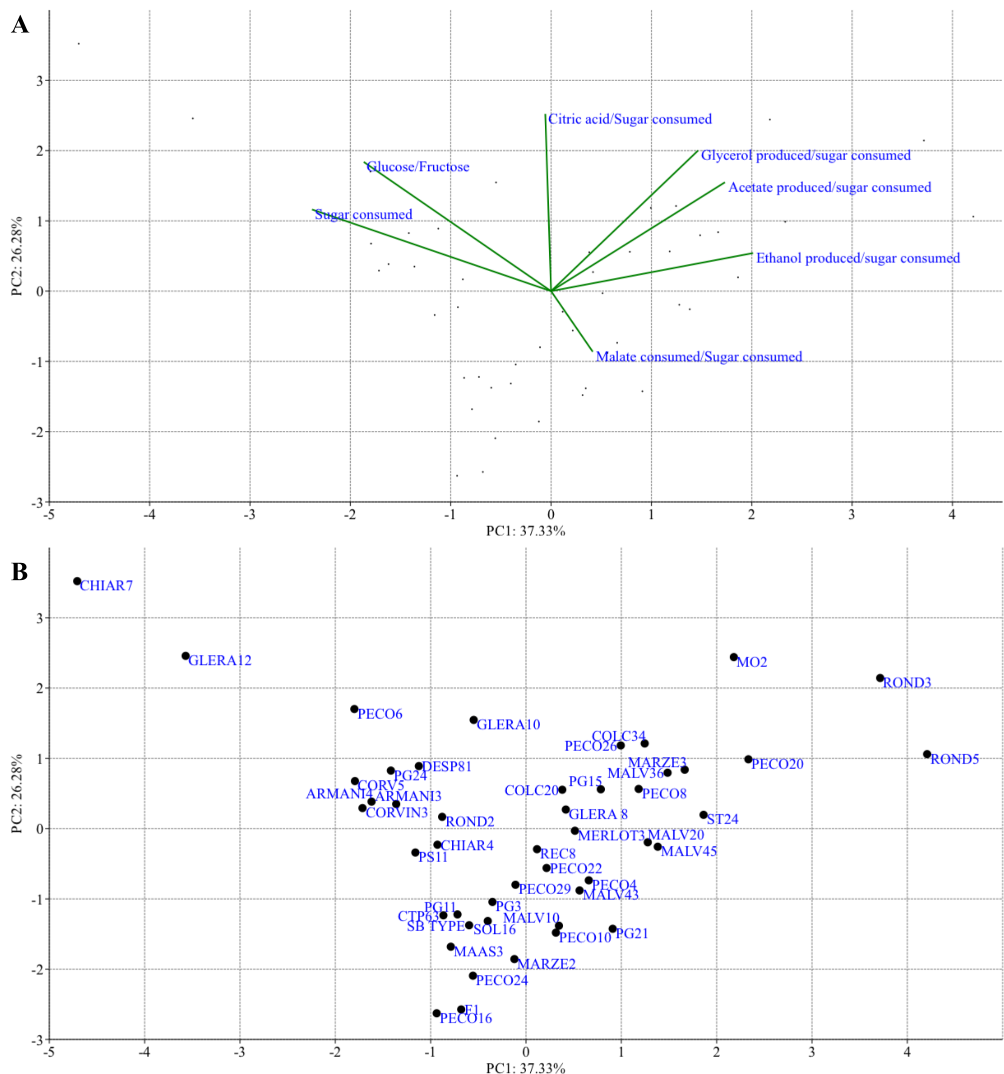

Figure 3.8. [A] Loading plot and [B] Score plot of the Principal Component Analysis of some important oenological parameters obtained after the single inoculation of Trebbiano grape must with isolates of *Starmerella bacillaris*.

3.4. CONCLUSIONS

The fingerprinting analysis have shown the presence of different strains among the isolates of the same species, which formed distinct clusters based in the similarity. No clear relations were observed between the geographical origin, variety of grape and the genotypic profiles. SAU-PCR was shown to be a useful tool for the genotyping of *S. bacillaris* and *L. thermotolerans* isolates. For *Metschnikowia* spp., the use of primer $(GTG)_5$ gave a higher discrimination power

than M13, and the combined analysis was interesting for the genotyping of this group.

This study has an in-depth and vast phenotypic characterization of around one hundred isolates belonging to three different genera, by means of stress tolerance assays, enzymatic activities trials and microvinifications, showing the potential of the non-*Saccharomyces* yeast species chosen to have a relevant impact in the wine quality.

The tests with five different components that are usually present on grapes and/or grape musts allowed the evaluation of the isolates' potential to survive to the stress caused by those elements, in order to establish the capacity of the yeasts to participate more actively and lastingly in the fermentation and therefore give a more relevant contribution to the wine quality. Isolates of *S. bacillaris* and *Lachancea* spp. were tolerant to all concentrations of ethanol tested, while *Metschnikowia* spp. was inhibited at concentrations higher than 8%. All isolates were tolerant to the high concentrations of sugar, but the increasing from 220 to 320 g/L caused an extension of the lag phase and slowing down of growth. An addition of 100 ppm of SO_2 was sufficient to inhibit all isolates. They were also resistant to copper at 10 mM, with the exception of some *L. thermotolerans* isolates which survived only until 5 mM. *Lachancea* spp. and *S. bacillaris* could barely grow on medium containing gluconic acid as carbon source, most of the *Metschnikowia* spp. showed a great potential to consume this substance usually present on grapes damaged by gray rot.

The screening of six enzymatic activities of oenological interest was performed to have an enzymatic profile of the studied species in order to evaluate the possible impact that the isolates could have in the wine aroma and other relevant technological steps of the winemaking process. The isolates of *S. bacillaris* and *L. thermotolerans* used in this study did not possess any of the tested enzymatic activities. On the other hand, *Metschnikowia* spp. showed a high potential for the modulation of wine aroma compounds by the presence of β-glucosidase, protease and esterase activity in many different isolates. The production of H_2S was highly variable among isolates of *Lachancea* spp. and *Metschnikowia* spp., while all isolates of *S. bacillaris* showed a medium production.

Microvinification trials in natural grape must with isolates of *S. bacillaris* and *Lachancea* spp. confirmed some interesting traits already reported before, as the lowering of ethanol and acetic acid when compared to *S. cerevisiae* and high production of glycerol. Particularly, the ability of the *Lachancea* spp. isolates to produce L(+)-lactic acid will gain further attention in the next chapter.

The genotypic and phenotypic characterization showed the presence of variability among the tested isolates, both inter- and intraspecies, and, moreover, did not give corresponding clusters. Some strains with similar fingerprinting showed different physiological responses and others very distant in the PCR-based dendrograms were placed very close when considering the phenotypic traits. Nevertheless, some isolates were similar in all analysis. It is very difficult to draw some conclusions about the factors influencing the variability among the different isolates, as they could be related with selective pressure caused by geographical influence, grape variety, specific health and ripeness conditions of the grapes during the sampling, human interventions. The same factors that were described in the previous chapter as having an impact in the different yeast population found in the diverse samples could also explain how isolates from the same species coming from different samples are able to have divergent phenotypic profiles.

This variability observed was used for the selection of a few isolates from each species that show different and interesting properties, in order to be tested alongside with *S. cerevisiae* in sequential inoculation, which results will be shown in Chapter 6.

3.5. REFERENCES

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Chapter 4

Antagonistic effect of alternative yeast strains against *Botrytis cinerea* for integrated vitivinicultural biocontrol strategies

CHAPTER 4. ANTAGONISTIC EFFECT OF ALTERNATIVE YEAST STRAINS AGAINST *Botrytis cinerea* FOR INTEGRATED VITIVINICULTURAL BIOCONTROL STRATEGIES

4.1. INTRODUCTION

Botrytis cinerea is an ascomycetous fungus that has adapted to be a parasite and is able to infect around 200 different dicotyledonous plants, being also capable of having a saprophyte life. This pathogen is very difficult to control, especially when the favorable environmental conditions are present, and it is considered one of the most relevant threats for the grapevine in temperate climate, usually known as gray rot (Williamson *et al*., 2007; Ferrari *et al.*, 2011).

The attack of *B. cinerea* on the vineyard may cause important quantitative and qualitative losses, especially for the grapes destined to winemaking. It could infect different parts of the plant, but the damage is usually more evident on the berries. They would initially present some brown spots in the black grapes or grey-yellow in the white grapes. Then, in case of substantial rainfall, the berries will become rotten and large portions of the bunch will be covered by the gray rot, which is the set of conidia and conidiophores (Vicenzini *et al*., 2009; Ferrari *et al.*, 2011). Considering the table grapes, this fungus also represents a problem during cold storage and shipment, since it can grow effectively at very low temperatures (Droby and Lichter, 2004). Indeed, *B. cinerea* is the most common postharvest pathogen of grapes in most regions of the world (Qin *et al*., 2010; Nally *et al*., 2012).

Considering the high risk of infection and the massive negative impact posed by *B. cinerea*, it is of ultimate importance to take some actions to avoid this disease, which are usually aimed to prevent the pathogen to start developing in the vineyard. Since the gray rot preferentially develops in habitats with high humidity, low temperature and limited light, the vine training system used could help to favor the aeration and light incidence and thus counteract the formation of the microclimate encouraging for the mold. The choice of the rootstock and grape variety have an influence in the degree of compaction within the bunches, which

could be a factor for the mold development (Williamson *et al.*, 2007). The green pruning technique is also used with the aim to give more exposition and aeration of the bunches (Gubler *et al.*, 1987). It is also fundamental to protect the vineyard from other grape diseases, like oidium and moths, and environmental hazards, like hailstorms, because they may cause damages to the integrity of the grapes and thus facilitate the infection with *B. cinerea* (Vicenzini *et al.*, 2009).

When the vines are infected by gray rot, the most common practice to save the grapes from the damage is the use of chemical agents. However, these products have been used indiscriminately, sometimes even when the disease is not yet present, causing environmental pollution over the time and possible harming the human health. The continuous use of those chemical fungicides could be also responsible for triggering resistance in the pathogens and thus the efficacy would be lost. The high costs accounted for the synthesis of new chemicals are also considered a disadvantage of their use (Tripathi and Dubey, 2004; Panebianco *et al.*, 2015; Parafati *et al*, 2015; Vitale *et al.*, 2016).

For those concerns, new methods have been developed for the protection from the pathogens, which should be sustainable for the environment, and also from the economic and scientific point of view. These proposed innovations have to assure the safety and promote an equilibrated integration of modern biotechnologies with traditional agri-food system (Capozzi and Spano, 2011). One of these alternative approaches is the use of other fungi and yeast species that have inhibitory activity against the plant pathogens. Some of these so-called biological control agents (BCA) already available in the market anti-*B. cinerea* include filamentous fungi (*Trichoderma* spp., *Ulocladium* and *Gliocladium*), bacteria (*Bacillus* and *Pseudomonas*) and yeasts (*Pichia* and *Candida*) (Jacometti *et al.*, 2010).

A series of features have been listed for the ideal application of a BCA: genetic stability, efficacy at low concentrations, reduced nutrient requirements, tolerance to adverse environmental factors, activity against a broad spectrum of pathogens, prolonged shelf-life, simplicity of use, compatibility with chemical and physical treatments, harmlessness for human health, innoxious for the plants and fruits on which would be applied (Droby *et al.*, 2009; Sharma *et al.*, 2009; Abano and Sam-Amoah, 2012).

The main mechanism through which a BCA is able to suppress a risk pathogen is the competition for nutrients and space (Zhang *et al.*, 2011). Other

proposed actions include also the parasitism, the release of secondary metabolites with inhibitory effect, the stimulation of defensive responses in the affected plant through elicitors secreted by the BCA, the production of ROS by the host and the antagonist. Some drawbacks of the biological control are the sensitivity to certain environmental conditions and the restricted inhibitory activity, usually specific (Janisiewicz and Korsten, 2002; Spadaro and Gullino, 2004; Spadaro and Droby, 2016). The antifungal activity varies according to the yeast species, substrate composition, culture mode, yeast growth stage, temperature and other environmental factors (Martins *et al.*, 2010).

Yeasts are the microorganisms most commonly used as BCA due to some interesting characteristics, such as their natural occurrence in fruits and vegetables, the ability to colonize large surface for long times, the absence of allergenic spores or mycotoxins, simple nutritional requirements, fast and cost-effective biomass production in bioreactors (El-Tarabily and Sivasithmparam, 2006; Chanchaichaovivat *et al.*, 2007; Bai *et al.*, 2008). Some examples of yeast strains that were registered for the use as BCA include *Candida oleophila* (Aspire™; Nexy™), *Candida sake* (Candifruit™), *Cryptococcus albidus* (Yieldplus™), *Metschnikowia fructicola* (Shemer™) and *Aureobasidium pullulans* (BoniProtect™), although certain products were already discontinued in the market (Spadaro and Droby, 2016; Droby *et al.*, 2016; Pretscher *et al.*, 2018).

Another important characteristic to consider is if the yeasts selected are generally regarded as safe (GRAS) (Banerjee, 2009). Even if the yeasts usually have this status, in recent years some cases of clinical infections caused by them have been reported, especially in immunocompromised patients (Okawa and Yamada, 2002; de Llanos *et al.*, 2006). For this reason, it is of major importance to evaluate the possible pathogenicity towards humans of BCA candidates. Some features frequently associated with pathogenicity are the growth at high temperatures, the adhesion to host cells, the secretion of hydrolytic enzymes that facilitate the invasion of host tissues (de Llanos *et al.*, 2006; Nally *et al.*, 2012).

Although many yeasts had already been recognized for the antifungal activity during the last 30 years of biocontrol research, the selection of new strains for the biocontrol on grapes is still challenging and studies concerning the interaction between species remain of great interest, since reliable commercial options for gray rot control are still lacking and important research effort is needed to develop

BCAs adapted to vineyard environment, effective in field conditions and with lower variability in disease control (Calvo-Garrido *et al*., 2018).

The microbial population dynamics in wine is very complex, in both vineyard and in the cellar, and so it is of major importance to establish if the biocontrol yeasts would remain in the grape musts and possibly actively participate during the fermentation process. The selection of new BCA should then consider the possible impacts that the strain could have in the natural or inoculated microbiota responsible for the fermentation and in the properties of the wine (Lemos Junior *et al*., 2016).

The microbiomes of many terrestrial environments represent a collection of a tremendous underexplored diversity, which remarkable scientific and industrial potential are still largely uncharacterized. The vineyard environment has received a growing attention in the past decades, and the hundreds of different bacteria and yeast species and strains living there could offer a world of biotechnological opportunities. There are various indigenous yeast genera living on the grape surface, such as *Candida*, *Hanseniaspora*, *Metschnikowia*, *Pichia*, *Torulaspora*, but the participation of this group known as "non-*Saccharomyces*" in the wine fermentation has been limited due to previous belief on their liability for spoilage and degradation of the wine quality (Rossouw and Bauer, 2016).

Nowadays, their role has been already completely reevaluated and a few strains are available in the market with the aim to improve the wine quality in many different aspects (Petruzzi *et al*., 2017; Supplementary Table S.1). Besides the many active extracellular molecules that have been highlighted due to the beneficial effect in wine quality and stability, some non-*Saccharomyces* can also produce metabolites active against wild spoilage organisms (Comitini *et al*., 2017).

Nevertheless, the potential use of non-*Saccharomyces* as BCA is still largely uncovered. Additionally, for the best of our knowledge, there are no data available on literature about the impact of non-*Saccharomyces* BCA in the later stages of winemaking after grape harvest, even if they could presumably persist on the grape surface at high concentrations after repeated applications. Moreover, there is only one reported study about the possibility of using non-*Saccharomyces* yeasts in an integrated approach as BCA and starter culture, performed with strains from the species *Starmerella bacillaris* (Lemos Junior *et al*., 2016).

S. bacillaris (synonym *Candida zemplinina*) is an important species of non-*Saccharomyces* yeast that stands out from the group due to many interesting oenological properties, such as the fructophilic character, the high tolerance to ethanol, high glycerol production, increase in aromatic complexity (Tofalo *et al*., 2016; Englezos *et al*., 2018). Another interesting species is *Lachancea thermotolerans*, which has been mainly investigated due to its particular ability to produce large quantities of lactic acid. Besides enhancing wine acidity, this yeast was also described as producing low levels of volatile acidity and ethanol, high levels of glycerol and beneficial aromatic compounds (Benito *et al*., 2016; Whitener *et al*., 2016). These species, as the other non-*Saccharomyces*, are not able to ferment the grape must to dryness, and for this reason they are coupled with *S. cerevisiae* in order to have a complete process resulting in increased quality, stability and complexity of the wine (Petruzzi *et al*., 2017).

The main goal of this study was to evaluate the biodiversity of strains from two oenological relevant species, *L. thermotolerans* and *S. bacillaris*, isolated from diverse grape varieties across many different Italian regions, in the potential use as BCA against grapevine pathogens, specifically the responsible for gray rot, *B. cinerea*. The ideal candidate should possess effective antagonism against the mold and, moreover, do not show possible harmful effects for the human health or for the outcome of the fermentation process, with the final aim of improving the wine quality. Those strains were extensively characterized for genotypic and phenotypic features and some of them were also tested in mixed inoculation with *S. cerevisiae*, as shown in different chapters of the present thesis.

4.2. MATERIAL AND METHODS

4.2.1. Yeast and mold strains and growth conditions

All isolates of *L. thermotolerans* (12) and *S. bacillaris* (46) listed in Supplementary Table S.2 were used for the pathogenicity tests, plus the type strains *L. thermotolerans* DBVPG 6232^T, *S. bacillaris* NCAIM Y016667^T, one commercial starter of *L. thermotolerans* (Viniflora® Concerto™) and one of *S. cerevisiae* (EC 1118). For *in vitro* antagonism trials, 16 isolates of *S. bacillaris* and the type strain of the species were used, alongside with seven isolates of *L.*

thermotolerans and the commercial strain, selected based on the characterization of the previous chapter and the pathogenicity tests. The list was shortened again for the *in vivo* antagonism assays, when two isolates for each species and the starter Viniflora® Concerto™ were used (Table 4.1). Two different strains of *B. cinerea* were chosen for the antagonism tests, the strain BM0510 from the VTT Culture Collection and one isolate (TOB62) from Nosiola withered grapes belonging to the collection of the Laboratory of Food Microbiology.

Table 4.1. List of the isolates used for the antagonism assays versus *Botrytis cinerea*. Bold names represent the isolates used for both *in vitro* and *in vivo* assays.

L. thermotolerans	***S. bacillaris***
COLC11, **COLC27**, DESP53, MALV17, LT15, LS15, **SOL13**, **Concerto™**	ARMANI3, **CHIAR4**, COLC20, COLC34, CORV5, DESP81, GLERA10, GLERA12, MALV45, **PECO4**, PECO6, PECO26, PECO29, PG15, RONDINELLA 3, SOL16, NCAIM Y016667^T

The activation of the yeast isolates followed the same procedures for the pathogenicity and antagonism tests. Starting from the frozen cultures kept at -80 ºC, the isolates were reactivated on WL (Sigma-Aldrich) agar plates incubated at 27 ºC for 72 hours. One single colony was then inoculated in YPD broth (yeast extract, 1.0%; bacteriological peptone, 2.0%; glucose, 2.0%; Sigma-Aldrich) and incubated under static conditions at 27 ºC for 48 hours. A fresh YPD-containing tube was then inoculated at 1% with the previous grown culture and put in agitation overnight at 27 ºC to reach the early stationary phase. The culture was then centrifuged at 3,000×*g* for 5 minutes, washed twice and resuspended in physiological solution 0.9% (w/v) NaCl (Sigma-Aldrich). The inoculum was standardized for all yeast cultures with an OD_{600} of 1.6, corresponding approximately to 1×10^7 cells/mL. Aliquots of the cellular suspension were used for the spot inoculation in the pathogenicity tests and the streak or spread inoculation for the tests of inhibition *in vitro*. A concentrated suspension at 1×10^8 cells/mL was prepared for the injection during the *in vivo* assays. The activation steps were repeated before each different trial.

The strains of *B. cinerea* were grown on PDA plates (potato extract, 0.4%; glucose, 2.0%; agar, 1.5%; Sigma-Aldrich) for 5 days at 27 ºC, and the mycelium

was used for the *in vitro* assays with contemporary growth. For the *in vitro* tests in opposite plates and the *in vivo* assays, a conidial suspension was used. It was prepared by the incubation of *B. cinerea* on PDA plates at room temperature for 15 days, exposed to sun light. A solution of 0.05%v/v Tween 80 (Sigma-Aldrich) was then used to recover the conidia from the plates, centrifuged at 14,000 rpm for 5 minutes and the pellet was resuspended in the same solution. The conidia were microscopically counted in order to have a suspension of 1×10^6 conidia/mL.

4.2.2. Tests of pathogenicity

All tests were carried out with spot inoculation on Petri dishes filled with the specific media. From the cell suspension prepared as described in section 4.2.1., a droplet of 10 μL was deposited on the agar surface and dried under biosafety cabinet. Ten spots were inoculated on each plate and all isolates were inoculated in quadruplicate, with the commercial strain *S. cerevisiae* EC 1118 as a control.

The plates were incubated at 27 ºC or 37 ºC for a specific time according to each test. The results were verified by changes in the aspect of the colony and/or the surrounding medium.

4.2.2.1. Growth at 37 ºC

The ability of the yeast isolates to grow at the human body temperature (37±4 ºC) was evaluated on YPD agar plates (yeast extract, 1.0%; bacteriological peptone, 2.0%; glucose, 2.0%; agar, 1.5%; Sigma-Aldrich), as described by de Llanos *et al.* (2006). The spot inoculated plates were incubated at 37 ºC for 3 days. The growth of the colonies was compared with a control incubated at 27 ºC.

4.2.2.2. Invasive growth

The same plates of YPD agar incubated at 27 ºC used as control for the growth at 37 ºC were then utilized for the verification of the invasive growth. They were incubated for additional 10 days at room temperature, after what the surface of the medium was washed with deionized water and a spatula was employed to rinse

off the colonies. This procedure allowed the visualization of the isolates' ability to penetrate and grow below the surface of the agar medium (de Llanos *et al*., 2006).

4.2.2.3. Formation of pseudohyphae

The medium SLAD (Synthetic Low Ammonium Dextrose) was applied for the assessment of pseudohyphae formation by the yeast isolates. It was prepared with 6.61 mg/L of $(NH_4)_2SO_4$ (Sigma-Aldrich), 6.7 g/L of Yeast Nitrogen Base (YNB) without amino acids (Sigma-Aldrich), 20 g/L of glucose and 20 g/L of agar. YNB was dissolved in sterile water and mixed with the other components after these were autoclaved. Following the spot inoculation, the plates were incubated for 10 days at 27 ºC. The results were reported as negative (-) when there were no visible pseudohyphae around the colony and the margins were plain, while irregular borders without pseudohyphae were accounted as +/- result and the clear presence of pseudohyphae was represented as positive (+) (de Llanos *et al*., 2006).

4.2.2.4. Proteolytic activity

The proteolytic activity was evaluated in medium containing 20 g/L of malt extract (Sigma-Aldrich), 0.2 g/L of $MgSO_4$ (Sigma-Aldrich), 2.5 g/L of K_2HPO_4 (Sigma-Aldrich), 2.5 g/L of Bovine Serum Albumin (BSA; Sigma-Aldrich), 5 g/L of NaCl, 1 g/L of yeast extract, 20 g/L of glucose and 20 g/L of agar. Malt extract and agar were sterilized in autoclave, while the other components were dissolved in sterile water, filtered and mixed together with the first two after the autoclave. The pH was adjusted to 5.0 with HCl (Sigma-Aldrich). The plates incubated at 27 ºC were observed after 10 days and a positive result would be indicated by a clear halo around the colony (de Llanos *et al*., 2006).

4.2.3. Tests of inhibition *in vitro* versus *Botrytis cinerea*

4.2.3.1. Contemporary growth on agar plates

For the inhibition assays on agar plates, the PDA medium was used, following the methods described elsewhere (Parafati *et al*., 2015; Lemos Junior *et al*., 2016).

The first approach was the contemporary inoculation of both yeast and mold on the same plate. From each of the yeast cultures of *S. bacillaris* and *L. thermotolerans* listed in Table 4.1, and prepared as stated in section 4.2.1., an aliquot of 10 µL was taken and orthogonally streaked following the diameter of the Petri dish. Subsequently, two fragments with 6 mm diameter were excised from the edges of a mycelial growth of *B. cinerea* and placed on the same plate in opposite positions, with a distance of 3 cm from the streaked yeast culture (6 cm distance between the two fragments) and 1.5 cm from the border of the plate. The plates were closed surrounded with Parafilm® to prevent air leakage and incubated at 27 ºC for 5 days. All yeast isolates were tested in duplicate for each of the two strains of *B. cinerea* (BM0510 and TOB62). Control plates were prepared with only the inoculation of each *B. cinerea*, without the yeast streak.

The inhibition of the mold growth caused by the presence of the yeast was compared with the control by using the following expression:

$$\%I = [(C - T) / C] \times 100$$

Where:

%I = percentage of inhibition of *B. cinerea* growth;

C = radial growth of *B. cinerea* in the control inoculation;

T = radial growth of *B. cinerea* in the presence of the yeast.

The mean and standard deviations of the four measurements for each yeast isolate with each mold strain were calculated, and the comparison between the different yeast isolates was performed based on One-way ANOVA (ANalysis Of VAriance) with post-hoc Tukey's HSD (Honestly Significant Difference) test ($p<0.05$), using software for statistical analysis PAST (Hammer *et al.*, 2001).

4.2.3.2. Separate growth on opposite agar plates

This test was also carried out using PDA medium and following protocol described in literature (Parafati *et al.*, 2015; Lemos Junior *et al.*, 2016). Two plates of PDA agar were necessary for each couple of non-*Saccharomyces*/*B. cinerea* tested. An aliquot of 100 µL of the yeast culture was spread plated on one

of the Petri dishes containing PDA, while on the second plate a spot of 10 μL from the conidial suspension of the mold was inoculated exactly in the center. The two plates were then positioned facing each other, the edges were docked and surrounded with Parafilm®. All pairs of plates were incubated at 27 ºC for 5 days. The control test was made with the plates with *B. cinerea* facing unseeded PDA.

The percentage of inhibition and the statistical analysis were calculated with the same procedure as described above.

4.2.4. Tests of inhibition *in vivo* versus *Botrytis cinerea*

For the *in vivo* assays, white table grapes were used. The strains of *S. bacillaris* PECO4 and CHIAR4, *L. thermotolerans* COLC27, SOL13 and Concerto™, and the two strains of *B. cinerea* BM0510 and TOB62 were used. Healthy undamaged grapes were cleaned and disinfected prior to the analysis. An artificial wound was made at the equator of each berry with a sterile needle and 10 μL of the conidial suspension (1×10^6 conidia/mL) were inoculated. After 2 hours air-drying, a new inoculation was performed in the same place with 10 μL of the concentrated yeast culture (1×10^8 cells/mL). The control was prepared by the inoculation of 10 μL physiological solution (0.09% NaCl) instead of the yeast. For each pair of non-*Saccharomyces*/*B. cinerea*, a group of eight homogenous berries randomly selected was used. They were incubated inside a plastic box for 5 days at 27 ºC, with a wet paper towel folded inside the box to maintain a high humidity. The effectiveness of the treatments were evaluated with the following expression (Parafati *et al*., 2015; Lemos Junior *et al*., 2016):

$$(DRI) = (C - T/C) \times 100$$

Where:

DRI = disease reduction incidence;

C = radial growth of the infection in the control;

T= radial growth of the infection in the presence of yeast.

The mean and standard deviations were calculated for the eight grapes of each treatment and the results analyzed as described above.

4.3. RESULTS AND DISCUSSION

4.3.1. Safety assessment of virulence factors

Before selecting the yeast strains with a potential utilization as BCA, it is important to verify some possible virulence factors, as a guarantee that they would not represent a risk for human health, especially the people responsible for applying the product in the field. The results for the four virulence factors tested are shown in Table 4.2.

Table 4.2. Pathogenicity tests with the yeast isolates.

Isolate	Phenotypic characteristic			
	37 ºC	SLAD	BSA	Invasive
Saccharomyces cerevisiae				
EC 1118	+	+/-	-	+
Lachancea thermotolerans				
COLC11	-	+	-	+
COLC27	-	+	-	+
DESP53	-	+	-	+
FIANO43	-	+/-	-	+
FIANO63	-	+/-	-	+
GLERA15	-	+	-	+
LS15	-	+/-	-	+
LT15	-	+/-	-	+
LT3	-	+/-	-	+
MALV13	-	+/-	-	+
MALV17	-	+	-	+
SOL13	-	+	-	+
Concerto™	-	+	-	+
DBVPG 6232^{T}	-	+/-	-	+/-
Starmerella bacillaris				
ARMANI3	-	-	-	-
ARMANI4	-	-	-	-
CHIAR4	-	-	-	-
CHIAR7	-	-	-	-
COLC20	-	-	-	-

Isolate	Phenotypic characteristic			
	37 ºC	SLAD	BSA	Invasive
COLC34	-	-	-	-
CORV5	-	-	-	-
CORVINONE3	-	-	-	-
CTP63	-	-	-	-
DESP81	-	-	-	-
F1	-	-	-	+/-
GLERA8	-	-	-	-
GLERA10	-	-	-	+/-
GLERA12	-	-	-	-
MAAS3	-	-	-	+/-
MALV10	-	-	-	+/-
MALV20	-	-	-	-
MALV36	-	-	-	-
MALV43	-	-	-	-
MALV45	-	-	-	-
MARZEMINO2	-	-	-	+/-
MARZEMINO3	-	-	-	+/-
MERLOT3	-	-	-	-
MO2	-	-	-	+/-
PECO4	-	-	-	-
PECO6	-	-	-	+/-
PECO8	-	-	-	-
PECO10	-	-	-	-
PECO16	-	-	-	-
PECO20	-	-	-	-
PECO22	-	-	-	-
PECO24	-	-	-	+/-
PECO26	-	-	-	-
PECO29	-	-	-	-
PINOTG3	-	-	-	-
PINOTG11	-	-	-	+/-
PINOTG15	-	-	-	+/-
PINOTG21	-	-	-	-
PINOTG24	-	-	-	+/-
PS11	-	-	-	+/-

Isolate	Phenotypic characteristic			
	37 ºC	SLAD	BSA	Invasive
RECIOTO8	-	-	-	+/-
RONDINELLA2	-	-	-	-
RONDINELLA3	-	-	-	-
RONDINELLA5	-	-	-	+/-
SOL16	-	-	-	-
ST24	-	-	-	-
NCAIM Y016667^T	-	-	-	-

37 ºC = growth after incubation at 37 ºC; SLAD = formation of pseudohyphae; BSA = proteolytic activity; Invasive = penetration of the YPD medium.

The first tests regarded the ability of the strains to survive under the temperature normally found inside the human body, around 37 ºC, and to penetrate in the growth medium. Any of the strains of *L. thermotolerans* or *S: bacillaris* was able to grow after the incubation in YPD at 37 ºC. Only the commercial strain of *S. cerevisiae* showed simultaneous growth in the control at 27 ºC and at 37 ºC.

The invasive growth assay showed some species and strain variation. All isolates of *L. thermotolerans*, but the type strain, were able to completely infiltrate within the YPD medium, although there was a partial invasive growth recorded for DBVPG 6232^T. For *S. bacillaris*, most of the strains were only able to develop on the surface of the medium, even though 15 out of 47 tested isolates had a partial ability to penetrate the agar. EC 1118 gave a positive result.

The test on SLAD agar offered the yeast isolates a minimal medium, without amino acids and with a very low concentration of ammonium sulfate. The lack of nitrogen caused a stress in the yeasts, triggering in some isolates the ability to form pseudohyphae, in order to help in the search for nutrients. This feature could be associated with the penetration of the human epithelium and damage of the endothelium, with consequent diffusion of the pathogen to the blood stream (Kumamoto and Vinces, 2005). The switching from normal growth to hyphal formation has been already associated with pathogenesis and virulence in *Candida albicans* and clinical isolates of *S. cerevisiae* (Gognies and Belarbi, 2002). Any of the isolates of *S. bacillaris* produced pseudohyphae and all of them grew with plain borders. On the other hand, for the isolates of *L. thermotolerans*,

half of them showed colonies with irregular margins and the other half clearly were able to produce pseudohyphae to chase for nutrients in the SLAD agar medium. The commercial *S. cerevisiae* had irregular borders, but without formation of pseudohyphae.

The proteolytic activity, examined in BSA agar, is the enzymatic hydrolysis of the peptide bond in the proteins. These hydrolytic enzymes are responsible for the cellular lysis and the destruction of molecules involved in the immunity response (Kumamoto and Vinces, 2005). Any of the isolates of the three species tested gave a positive result for the proteolytic activity in BSA. These results are in accordance with the assay reported in Chapter 3, when all of those isolates also showed a negative result for the protease activity in YPD containing skim milk.

As a comparison, Ponsone and co-workers (2016) tested the same phenotypic characteristics related to human pathogenicity with two strains of *L. thermotolerans*. Both strains gave positive results only for the growth at 37 ºC, but did not possess any of the other virulence factors.

Previously, Nally and colleagues (2012) studied the same traits in 15 strains of *S. cerevisiae* and one *Schizosaccharomyces pombe*. They found that most of the strains were able to grow at 37 ºC, but only one positive result was observed in the tests for proteolytic activity, pseudohyphae formation and invasive growth. One of the strains of *S. cerevisiae* scored three positive results in the four tests, while the *S. pombe* resulted negative for all assays.

4.3.2. Biocontrol activity against grey rot

Considering the results of the previous molecular and physiological characterization of the isolates and the fermentation trials with single inoculation (Chapter 3), looking then also the pathogenicity tests described above, a limited number of 17 isolates of *S. bacillaris* and eight of *L. thermotolerans* were further characterized for the antagonism capacity. The ability of *L. thermotolerans* and *S. bacillaris* strains to limit the growth of the pathogen *B. cinerea* was evaluated with *in vitro* and *in vivo* assays. The tests in PDA plates aimed to give a first picture in the potential of those species to act as BCA, but also the use of two different protocols allowed the comparison between two of the proposed mechanisms of antagonism. The first trial focused in the competition for nutrients

and space, since the pathogen and the BCA were contemporary inoculated at the same plate. The results of the inhibition of *B. cinerea* are shown in Figure 4.1.

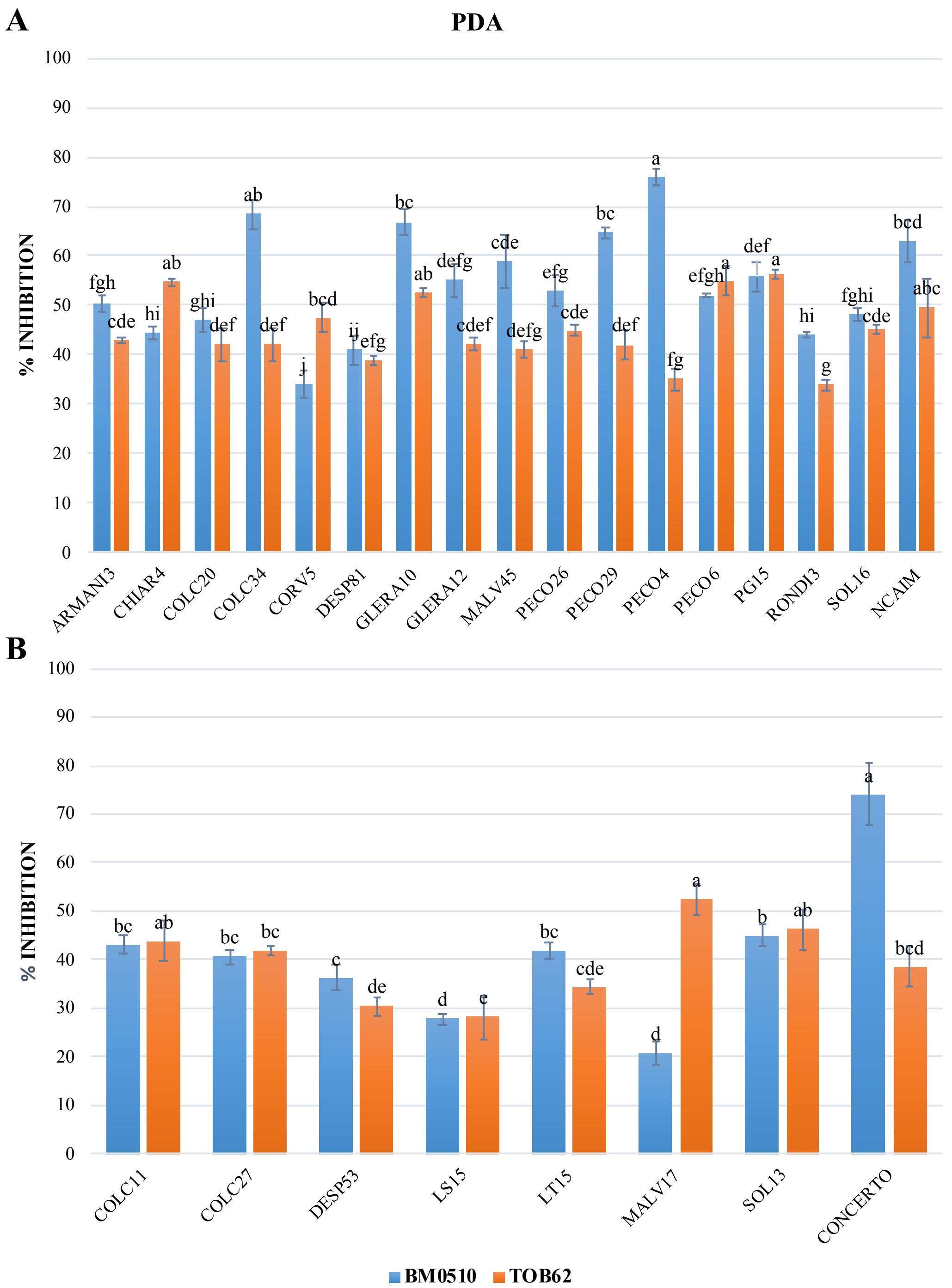

Figure 4.1. Inhibition of *B. cinerea* growth by antagonism of yeast isolates in the same plate. [A] *S. bacillaris*; [B] *L. thermotolerans*. Different letters for the same series of data indicate significant difference in Tukey's HSD test ($p<0.05$).

All isolates were able to exert inhibition of *B. cinerea* to some extent, ranging from around 20% (MALV17 versus BM0510) to 75% (PECO4 against BM0510). In the average, isolates of *S. bacillaris* were more effective than *L. thermotolerans*, for both the *B. cinerea* strains. Moreover, for both the non-*Saccharomyces* species the inhibition was stronger towards BM0510 than TOB62, but the differences were very slight.

For most of the strains, the levels of inhibition were similar in tests with BM0510 and TOB62, but some diverging profiles were observed. The isolates COLC34, GLERA10, GLERA12, MALV45, PECO29, PECO4, NCAIM Y016667^{T} and Concerto^{TM} had a much stronger inhibitory effect against BM0510 than TOB62, while CHIAR4, CORV5 and MALV17 resulted in higher inhibition of TOB62 than BM0510.

In the same assays performed by Junior Lemos and colleagues (2016), using different strains of *S. bacillaris* isolated from withered Raboso Piave grape variety, they have observed a range between 12 and 33% for the inhibition of radial mycelial growth of *B. cinerea* BM0510, thus less effective for the biocontrol than the strains of the present study.

In a study with more than 200 different strains of *S. cerevisiae* and non-*Saccharomyces*, Nally and co-workers (2012) observed that around one quarter of them showed antagonistic activity against *B. cinerea* under *in vitro* conditions, co-cultured with the pathogen in the same Petri dish. However, the two isolates of *Kluyveromyces thermotolerans* (synonym *L. thermotolerans*) were among the yeasts not presenting antagonism effect. Any isolates of *S. bacillaris* were included in the study. Applying a similar approach, Pretscher and colleagues (2018) tested 38 yeast strains against 12 different fungal phytopathogens strains, and the only isolate of *K. thermotolerans* in the screening did not caused inhibition of any of these pathogens, including five strains of *B. cinerea*.

The second *in vitro* trial put the isolates of non-*Saccharomyces* and the strain of *B. cinerea* in opposite plates, so they could not have a physical contact and were not competing for nutrients or space, but the inhibitory activity may be due to the action of volatile organic compounds (VOCs), since the two opposite agar plates were sharing the same air space. The results on the percentage inhibition of the radial growth of *B. cinerea* are shown in Figure 4.2.

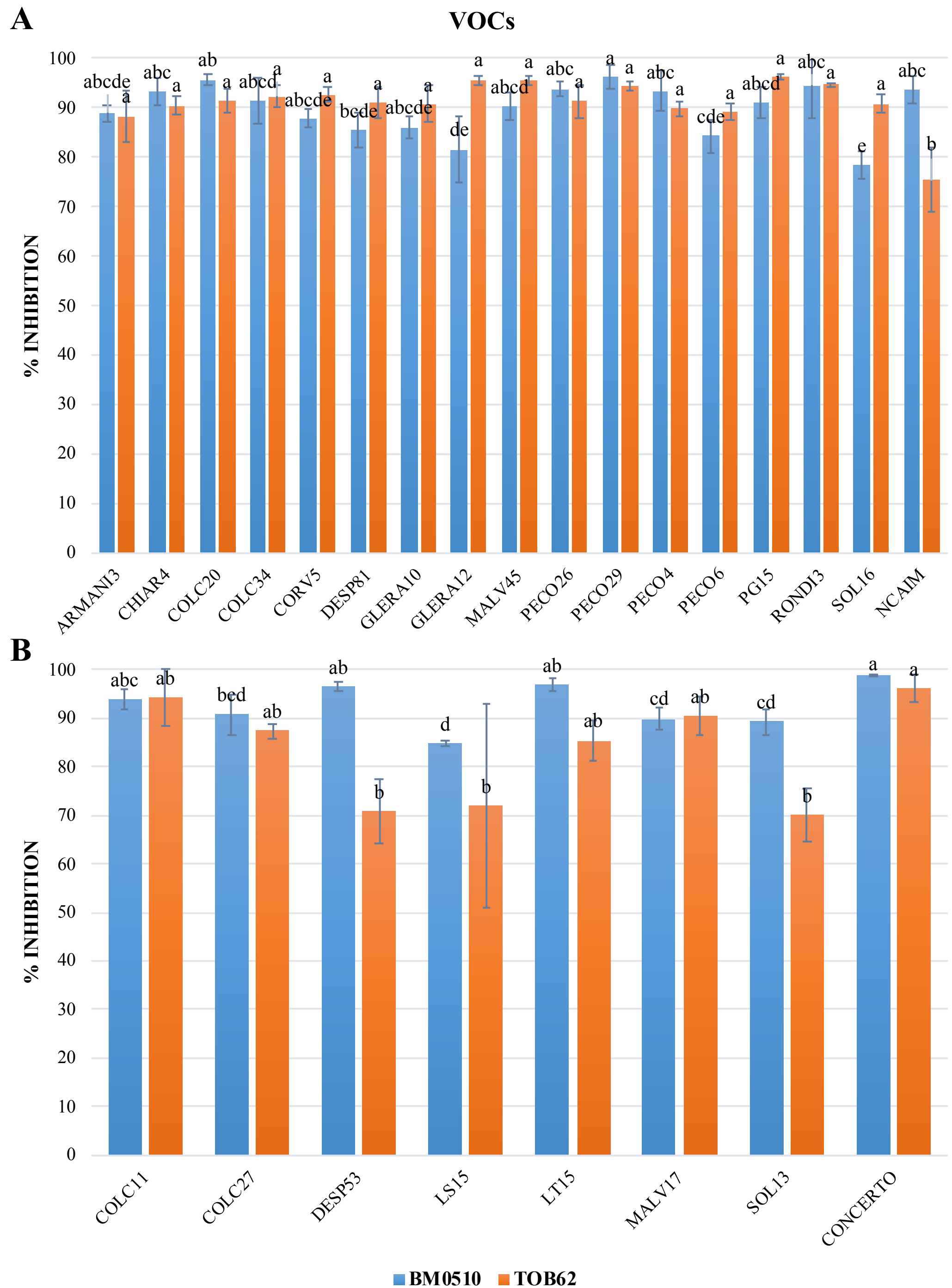

Figure 4.2. Inhibition of *B. cinerea* growth by antagonism of yeast isolates in opposite plates. [A] *S. bacillaris*; [B] *L. thermotolerans*. Different letters for the same series of data indicate significant difference in Tukey's HSD test ($p<0.05$).

As for the previous assay, again all tested isolates showed antagonistic activity versus *B. cinerea*. For both species, it is clear that the inhibition caused by the VOCs was much stronger than that recorded above in the contemporary growth,

reaching values above 70% for all tested yeasts. The percentage inhibition ranged from around 75% (NCAIM Y016667^{T} versus TOB62) to 96% (PECO29 versus BM0510) for *S. bacillaris*, and from 70% (SOL13 versus TOB62) to 99% (Concerto^{TM} versus BM05010) for *L. thermotolerans*.

In the average, *L. thermotolerans* isolates were more effective against BM0510, while *S. bacillaris* inhibited more the growth of TOB62, but the differences were very small. Again the differences in the inhibition of the two *B. cinerea* were not significant for most strains, but some cases of greater inhibition of one strain rather than the other were observed, such as NCAIM Y016667^{T}, DESP53 and SOL13, who were much more active against BM0510; and GLERA12 and SOL16, who provoked higher inhibition of TOB62.

In the previous assay, GLERA12 was more effective against BM0510, while in the VOCs assay it inhibited more TOB62. On the other hand, NCAIM Y016667^{T} showed a stronger antagonism towards BM0510 in both trials.

The study of Lemos Junior and colleagues (2016) showed the same trend of the present study, with the inhibition caused by the VOCs higher than the co-cultivation in the same plate. Their values ranged from 44 to 79%, hence lower than the inhibitions found for our strains of *S. bacillaris*.

Also applying the same protocols as in the present study, but using different yeasts strains, Oro and co-workers (2018) verified the successful biocontrol activity of all species tested (*M. pulcherrima*, *S. cerevisiae* and *Wickerhamomyces anomalus*) against different species of pathogens, including *B. cinerea*, with percentages of inhibition ranging from 56 to 87%. Parafati and collaborators (2015) tested the effect of VOCs and also the contemporary growth on the same plate with *B. cinerea*, including the same yeast species cited above plus *A. pullulans*, reporting an inhibition ranging from 20 to 99% in the different trials. However, on the contrary of the present study, they have reported a higher antagonism in the co-culture test, compared to the effect of VOCs, suggesting that different mechanisms of action could be more or less important for different species, such as iron depletion, production of cell wall-degrading enzymes, and a cumulative effect of several mechanisms.

These results show how the interaction between the yeast isolates and the mold is both species- and strain-dependent. Among the different mechanisms of inhibition that were tested in the present study, there was a good evidence that the

production of VOCs is a very effective tool of the examined yeast species to inhibit *B. cinerea*. Additional previous studies that have shown the antifungal effects of such compounds towards *B. cinerea* include yeast species such as *S. cerevisiae*, *W. anomalus*, *M. pulcherrima*, *A. pullulans*, *Candida intermedia*, *Sporidiobolus pararoseus*, *Hanseniaspora uvarum*, *Meyerozyma guilliermondii* (Druvefors and Schnürer, 2005; Huang *et al*., 2011, 2012; Hua *et al*., 2014; Di Francesco *et al*., 2015; Cordero-Bueso *et al*., 2017).

Approximately 250 different VOCs produced by fungal metabolism have been identified, and they are produced as mixtures of simple hydrocarbons, heterocyclics, aldehydes, ketones, alcohols, phenols, thioalcohols and thioesters, and/or their derivatives (Korpi *et al*. 2009; Ortiz-Castro *et al*. 2009). Numerous studies have been carried out about the potential biotechnological applications of VOCs in agriculture, food industry and pharmacology, particularly as biocontrol factors, due to the small sizes of these molecules and their diffusion through the atmosphere and soil (Liu *et al*. 2008; Arrebola *et al*. 2010; Morath *et al*. 2012; Giorgio *et al*. 2015).

Since the production of inhibitory compounds in culture medium is not a guarantee of their production on fruit surface (Dal Bello *et al*., 2008), a few isolates were selected to confirm the antagonistic activity on *in vivo* trials. Three isolates of *L. thermotolerans* (COLC27, SOL13 and Concerto^{TM}) and two of *S. bacillaris* (CHIAR4 and PECO4), chosen due to the interesting results on the pathogenicity and *in vitro* antagonism assays, were used for the competition with the two strains of the pathogen *B. cinerea* (BM0510 and TOB62) in white grape berries.

All isolates were able to reduce the visible symptoms of the disease, i.e. the size of decay and the mycelial growth of *B. cinerea* (data not shown). The percentage reduction on the diameter of the lesion on the surface of the infected grape berries, compared to a control, ranged from around 30% (COLC27 and Concerto^{TM}) to 64% (CHIAR4) in the challenge against BM0510 and from 35% (Concerto^{TM}) to 63% (COLC27) versus TOB62. It can be underlined that the responses were similar in the two pathogen strains, even if COLC27 inhibited more BM0510 than TOB62 and CHIAR4 had the opposite behavior. In the average, *L. thermotolerans* was more effective against TOB62 and *S. bacillaris* against BM0510, although it is difficult to draw conclusions with a limited

number of strains. What can be clearly highlighted is the high potential of both species to contrast the growth of *B. cinerea* in these *in vivo* assays.

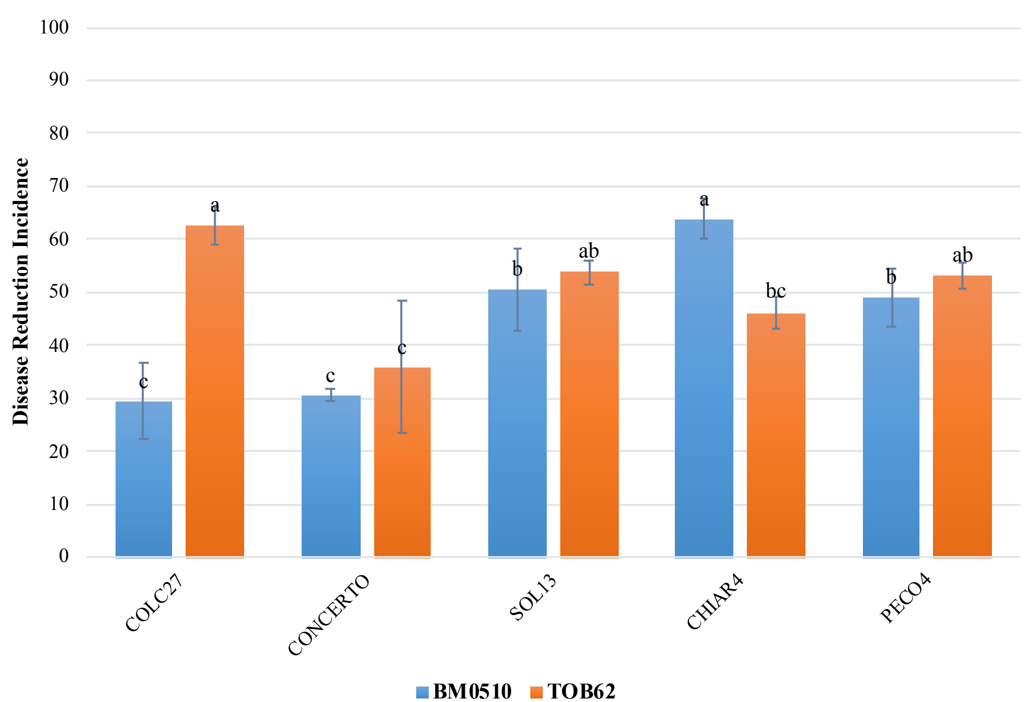

Figure 4.3. Inhibition of *B. cinerea* growth by antagonism of yeast isolates inoculated within healthy grape berries.

Similar values to our study were found by Lemos Junior and colleagues (2016) using *S. bacillaris* isolates, with DRI ranging from 39 to 85%. Other studies confirmed the great potential of different yeast species to reduce gray rot in grape berries inoculated simultaneously with the mold and antagonist yeast, correlating well with *in vitro* assays (Parafati *et al*., 2015; Cordero-Bueso *et al*., 2017).

4.4. CONCLUSIONS

Considering only the pathogenicity tests, the isolates of the species *S. bacillaris* would presumably be safer for the application in vineyard to the biocontrol of *B. cinerea*, since they presented mostly negative results for the virulence factors, while isolates of *L. thermotolerans* gave positive results for the invasive growth and ability to generate pseudohyphae. Nevertheless, any isolates

gave all positive results for the characteristics associated with pathogenicity in humans at the same time.

Successively, some selected isolates of *S. bacillaris* and *L. thermotolerans* were submitted to *in vivo* and *in vitro* assays in order to assess their biocontrol activity against the fungal pathogen responsible for the gray rot disease in grapes, *B. cinerea*. All trials have evidenced a remarkable capacity of all isolates to constrain the development of the mold, particularly higher for *S. bacillaris* than *L. thermotolerans*.

The *in vitro* tests showed that the production of VOCs is very likely more relevant to the antifungal ability, although the competition for nutrients and space are also probably involved, with a lesser effect. The *in vivo* analysis confirmed the potential observed before and moreover clearly highlighted the beneficial action of the yeast isolates to reduce the symptoms caused by the infection.

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Chapter 5

Unraveling molecular and physiological divergence among strains of *Lachancea thermotolerans* in the production of lactic acid

CHAPTER 5. UNRAVELING MOLECULAR AND PHYSIOLOGICAL DIVERGENCE AMONG STRAINS OF *Lachancea thermotolerans* IN THE PRODUCTION OF LACTIC ACID

5.1. INTRODUCTION

Lachancea thermotolerans, formerly known as *Kluyveromyces thermotolerans*, is a species of non-*Saccharomyces* yeast with a remarkable technological potential, yet underexplored. This group of "non-conventional" yeasts is always receiving more attention, not only by the scientific community but also by the industry searching for new biotechnological applications, due to their uncommon metabolic and physiological features (Kurtzman, 2003; Hranilovic *et al*., 2017).

The proposal of the new genus *Lachancea* in 2003 by Kurtzman was based on relationships among species of the '*Saccharomyces* complex' from a multigene sequence analysis (18S, ITS, 5.8S and 26S rDNAs, translation elongation factor 1-K(EF1-K), mitochondrial small-subunit rDNA and cytochrome oxidase (COX II)), when the family *Saccharomycetaceae* was resolved into 11 well-supported clades (Kurtzman, 2003). Since then, the former *K. thermotolerans* was renamed to *L. thermotolerans* and considered the type species of the genus, however there are still some commercial products using the previous genera classification (Lachance and Kurtzman, 2011; Benito, 2018). To date, the complete genome of the type strain *Lachancea thermotolerans* CBS 6430^{T} is available in the public databases, showing a genome of 10.6 Mb organized in eight chromosomes and containing 5,350 annotated genes (Malpertuy *et al*., 2000; Talla *et al*., 2005; Souciet *et al.*, 2009). However, there is still no consensus about the ploidy of *L. thermotolerans*, whereas some authors report it being diploid (Malpertuy *et al*., 2000; Souciet *et al.*, 2009) while others argue it to be haploid (Freel *et al*., 2014; Banilas *et al.* 2016; Hranilovic *et al*., 2017).

Most species belonging to the ascomycetous genus *Lachancea* are ubiquitous, it is included among the 20 most frequent foodborne yeast genus. *L. thermotolerans* is usually found in diverse natural habitats, such as soil, insects,

plants, fruits, fermented foods, in particular grapes and musts in several viticultural regions worldwide (Torija *et al*., 2001; Jolly *et al*., 2003; Ganter, 2006; Naumova *et al*., 2007; Deák, 2008; Lachance and Kurtzman, 2011; Freel *et al*., 2014).

As it can persist during the grape must spontaneous fermentation and have an impact in the process, *L. thermotolerans* was already studied during single and mixed fermentations with *S. cerevisiae*, and some interesting oenological traits have been reported, such as low production of volatile acidity, reduction of ethanol content, increase of glycerol, improved wine aroma, flavor and mouthfeel (Kapsopoulou *et al*., 2005; Gobbi *et al*., 2013; Benito *et al*., 2016).

One of the most important features of *L. thermotolerans* is its particularly high capacity to produce L(+)-lactic acid (LA) during fermentation, a very uncommon metabolic activity among yeasts, that arise great biotechnological interest (Witte *et al.*, 1989; Dequin and Barre, 1994; Sauer *et al*., 2010). LA is considered one of the most industrially important organic acids, due to many versatile applications, in food, pharmaceutical, textile, chemical industries (Datta and Henry, 2006; Sauer *et al*., 2008; Chen *et al*., 2013; Martinez *et al*., 2013). It is widely distributed in nature in two enantiomeric forms: L(+) and D(-), and the production through lactic fermentation by microorganisms leads selectively to one of two stereoisomers or their racemic mixture (Axelsson, 2004; Martinez *et al.*, 2013).

Although the efficiency of *L. thermotolerans* to produce LA is very low for the industrial bulk chemical manufacture of this substance and not much effort was put into the use of this yeast as a producer organism, in processes in which the concomitant acidification with alcoholic fermentation is a benefit, notably in the oenological industry context, it could provide an effective acidification of wines, important for some grape cultivars and some wine-producing regions where the acidity is insufficient (Mora *et al*., 1990; Kapsopoulou *et al*., 2007; Sauer *et al*., 2010; Hranilovic *et al*., 2017). The biological acidification can have a positive effect in the sensorial profile and also provide a higher microbial stability of the wines (Jolly *et al*., 2014). This attribute, coupled with the reduction of ethanol content, could be very useful to address some recent concerns of the wine industry regarding climate change, which is causing an increase in sugar levels

(and consequent ethanol increase) and loss of acidity of the grapes (Balikci *et al*., 2016; Benito *et al*., 2016).

In yeasts, the major flux of pyruvate metabolism is to ethanol, by way of pyruvate decarboxylase (PDC; EC 4.1.1.1) and alcohol dehydrogenase (ADH; EC 1.1.1.1). Providing an alternative route during anaerobic respiration for regenerating NAD+ through lactate dehydrogenase (LDH; E.C. 1.1.1.27), which catalyzes the reduction of pyruvate to lactate, they could theoretically replace ethanolic fermentation (Everse and Kaplan, 1973; Skory, 2003).

Several recent studies have underlined the significant variability among strains of *L. thermotolerans*, especially when important oenological parameters are compared, indicating that a proper selection has to be carried out in order to find the most appropriate isolates for the wine industry, with the goal of overall product enhancement (review by Benito, 2018).

Despite the great interest in the production of LA and the availability of genomic information brought by the complete sequencing of *L. thermotolerans* genome, the knowledge about the lactate metabolism, either at genotypic or phenotypic level, is rather limited. Aiming to shed light on this important metabolic pathway, our collection of *Lachancea* spp. isolates was submitted to a physiological characterization of their ability to produce lactic acid and the extension of the biological acidification in grape must. Subsequently, the presence of the putative genes responsible for this activity and their levels of expression was evaluated by means of molecular methods.

In order to unravel the genomic differences such as genes translocation and SNPs numbers, for the first time it was proposed the whole-genome sequencing of *L. thermotolerans* strains to be compared. Bioinformatics tools were used for the alignment of the reads obtained from the genome sequences, the prediction of protein-coding sequences, regulation sites and protein structures. These analysis allowed the annotation of the genome from an important yeast species and more specifically could help to clarify a very technologically relevant pathway associated also with other yeast species, which is the metabolism of lactic acid. Finally, this study had a focus on genes responsible for wine technological characteristics.

5.2. MATERIAL AND METHODS

5.2.1. Yeast strains

From the 104 yeast isolates characterized in the previous chapters (Supplementary Table S.2), 11 isolates of the species *Lachancea thermotolerans* were taken for the molecular and technological characterization of the LA metabolism, with the addition of the type strain (DBVPG 6232^T = CBS 6340^T) and a commercial starter of the same species (Viniflora® Concerto™). One isolate belonging to the species *L. fermentati* and one *L. kluyveri* were also used.

5.2.2. Microfermentations

The quantification of LA production was made simultaneously with the physiological characterization carried out in Chapter 3. The supernatant of the stress tolerance assay made in YPD broth containing 220 g/L of glucose, after 72 h growth (par. 3.2.5.2.), and the wine obtained from the single culture inoculation in Trebbiano grape must (par. 3.2.7.), were used for the LA analysis.

For the gene expression analysis, a new microfermentation was set up with only two isolates of *L. thermotolerans*, the one with the highest and the one with the lowest LA production in the previous microfermentations. They were inoculated in glass bottles containing 100 mL of a biological white grape juice (160 g/L of sugar, Folicello), pasteurized at 70 ºC. The bottles were equipped with silicon stoppers and a folded Pasteur pipette to let the carbon dioxide release. The bottles were kept at 22 ºC under static conditions and weight loss was measured daily until the end of fermentation (constant weight). Samples were taken during the fermentation for the RNA isolation and at the end for the LA quantification.

5.2.3. L(+)-Lactic acid analysis

Two different methods were used for the quantification of LA. The concentrations in the YPD broth modified and in biological grape juice at the end of fermentation were quantified by using an enzymatic assay (Megazyme),

following the manufacturer's protocol. The quantity of LA in the wines obtained with Trebbiano grape must was evaluated by HPLC, as explained in paragraph 3.2.7.2.

5.2.4. DNA extraction

Total genomic DNA was isolated and purified using the commercial kit Wizard Genomic DNA Purification (Promega) following the manufacturer's protocol, from a 2-mL aliquot of cultures grown in YPD broth for the DGGE analysis and from a volume of 50 mL to have a sufficient concentration for the genome sequencing. The quality assessment of the DNA obtained was performed with a NanoDrop ND1000 UV-Vis Spectrophotometer (Thermo Scientific) and dilution in DNAse-free water was carried out when necessary.

5.2.5. DGGE and band sequencing

In order to check the presence of the genes encoding for LDH and eventual sequence differences among the isolates, the primers listed in Table 5.1 were designed for the amplification of such fragments and subsequent separation in DGGE, based on the genome sequence of the type strain of *L. thermotolerans* CBS 6340^T. A GC-clamp was added at position 5' of the forward primers.

Table 5.1. List of primers used for LDH genes amplification.

Primer	Sequence (5'-3')	Amplicon size (bp)	T_m (ºC)
LDH1-F	ATCCGGTCGGAAGTACCAAC	195	59.4
LDH1-R	TACTGGTGACTGACAGCGC		58.8
LDH2-F	TTCAAGTACCAGCATTCGG	258	54.5
LDH2-R	CGGGTCAGCTAAACAGTA		53.7
LDH3-F	AGATACCTTGCGTAAGACTAGT	287	56.5
LDH3-R	CTAACTCGTAGACCTCTAGT		55.3

The conditions of the reaction for a final volume of 40 µL were as follows: 1× DreamTaq Green Buffer (Thermo Scientific); 2.0 mM $MgCl_2$; 0.2 mM dNTPs; 1 µM GC-clamp forward primer; 1 µM reverse primer; 0.025 U/µL DreamTaq

DNA Polymerase (Thermo Scientific) and 50 ng genomic DNA. The amplification program started with an initial denaturation at 94 ºC for 4 minutes, followed by 30 cycles of denaturation at 94 ºC for 1 minute, annealing at the respective melting temperature (T_m) of each pair of primers reported in Table 5.1 for 30 seconds and extension at 72 ºC for 1 minute, and concluded with a final extension of 8 minutes at 72 ºC. The amplification was conducted in Thermal Cycler 2720 (Applied Biosystems).

The separation of GC-clamped amplicons was carried out in a D-Code^{TM} Universal Mutation Detection System (Bio-Rad). The PCR samples were added with the same volume of loading buffer 2.0× and applied into an 8% polyacrylamide gel (acrylamide:bis-acrylamide ratio of 37.5:1) of 20x20x0.1 cm with a denaturing gradient from 30–50% of urea and formamide. The electrophoretic run was performed in a running buffer of 1% TAE at a constant voltage of 50 V for 16 hours at 60 ºC. After the end of the run, the gel was stained in solution containing EuroSafe colorant (Euroclone). The visualization and image capturing were made under UV light with UVITEC Gel Documentation System (Cleaver Scientific).

Some interesting bands were excised from the polyacrylamide gel and transferred to a microtube containing 50 µL of TE buffer (pH 8.0). Subsequently, 1 µL of the suspension was used for the reamplification using the same primers listed in Table 5.1, without the GC-clamp, and following the same protocol described above. The PCR products were purified and sent to GATC Biotech (Konstanz, Germany) for the sequencing. The sequences obtained were aligned with Clustal Omega 1.2.4 multiple sequence alignment tool (http://www.ebi.ac.uk/Tools/msa/clustalo/) and the relations observed through MEGA5 (http://www.megasoftware.net).

5.2.6. Genome sequencing

The shotgun sequences were generated using an Illumina NextSeq 500 platform (1-kb mate-pair libraries) at the CRIBI Biotechnology Center (Padua, Italy). The assembly and annotation were carried out following the same protocols described by Lemos Junior and colleagues (2017). Genome sequences were *de*

novo assembled using CLC Genomic Workbench software (version 9.5). Coding sequences (CDS) were predicted by GeneMark-ES (Besemer and Borodovsky, 2005) and gene annotation was obtained using BlastKOALA tool (Kanehisa *et al*., 2016), in order to access the non-redundant set of KEGG genes, with the selection of the family *Saccharomycetaceae* as a taxonomy group and 4893 as taxonomy ID, and RPS BLAST, to compare protein sequences with eukaryotic orthologous groups of proteins (KOG) (Tatusov *et al.*, 2003).

SnpEFF was used to identify variants between the two strains using SOL13 as reference genome. Only variants with predicted "high" or "moderate" effect were selected. Function of the genes with variants was obtained by annotating proteins with BlastKOALA software (Kanehisa *et al*., 2016).

Progressive MAUVE (Darling *et al*., 2004) was used for genome alignment where the presence of translocations between *L. thermotolerans* CBS 6340^T and the examined strains in the LDH genes was identified by visual inspection. The comparison of the sequences of LDH genes among COLC27, SOL13 and CBS 6340^T was carried out in MEGA5 software (Molecular Evolutionary Genetics Analysis) (Kumar *et al*., 2008).

5.2.7. RNA extraction and cDNA synthesis

The sampling for the RNA isolation was taken during the fermentation in grape juice with the two isolates representative of the highest and lowest LA production at three different times: T0 (time of inoculation; 10 mL), T1 (half fermentation; 1 mL) and T2 (end of fermentation; 1 mL), in triplicate.

The samples were centrifuged at 8,000 rpm for 5 minutes, the pellets were washed twice with (diethylpyrocarbonate) DEPC-treated water and centrifuged again. The pellets were then resuspended in 500 μL of LETS (200 mM LiCl, 20 mM EDTA, 20 mM Tris, 0,4% SDS; stock 5×), followed by the addition of 300 μL of glass beads (∅ 0.45 mm), 300 μL of solution phenol-chloroform-isoamyl alcohol (25:24:1) and 1 μL of DEPC. The cellular lysis was performed with five cycles of agitation on vortex during 1 minute followed by 1 minute in ice. After centrifugation at 13,000 rpm for 10 minutes at 4 ºC, the upper phase of the supernatant, containing the RNA, was transferred to a new tube with 600 μL

of a solution chloroform-isoamyl alcohol (24:1) and mixed by inversion. A new centrifugation was made with the same conditions and the upper phase was again transferred to new tubes, containing 60 µL of potassium acetate 3 M. After gentle agitation, 1 mL of cold ethanol 100% was added and the tubes incubated at -80 ºC for one hour. Afterwards, the pellet obtained by centrifugation (13,000 rpm, 15 minutes, 4 ºC) was washed with cold ethanol 70%, and dried. The RNA was finally resuspended in 50 µL of DEPC-treated water.

The concentration and purity of the resulting RNA were measured with a NanoDrop ND1000 UV-Vis Spectrophotometer (Thermo Scientific). All samples were then diluted in DEPC-treated water to reach a concentration of 100 ng/µL and submitted to DNAse treatment. This purification was carried out with the kit Turbo DNA-free (Life Technologies), following the manufacturer's instructions. The purified RNA was kept at -80 ºC freezer until the synthesis of cDNA, using the kit ImProm-II™ Reverse Transcriptase (Promega) according to the manufacturer's protocol.

5.2.8. qPCR

The standard curve for the quantification of the number of transcribed genes was prepared by the cloning of the amplification products of LDH1 and LDH2 from the genome of type strain *L. thermotolerans* DBVPG 6232^T in plasmid pGEMT-easy (Promega), followed by transformation of JM109 cells (Promega) and isolation of plasmid DNA with GenElute™ Plasmid Miniprep kit (Sigma-Aldrich), always following the manufacturer's guidelines.

Real-time PCR (qPCR) was carried out for the cDNA obtained from the microfermentations with two isolates of *L. thermotolerans* and the plasmid DNA prepared for the standard curve. For the amplification reaction of LDH1 and LDH2 (Table 5.1) in a final volume of 24 µL, the following components were added: 10 µL of SYBR Green Master Mix (Life Technologies); 0.4 µL forward primer (10 µM); 0.4 µL reverse primer (10 µM); 6 ng DNA. The amplification program included an initial denaturation at 95 ºC for 5 minutes, followed by 40 cycles of denaturation at 95 ºC for 1 minute, annealing at the respective melting temperature (T_m) of each pair of primers reported in Table 5.1 for 30 seconds and

extension at 72 ºC for 40 seconds, and concluded with a final denaturation of 10 seconds at 95 ºC. The melting curve was conducted by raising the temperature from 60 to 95 ºC with a gradient of 0.1 ºC/s. The reactions were performed with technical replicate in Light Cycler Nano (Roche).

5.3. RESULTS AND DISCUSSION

5.3.1. L(+)-Lactic acid production

Eleven isolates of the species *L. thermotolerans* recovered during this PhD work, plus one type strain and one commercial starter of the same species, one *L. fermentati* and one *L. kluyveri*, were submitted to an extensive molecular and physiological characterization. During these tests, it came out that a remarkable variability in the production of L(+)-lactic acid was present among the isolates of *Lachancea* spp., an important feature already associated with this group. Figure 5.1 shows the LA production during growth in synthetic laboratory medium containing 220 g/L of glucose (modified YPD) and in natural grape must containing also approximately 200 g/L of sugar (almost equally split between glucose and fructose).

The results for *L. fermentati* and *L. kluyveri* were omitted from the graphic, since we chose to focus on *L. thermotolerans* due to the interesting potential of this last species to the use as starter culture in sequential fermentations of grape must. It is worth to point out that *L. fermentati* produced around 13 g/L of LA in both YPD and grape must, a value significantly higher than all *L. thermotolerans* isolates that were reported here. Future studies could be done for an in-depth characterization of this species, since it was never exploited before, for the best of our knowledge. On the other hand, *L. kluyveri* produced just 0.3 g/L of LA in YPD and no production could be detected by the HPLC method in the grape must assay.

As it can be seen, significant differences were found in the LA production among the diverse isolates. The production ranged from 1.15 g/L (COLC27) to 5.24 g/L (SOL13) after 72 hours of growth in modified YPD broth, and from 0.26 g/L (CONCERTO) to 10.54 g/L (SOL13) after 10 days of growth in

Trebbiano grape must. The isolates can be divided into three separate groups according to their LA metabolism, namely: Low producers (COLC11, COLC27, CONCERTO and DBVPG 6232^{T}); Medium producers (FIANO43, GLERA15, MALV13, MALV17, LS15 and LT3) and High producers (LT15 and SOL13). The isolate DESP53 is among the medium producers when the grape must is considered, but it is a low producer when looking at the results in YPD broth.

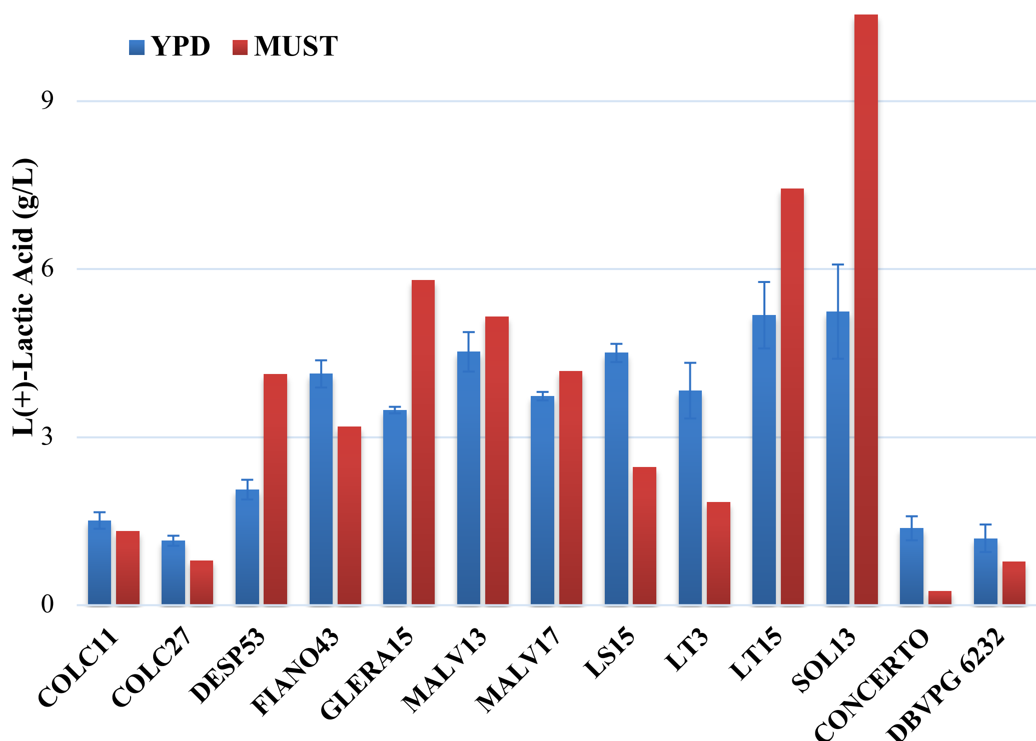

Figure 5.1. L(+)-Lactic acid production by isolates of *L. thermotolerans* during fermentation in YPD broth and natural grape must.

Only to cite the most recent works, in a study with 24 non-*Saccharomyces* isolates of eight different species, some oenological features were evaluated after pure culture fermentations of Viura pasteurized grape must (206 g/L of reducing sugars). At the end of fermentation, the four isolates belonging to *L. thermotolerans* have shown very different concentrations of LA, ranging from 0.90 to 4.20 g/L, corresponding to the low to medium producers in the present study. Any of the other species could produce more than 0.15 g/L (Escribano *et al.*, 2018).

Another screening with 23 isolates (10 non-*Saccharomyces* and 13 *S. cerevisiae*) published in 2016 by Aponte and Blaiotta, in Aglianico grape must (240 g/L of reducing sugars), found the three isolates of *L. thermotolerans* producing 1.24, 1.29 and 2.56 g/L of LA, so a similar value to the strains in the group of low producers in our study. One strain of *Pichia kudriavzevii* produced 0.84 g/L of LA, while any of the other isolates could produce detectable amounts of this organic acid.

A total of 25 *L. thermotolerans* isolates obtained from different vineyards in Greece were tested in microfermentations of pasteurized grape must (215 g/L of sugars) and the content of LA was quantified at the end of fermentation by enzymatic kit (Banillas *et al.*, 2016). As in the present study, a huge diversity was found among the strains. The production of LA ranged from 1.0 to 16.6 g/L, where 56% of the isolates were able to produce more LA than the highest producer found in our study (SOL13, 10.54 g/L) and the other 44% produced less than 2.6 g/L, so similar to the study of Aponte and Blaiotta (2016) and corresponding to our group of low producers.

Aiming to further analyze the possible reasons for the huge phenotypic divergence in the LA production, a genotypic investigation of the genes codifying for lactate dehydrogenase (LDH), the putative enzymes responsible for the lactic acid metabolism, was carried out using bioinformatics tools and molecular biology protocols.

5.3.2. LDH genes

In the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/), the production of LA was identified in the pyruvate metabolic pathway of *L. thermotolerans* as the resultant activity of an enzyme classified as EC 1.1.1.27. The gene codifying for the LDH enzyme was present in three different copies within the deposited genome of the type strain CBS 6340^T, denominated by us as LDH1, LDH2 and LDH3, as it can be seen in Figure 5.2.

To verify the presence of those genes in the isolates of our collection, three pairs of primers were designed, one for each copy of LDH (LDH1, LDH2, LDH3)

found in the type strain genome. The presence of LDH was evaluated in all 15 isolates, described before in the LA quantification, by the amplification with the primers listed in Table 5.1, using the genomic DNA purified from each isolate.

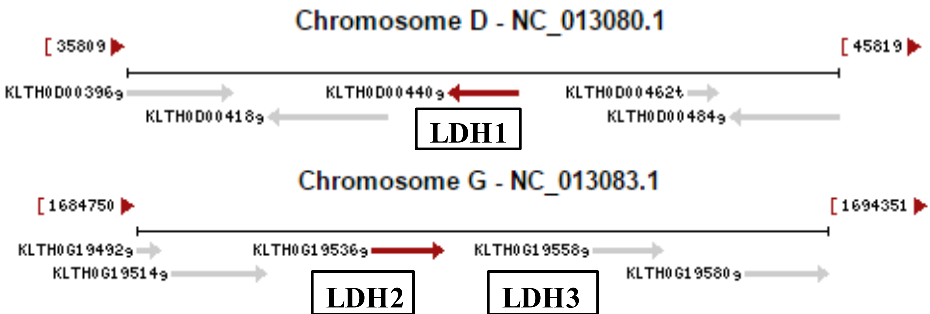

Figure 5.2. Genomic context in which the lactate dehydrogenase (LDH) genes are positioned within the genome of type strain *L. thermotolerans* CBS 6340^{T}.

The results of the electrophoresis run with the amplification products confirmed the presence of LDH1 and LDH2 in the genome of the 13 *L. thermotolerans* isolates, while the amplification of LDH3 did not result in visible bands for any of the 15 *Lachancea* spp. isolates tested. Neither *L. kluyveri* nor *L. fermentati* had positive results for any of the three copies of LDH (data not shown). It could be argued that our isolates simply do not have LDH3 in their genomes, however, also the type strain DBVPG 6232^{T} belongs to our collection and it is supposed to be the same strain as CBS 6340^{T}, so it should be expected that they have the same genome and LDH3 must have been amplified at least in this strain, what did not happen. Other possible reasons for the failure in the amplification of LDH3 are the wrong design of the pair of primers or the presence of mistakes in the sequenced genome of CBS 6340^{T} available in public databases.

Afterwards, DGGE runs were carried out with the products of amplification of LDH1 and LDH2. The analysis of LDH1 confirmed that only one band was present in the amplified DNA of each *L. thermotolerans* isolates and they were all at the same position in the gel, thus it could be concluded that the designed primers were specific for the target gene, all tested *L. thermotolerans* isolates have the copy LDH1 in their genomes and they all have presumably the same sequence for this specific copy of the LDH gene (data not shown).

As regarding LDH2, an interesting outcome was observed with the DGGE analysis (Figure 5.3). Also only one band was present for each of the *L. thermotolerans* isolates, but these bands were at three different positions, suggesting that there are differences in the LDH2 sequence. Moreover, it can be seen how the different variations in LDH2 sequence were related to the groups formed in the phenotypic evaluation of LA production (Figure 5.1).

All medium and high producers share the same LDH2 (yellow circle), while COLC11 and CONCERTO (low producers; green circle) show a different sequence and COLC27, DESP53 and the DBVPG 6232^T (also low producers; gray circle) possess a third variation in the LDH2 sequence.

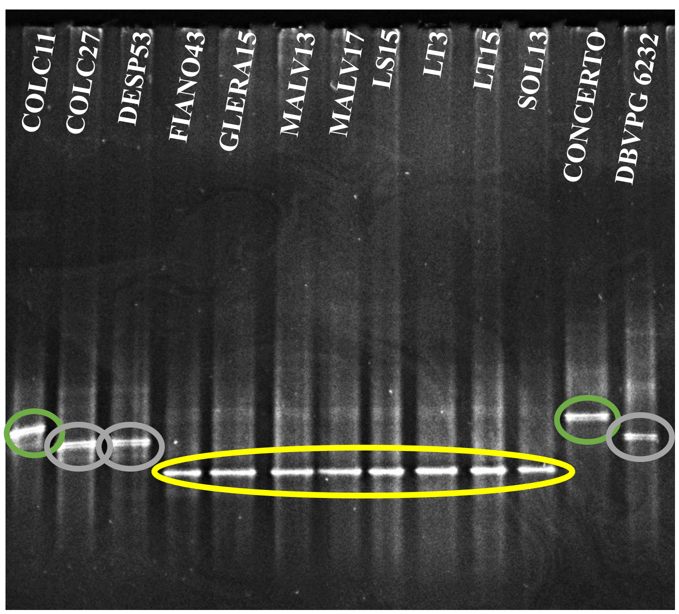

Figure 5.3. PCR-DGGE analysis (gradient 30-50%) of the amplified LDH2 gene in the genomic DNA extracted from *L. thermotolerans* isolates. Colored circles represent bands at the same position in the gel.

The bands from three representative (CONCERTO, DESP53 and FIANO43) of these variants in the sequence were excised from the gel and the product of the reamplification was sequenced. The alignment of sequences with Clustal Omega is shown in Figure 5.4, together with the reference sequence of CBS 6340^T.

The results of the sequence alignment reflected exactly what could be expected from the PCR-DGGE analysis. The LDH2 sequence of DESP53 matched perfectly with CBS 6340^T (DBVPG 6232^T), as they showed the same

band height in the polyacrylamide gel. CONCERTO has only one nucleotide of difference, while the sequence of FIANO43 had ten nucleotides diverging from the other three strains analyzed. This observation makes sense when the phenotypes are confronted, as DESP53, CONCERTO and DBVPG 6232^{T} have shown very low and similar production of LA, whereas FIANO43 belongs to the group of medium producers.

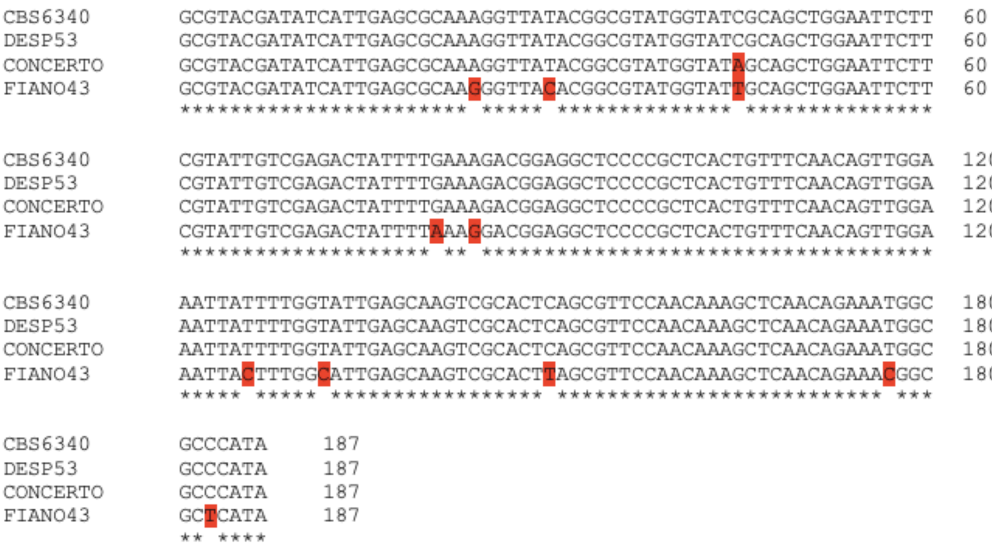

Figure 5.4. Sequence alignment of the gene LDH2 in three strains of *L. thermotolerans* (CONCERTO, DESP53 and FIANO43) and the type strain CBS 6340^{T}.

However, these DNA sequences were converted to the aminoacidic sequence and compared again with Clustal Omega 1.2.4., where it was shown that all those point mutation were actually silent, as no differences could be observed in the sequence of amino acids obtained (data not shown).

As there was a strong indication that the differences in LA metabolism were related to the differences at the genomic level of the enzyme LDH, but since the divergences found in the sequence of one copy of the gene LDH could not explain changes in the protein, an in-depth investigation of molecular mechanisms that could be related with LA metabolism involved the complete genome sequencing and the analysis of expression of the LDH1 and LDH2 genes, with the highest (SOL13) and lowest (COLC27) producers of LA.

5.3.3. Genome assembly and annotation

The results of assembly and annotation of whole-genomes from *L. thermotolerans* strains COLC27 and SOL13 are shown in Table 5.2.

Table 5.2. Whole-genome information of the two *L. thermotolerans* strains COLC27 and SOL13.

Feature		COLC27	SOL13
Nucleotide distribution	**GC (%)**	47.34	47.34
Contig measurements	**N75**	478,272	364,893
	N50	788,172	946,139
	Scaffold	43	71
Genome size		10.24 Mb	10.28 Mb

The Table 5.2 contains the following information:

Nucleotide distribution (GC (%)): fraction of the assembly covered by nucleotides C and G.

Contig measurements: this section includes statistics about the number and lengths of contigs. A contig is a continuous stretch of genomic sequence containing only A, C, G, or T bases without gaps, in which the order of bases is known to a high confidence level. N75 and N50: the N50 contig set is calculated by summarizing the lengths of the longest contigs until reach 50% of the total contig length, and the minimum contig length in this set is the N50 value of a *de novo* assembly. N75 values is computed in a similar way. Scaffold: Scaffolds are composed of contigs and gaps, created by chaining contigs together using additional information about the relative position and orientation of the contigs in the genome.

Genome size: The total number of bases in the result. This can be used for comparison with the estimated genome size to evaluate how much of the genome sequence is included in the assembly.

High-throughput sequencing technology has afforded the sequencing of a great number of yeast genomes. Currently, tools from the comparative genomic help us in the comprehension of why some phenotypic differences rise among similar fungal species (Dujon, 2010; Mohanta and Bae, 2015).

L. thermotolerans has a highly conserved mitochondrial genome with the coding regions characterized by low rates of non-synonymous substitutions. The genetic structure of *L. thermotolerans* genomes, in features such as the number of genes encoding enzymes, synthesis and metabolic pathways, has to be elucidated (Freel *et al*., 2014). Genomes of *L. thermotolerans* strains COLC27 and SOL13 were not aligned in a unique consensus sequence, and, besides that, SNP (single-nucleotide polymorphism) between the two strains was 87,968 and genome sizes of 10.24 and 10.28 Mb were generated, respectively. In a review about the diversity of fungal genome, Mohanta and Bae (2015) reported that reduced genome sizes, as found for *L. thermotolerans* strains, are associated with a rapid evolution in terms of phenotypic diversification motivated by adaptive exigencies or due to natural selection.

The GC content of 47.34% is compatible with close related yeasts that belong to the same clade. Regarding the comparison between *L. thermotolerans* COLC27 and SOL13 genomes by alignment using MAUVE software, it was not evidenced any translocations in the LDH genes.

According to the BlastKOALA annotation tool, the annotated genomes of the two *L. thermotolerans* isolates were grouped in functional categories, based on the biological roles that are played, and the distribution of the genes among the different categories is shown in Figure 5.5, alongside with the annotation of the reference genome of type strain CBS 6340^{T}.

The annotation showed very similar results for the three analyzed strains. The most abundant category for all genomes was the Genetic Information Processing, followed by Cellular Processes and Carbohydrate metabolism. Nucleotide metabolism had a higher distribution in the type strain CBS 6340^{T} than in the two strains isolated.

To understand in more detail the genetic basis in the LA metabolism differences, the LDH genes were further analyzed. Three copies of the LDH gene were found in the genome of SOL13, COLC27 and CBS 6340^{T}. The similarity between these copies was analyzed with MEGA5 software and the Neighbour-joining tree is reported in Figure 5.6.

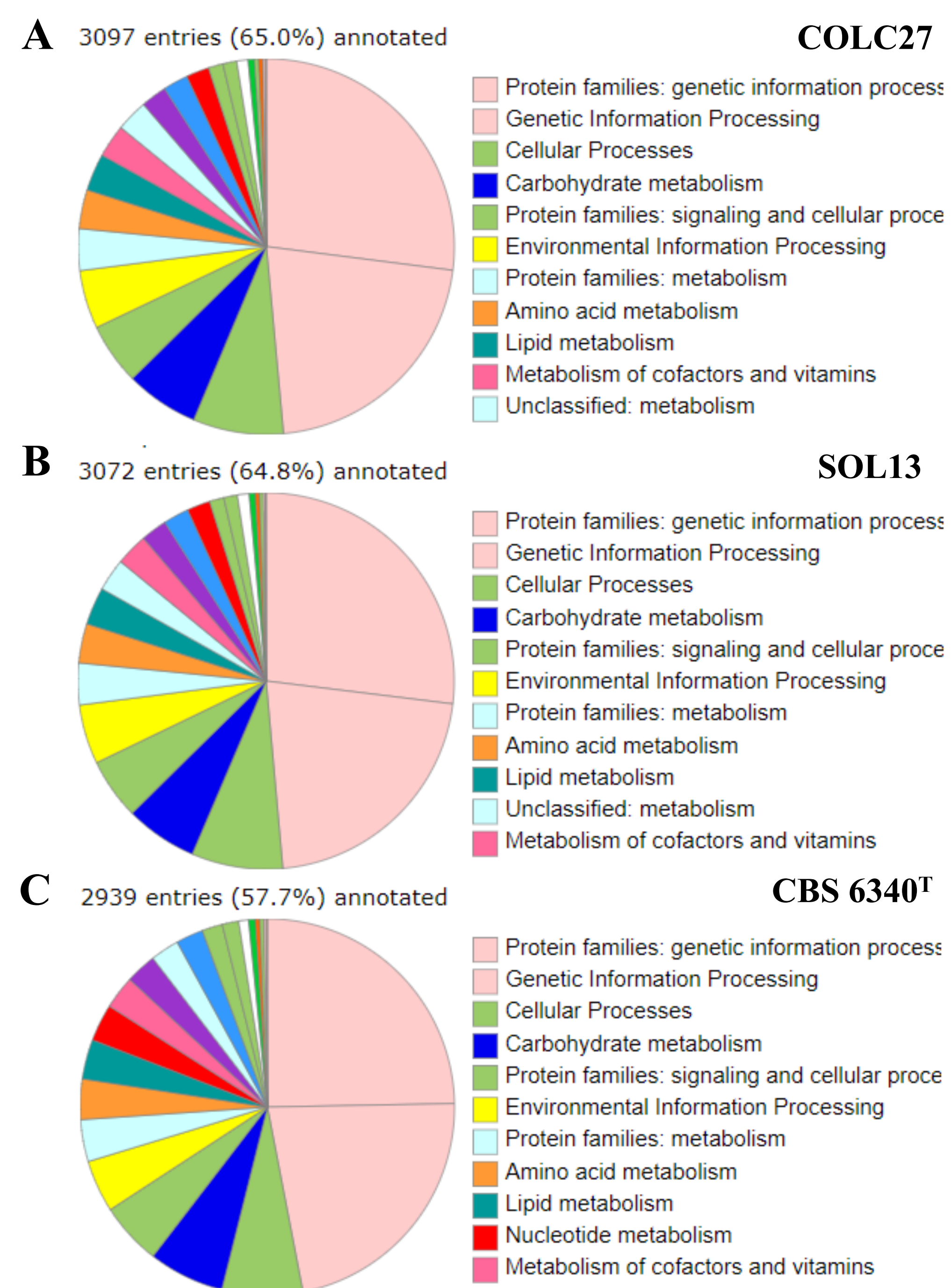

Figure 5.5. Functional annotation of the genome sequence of three *L. thermotolerans* strains: (A) COLC27; (B) SOL13 and (C) CBS 6340^T, obtained with BlastKOALA tool.

It can be noted in the tree that the alignment of LDH1 was very close for the three strains, while of LDH2 a slight difference was observed for SOL13 in comparison with the other two strains, and LDH3 was similar between SOL13 and COLC27, with the type strain positioned separately.

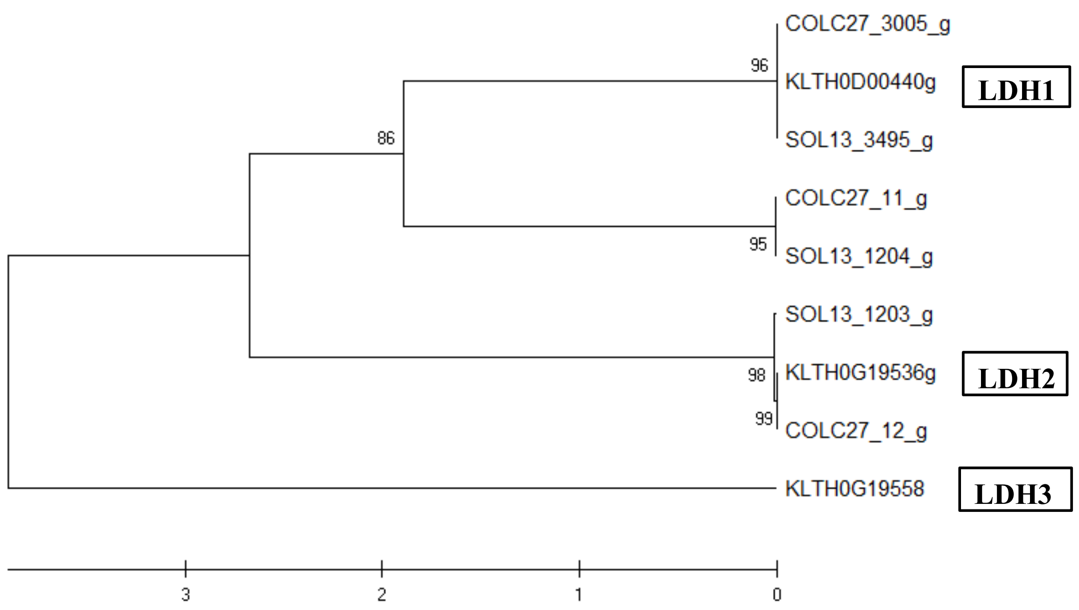

Figure 5.6. Phylogenetic tree based on the aminoacidic sequence from the LDH genes of three strains of *L. thermotolerans*: COLC27, SOL13 and CBS 6340^T (KLTH0). The codes following the names represent the position of the genes within the genome.

The results of the PCR-DGGE (Figure 5.3) have shown that COLC27, DESP53 and DBVPG 6232^T have all the same band of the amplification of LDH2, and indeed the alignment of the amplicon sequenced from DESP53 (Figure 5.4) and the whole-genome sequencing of COLC27 (Figure 5.6), when compared to the reference genome of CBS 6340^T, have all a very strong similarity for this gene. The LDH2 amplicon of SOL13 was at the same height as FIANO43, which sequencing showed multiple differences with the sequences of DESP53 and CBS 6340^T, and so also in accordance with the results of the whole-genome sequencing. Moreover, the DGGE analysis also showed that the amplification of LDH1 resulted in the same band for all isolates, and this was the case for COLC27, SOL13 and CBS 6340^T in the phylogenetic tree. LDH3 could not be observed in the DGGE analysis, since it was not successfully amplified in any tested strains.

The findings seem to be in accordance with the phenotypic results observed before. COLC27 and DBVPG 6232^T (CBS 6340^T) produced almost the same quantity of LA, in both YPD broth and grape must, being considered in the group of low producers, while SOL13 was the highest producer of all isolates tested,

with 4.5 times more than COLC27 and DBVPG 6232^T in YPD broth and 13 times more in the grape must (Figure 5.1).

5.3.4. Expression analysis

The samples for the RNA extraction from the two representative strains that were chosen for the expression analysis (COLC27: lowest producer; SOL13: highest producer) were taken at the beginning, middle and end of the fermentation, determined by the weight loss. By using the standard curve built with the products of LDH1 and LDH2 amplification from the type strain CBS 6340^T cloned in plasmidial vector, it was possible to quantify the number of transcripts produced by the two *L. thermotolerans* isolates.

LA production was measured at the same time of the sampling for the gene expression analysis. As it can be seen in Figure 5.7, the production by SOL13 was approximately 10 times higher than COLC27. Moreover, it is interesting to note how the LA metabolism happened during the first days of fermentation, statistically the concentration did not change from to middle (T1) to the end (T2) of the process.

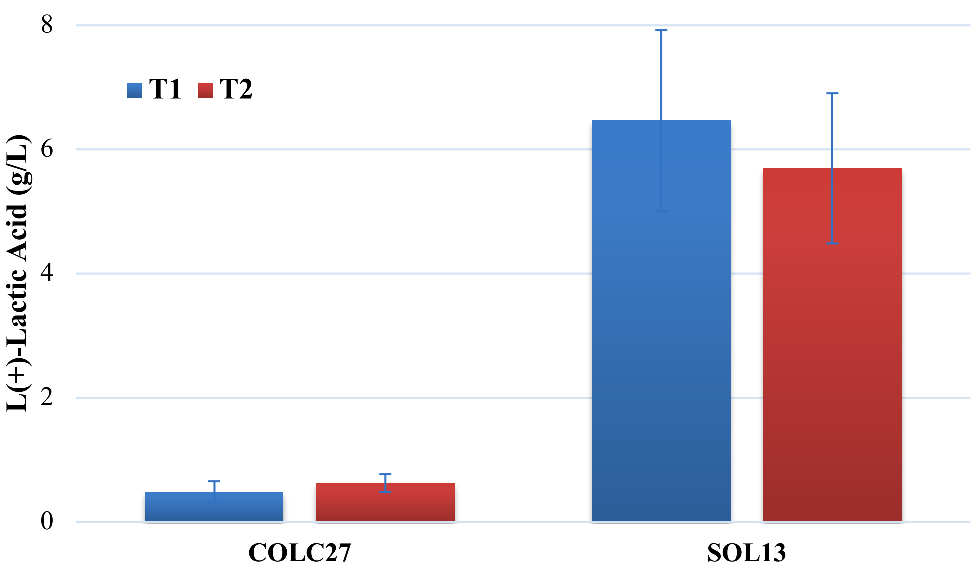

Figure 5.7. L(+)-Lactic acid production during fermentation of pasteurized grape juice by the two isolates of *L. thermotolerans* COLC27 and SOL13.

The level of transcription of the genes LDH1 and LDH2 for the two isolates are represented in Figure 5.8.

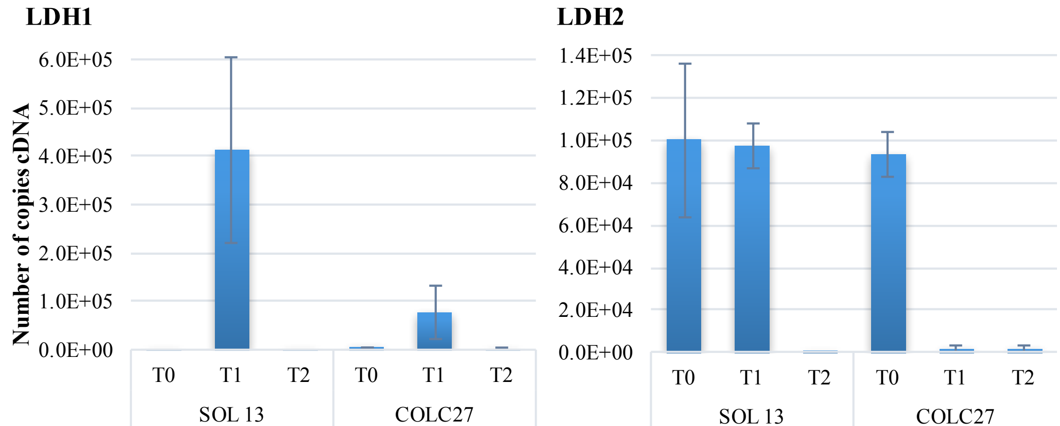

Figure 5.8. Gene expression level by qPCR analysis of LDH1 and LDH2 in the two isolates of *L. thermotolerans* COLC27 and SOL13 after inoculation in grape juice, at the beginning (T0), middle (T1) and end (T2) of fermentation.

For the expression of LDH1, both strains only showed relevant amount of transcripts at the middle of fermentation, suggesting that the expression of this gene was activated in the first days of fermentation and then it was repressed towards the end, when the metabolism of LA was no longer necessary for the cells. Interestingly, the expression of LDH1 in the isolate SOL13 was significantly higher than COLC27, in accordance with the higher production of LA by the first.

As regards LDH2, the same observation for the higher transcription in SOL13 than COLC27 in the sample collected at middle fermentation was true, and the repression of transcription from halfway to the conclusion of fermentation. However, differently than LDH1, it can be noted that an elevated number of transcripts was already present at the beginning of fermentation. The inoculation was made with cultures grown in YPD medium at the exponential phase of growth, so possibly the LA metabolism was already active in the inocula.

Both genotypic analysis are in accordance with the phenotypic outcome shown in Figure 5.4, as LA production by SOL13 was much higher than COLC27 and this metabolic pathway was active until the middle of the fermentation process, after what the concentration of LA did not change.

5.4. CONCLUSIONS

Isolates of *Lachancea* spp. showed diverging profiles in LA production, both in synthetic medium and natural grape must, in some cases reaching notably high values, thus this characteristic received a more detailed attention. For the best of our knowledge, this was the first time when a molecular characterization of the lactate dehydrogenases (LDH), the putative enzymes responsible for the LA metabolism, was carried out.

Making use of bioinformatics tools, it was possible to identify three copies of the gene codifying for LDH (LDH1, LDH2 and LDH3) in the available genome of the type strain from the species, *L. thermotolerans* CBS 6340^T, and later the whole-genome sequencing of two isolates from our collection allowed the individualization of also three copies in both of them (COLC27 and SOL13). The phylogenetic analysis also showed a very high similarity between the copies of LDH2 in COLC27 and CBS 6340^T, while some differences were present for SOL13. LDH1 was similar to all three strains.

Primers were designed based on the genome of CBS 6340^T and the LDH genes were amplified in the isolates of *L. thermotolerans* from our collection. The Denaturing Gradient Gel Electrophoresis (DGGE) PCR analysis showed three different sequences among the tested isolates for the isoform LDH2, distributed in the isolates according to the quantity of LA produced.

The two isolates with the highest and lowest production of lactic acid, SOL13 and COLC27, respectively, were inoculated in grape juice on a subsequent fermentation trial and the expression of the genes LDH1 and LDH2 was evaluated by real-time PCR. For both genes, the qPCR analysis showed a significant higher expression present in the isolate that produced more lactate, suggesting a possible correlation of this metabolism with the transcriptional regulation of LDH genes.

Even considering a relatively low number of isolates studied, a remarkable diversity was presented in the character studied and some isolates have a potential to be further exploited for their biological acidification of wines, especially for grape musts from warmer regions that could lack on natural acidity. The molecular mechanisms elucidated here could help the optimization of screening

protocols for the selection of isolates with a higher potential for the production of large amounts of LA.

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Chapter 6

Improvement in the quality of wines elaborated with mixed fermentations of non-*Saccharomyces* yeasts and *Saccharomyces cerevisiae*

CHAPTER 6. IMPROVEMENT IN THE QUALITY OF WINES ELABORATED WITH MIXED FERMENTATIONS OF NON-*Saccharomyces* YEASTS AND *Saccharomyces cerevisiae*

6.1. INTRODUCTION

The term "non-*Saccharomyces* yeasts" was used in the past to refer a group of species with secondary relevance during the fermentation of grape musts to wine, considered even as spoilage organisms in some cases. Their influence was usually repressed and even eliminated by the inoculation of selected pure cultures of *S. cerevisiae*. More recently, they are not only recognized by their important contributions to the wine quality, due to the metabolites produced, but also desired and screened for being used as starter cultures themselves. They are usually proposed for their contribution to the aroma profile of wines, but could give more important inputs, such as lowering the alcohol level, modulating the acidity, improving other aspects related to the wine quality, controlling spoilage organisms, optimizing steps along the winemaking process (Andorrà *et al*., 2010; Padilla *et al*., 2016; Wang *et al*., 2016; Petruzzi *et al*., 2017).

The reduction of the ethanol content in wines is a demand from the market, because of consumers' preferences and also from a tributary point of view, since wines with more alcohol are subject to higher taxes in some countries (Pickering, 2000; Kutyna *et al.*, 2010). The use of non-*Saccharomyces* yeasts in multi-starter fermentations could be interesting for the lowering of ethanol by taking advantage of the differences in energy metabolism, altered biomass synthesis, byproduct formation and/or alternative regulation of respiration among the diverse wine species (Gonzalez *et al*., 2013; Mateo and Maicas, 2016; Varela *et al*., 2017).

The contributions to the wine aroma by non-*Saccharomyces* yeasts can occur by different forms. The most important is probably the direct biosynthesis of volatile aroma compounds and a large variety of molecules produced by these yeasts is known (Swiegers *et al*., 2005; Fleet, 2008; Styger *et al*., 2011). Non-*Saccharomyces* can be divided into two groups, those that produce little or no flavor compounds, neutral yeasts, and those that are flavor-producing (Clemente-Jiménez *et al*., 2004). Other metabolites from the non-*Saccharomyces* are

odorless, but can be used as intermediaries in metabolic pathways by *S. cerevisiae* for the release of different aromas, as for example the conversion of acetoin to diacetyl and 2,3-butanediol (Romano and Suzzi, 1993; Mateo *et al*., 2011).

Some non-*Saccharomyces* can produce enzymes that release volatile compounds from glycosidic precursors (Zironi *et al*., 1993; Sarry and Gunata, 2004), other metabolic products include terpenoids, esters, higher alcohols, acetaldehyde, acetic acid, succinic acid, volatile fatty acids, carbonyl and sulfur compounds (Lambrechts and Pretorius, 2000; Capozzi *et al*., 2015; Swiegers and Pretorius, 2005;). Moreover, some of these aroma compounds derived from non-*Saccharomyces* activity are not associated with *S. cerevisiae* metabolism, such as particular terpenoid compounds and monoterpenes (Rossouw and Bauer, 2016).

Acetaldehyde is an important volatile flavor compound found in many food and beverages, that in wine at low levels (below 70 mg/L) can give pleasant fruity aroma, but at high concentrations (above 100 mg/L) produces a green, grassy, nutty or apple-like off-flavor and could be a pungent irritating odor (Dittrich and Barth, 1984; Henschke and Jiranek, 1993; Miyake and Shibamoto, 1993). Considered to be a leakage product of the yeasts alcoholic fermentation, acetaldehyde is extremely reactive and can react with amino acids to generate various flavor compounds (Margalith, 1981; Griffith and Hammond, 1989).

The binding of SO_2 by acetaldehyde limits the sensory effect of acetaldehyde, but also reduces the effectiveness of SO_2 antimicrobial activity and its antioxidative effect. Therefore, due to this phenomenon, more SO_2 is usually added to a wine containing high concentrations of acetaldehyde (Liu and Pilone, 2000). However, as a result of escalating consumer awareness of the adverse health risks related to SO_2, efforts have been prioritized to reduce its contents in wines (Osborne *et al*., 2006), and so the reduction of acetaldehyde levels by use of non-*Saccharomyces* that are lower producers in mixed fermentations could be an alternative for the reduction of SO_2 additions to wine.

Extracellular enzymatic activities, such as proteolytic and pectinolytic, could also be interesting for the winemaking process, helping in technological steps such as clarification, filtration and color extraction (Van Rensburg and Pretorius, 2000; Strauss *et al*., 2001). Regarding other extracellular active molecules released by yeast metabolism, some have been regarded as able to counteract the

development of undesired spoilage microorganisms and thus help to improve the wine stability (Comitini *et al*., 2017).

Glycerol, polysaccharides and mannoproteins can positively influence the wine taste by affecting smoothness, sweetness, mouth-feel and complexity, and several non-*Saccharomyces* have been reported to alter the concentration of those compounds (Ciani and Maccarelli, 1998; Prior *et al*., 2000; Vidal *et al*., 2004; Domizio *et al*., 2014). Glycerol is the second most abundant compound produced during yeast fermentation of grape must, after ethanol, being considered as one of the main contributions of the non-*Saccharomyces*, and plays a role in their metabolism for the regulation of redox potential in the cell (Scanes *et al*., 1998).

Usually present on the skin of grape berries and surface of the winery equipment, the non-*Saccharomyces* are able to initiate the fermentation process, but their persistence during the course of fermentation depends on the winemaking practices, grape must composition and the type of inoculation employed (Bisson and Kunkee, 1993). Nevertheless, these alternative yeasts generally present low fermentation performances and cannot be relied on to complete the alcoholic fermentation in the winemaking process, due to their weak ethanol tolerance. Since their dominance during the early stages of the process can be sufficient to leave their imprint in the wine final composition, they are suitable for the inoculation as mixed starters with strains of *S. cerevisiae*, in order to exploit their positive contribution and avoid the risk of stuck/sluggish or spoiled fermentations (Romano *et al.*, 1997; Jolly *et al*., 2003; Padilla *et al*., 2016).

The metabolic characteristics of non-*Saccharomyces* are very species- and strain-dependent, making the selection of the most suitable strains, starting from a great diversity, an essential step for their biotechnological exploitation, in the same way as it has been for *S. cerevisiae* a few decades before. It should be taken into account that the criteria usually used for the selection of *S. cerevisiae* strains could not be the most adequate for non-*Saccharomyces* though. Features such as fermentation power, high ethanol tolerance, positive killer activity, although desirable for *S. cerevisiae*, are less important for non-*Saccharomyces* than traits like efficient sugar utilization, high enzymatic activity, high production of glycerol and other secondary compounds (Mateo and Maicas, 2016).

The practice of mixed culture fermentation implies a lot of work that has to be done by researchers and oenologists, such as selection of the most suitable strains,

considerations about the interactions between them, decision on the timing and load of inoculation. The proper management of the mixed fermentations is one of the main challenges for the wine industry nowadays and the optimization of this important innovative solution is a key for the production of quality wines with remarkable stylistic distinctions (Ciani *et al*., 2010; Padilla *et al*., 2016).

During the last decades, many studies have been done in mixed fermentations with *S. cerevisiae* and non-*Saccharomyces*, in order to better understand the effects of this interaction in the wines obtained (Ciani *et al*., 2006; Comitini *et al*., 2011; Padilla *et al*., 2016). When the cultures of non-*Saccharomyces* grow together with *S. cerevisiae* strains, it is believed that some possible negative metabolic activities may be modified or even non-expressed due to their interaction (Ciani and Comitini, 2011; Mateo and Maicas, 2016). The impact of the mixed culture in the wine quality will depend on the strains that are used and the inoculation strategies (Maturano *et al*., 2012; Sadoudi *et al*., 2012). During the fermentation process, even the early death of the non-*Saccharomyces* could be beneficial, due to the release of specific nutrients for the ideal growth of *S. cerevisiae*, thus helping for the optimal continuation of the process. However, it is necessary that any killer or other inhibitory compounds, such as medium chain fatty acids, would not be released alongside with the useful nutrients, otherwise the fermentation would instead be adversely affected (Fleet *et al*., 2002; Mateo and Maicas, 2016). The competition for nutrients, especially nitrogen, during the first days of fermentation may also limit the growth and metabolism of *S. cerevisiae* (Medina *et al*., 2012).

Two different approaches have been proposed and tested for the mixed fermentations (Whitener *et al.*, 2016). The co-inoculation involves the addition of the non-*Saccharomyces* and *Saccharomyces* at the same time and the concentration could vary between them. The second strategy, sequential inoculation, implies the addition firstly of the non-*Saccharomyces* species and subsequently *S. cerevisiae*, so the previous can ferment on their own for a given amount of time before *S. cerevisiae* takes over the fermentation. (Padilla *et al*., 2016). The second technique gives more time to the non-*Saccharomyces* to express their metabolic reactions without the competition of *S. cerevisiae* (Whitener *et al*., 2015), but the extent of their effect in the wine quality will greatly depend on the concentration of cells inoculated and the ratio non-

Saccharomyces/*S. cerevisiae* (Comitini *et al*., 2011). Both strategies were able to mimic the natural process of spontaneous fermentation in the goal of having wines with improved complexity (Whitener *et al.*, 2016); nevertheless, most studies claim that sequential inoculation would be the best option (Benito *et al*., 2016).

With the goal of evaluating the potential of three different species of non-*Saccharomyces* species, series of fermentation trials were set up applying sequential inoculation with a commercial strain of *S. cerevisiae* and some of the isolates identified, characterized and selected in the previous chapters, using grape juice and must with different nutritional composition.

The fermentation kinetics and growth profile of the yeasts were followed during the whole process, and an extensive analysis of important chemical parameters was carried out at the end, in order to help understanding how the interactions between the different species in fermentation media with different composition affected the wine style and quality. As one of the main reasons to the use of non-*Saccharomyces* yeasts in wine fermentations is their possible contribution to the aroma, it was also very important to obtain the complete profile of volatile compounds produced during the different fermentations.

6.2. MATERIAL AND METHODS

6.2.1. Yeast strains and culture conditions

From the isolates of three oenologically important genera belonging to the yeast culture collection organized as explained in Chapter 2 and characterized in Chapters 3, 4 and 5, nine were selected for the sequential microfermentations, three of each genera. The commercial strain EC 1118 of *Saccharomyces cerevisiae* was used as control and to complete the fermentations.

The isolates maintained in the freezer at -80 ºC were reactivated on WL (Sigma-Aldrich) agar plates, incubated at 27 ºC for 72 hours. One single colony was then inoculated in YPD broth (yeast extract, 1.0%; bacteriological peptone, 2.0%; glucose, 2.0%; Sigma-Aldrich) and incubated under static conditions at 27 ºC for 48 hours. A fresh YPD-containing tube was then inoculated at 1% with the previous grown culture and put in agitation overnight at 27 ºC to reach the

early stationary phase. The cultures were then centrifuged at 5,000×*g* for 10 minutes, washed twice in physiological solution 0.9% (w/v) NaCl (Sigma-Aldrich) and resuspended in the pasteurized grape juice or untreated must used for the microvinification. The cells were microscopically counted in order to calculate the volume for inoculation.

6.2.2. Grape musts and sequential inoculation

Two sets of microvinification trials were carried out, with the same scheme of inoculation but using two different fermentation media. Firstly, a commercial biological red grape juice, filtered and pasteurized at 70 ºC (Folicello), made with Lambrusco Grasparossa di Castelvetro e Sangiovese grapes (225 g/L of sugar, 46.5 mg/L of YAN (Yeast Available Nitrogen) and 18 mg/L of SO_2). Subsequently, a second trial was performed in fresh untreated must obtained by the pressing of Pinot Grigio grapes (236 g/L of sugar, 235.5 mg/L of YAN and 2.5 mg/L of SO_2).

The nine isolates were inoculated at a concentration of 1×10^6 cells/mL in four replicates in sterile 200-mL glass bottles filled to the top, equipped with perforated silicon stoppers combined with 0.45-mm filters (Millipore) to let the carbon dioxide release and prevent contamination, kept under static conditions at 22 ºC. The strain EC 1118 was inoculated at the same concentration in all bottles containing the non-*Saccharomyces* yeasts, after 48 hours. Control fermentations were prepared with single inoculation of EC 1118, one set of inoculations at the same time as the non-*Saccharomyces* and another contemporary with the sequential inoculations.

6.2.3. Analytical determinations

The weight loss was measured daily in three of the replicates, while the fourth replicate was used for taking samples to the microbiological analysis. Cell concentration was followed throughout the fermentation by means of plate counts. Serial dilutions were prepared in physiological solution (0.9% NaCl; Sigma-Aldrich) and plated on WL nutrient agar (Sigma-Aldrich). After 48 hours

incubation at 27 ºC, the cells could be differentially counted based on the morphological particularities presented by most non-*Saccharomyces* species that distinguish them from *S. cerevisiae* (Pallman *et al.*, 2001).

When the daily weight loss was less than 0.05 g/L, the fermentations were finished by the addition of 100 mg/L of potassium metabisulfite ($K_2S_2O_5$; Sigma-Aldrich), the bottles were hermetically closed and kept at 4 ºC until further analysis. Before the chemical analysis, the wines were centrifuged at 5,000 rpm for 10 minutes and the clarified supernatants were then loaded in an automated enzymatic analyzer (Y15, BioSystems). Specific enzymatic kits were employed for the optical measurements of absorbance in the following determinations: residual sugars (glucose/fructose), acetic acid, primary amino nitrogen (PAN), ammonia, free sulfite, total sulfite, acetaldehyde, glycerol and ethanol. YAN is calculated by the sum of PAN and ammonia. L(+)-Lactic acid was measured manually with an enzymatic kit (Megazyme), only for the bottles inoculated with *L. thermotolerans* and *S. cerevisiae*.

Volatile compounds were quantified as described by Slaghenaufi and Ugliano (2018). An aliquot of 50 mL of the wine was diluted 1:1 with distilled water and 100 μL of internal standard solution (octen-2-ol at 42.0 mg/L in ethanol, Sigma-Aldrich) were added. Subsequently, solid phase extraction (SPE) was performed with Bond Elut ENV cartridges (Agilent Technologies) and the free volatile compounds were eluted with dichloromethane (CH_2Cl_2, Sigma-Aldrich). They were then evaluated by GC-MS (Gas Chromatography-Mass Spectrometry) analysis, performed in an HP 7890A gas chromatograph coupled to a 5977B quadrupole mass spectrometer (Agilent Technologies), equipped with a MPS3 autosampler (Gerstel). Separation was performed using a DB-WAX capillary column (30m × 0.25, 0.25 μm film thickness, Agilent Technologies) and helium as carrier gas at 1.2 mL/min of constant flow rate. GC oven was programmed as follow: started at 40 ºC for 5 min, raised to 240 ºC at 4 ºC/min and maintained for 10 min. Mass spectrometer operated in electron ionization at 70 eV with ion source temperature at 200 ºC and quadrupole temperature at 150 ºC, scanning from 47 to 400 m/z. For quantification, mass spectra were acquired in Selected Ion Monitoring mode.

The comparison between the different treatments was performed based on One-way ANOVA (ANalysis Of VAriance) with post-hoc Tukey's HSD (Honestly Significant Difference) test. The Principal Component Analysis (PCA) were calculated with the results of the chemical determinations and the volatile compounds quantification by using the software for statistical analysis PAST (Hammer *et al*., 2001).

6.3. RESULTS AND DISCUSSION

6.3.1. Fermentation kinetics

The molecular and physiological characterization carried out with 104 isolates from the genera *Lachancea*, *Starmerella* and *Metschnikowia* enabled us to see some significant differences between them. Considering the most tolerant yeasts in the stress assays, the presence of interesting enzymatic activities, lower production of H_2S, fermentation performance and the profiles of wines obtained with single inoculation, it was possible to draw some characteristics that would be more desirable from the oenological point of view. Among the isolates possessing those characteristics, it was taken into account then the similarities in the molecular typing, in order to select different strains. This way, three strains of each of those genera were chosen for the sequential fermentation assays, having interesting profiles but also still some diversity, aiming to confirm how the preliminary features evaluated would have an impact in the wines obtained after interaction with *S. cerevisiae*, responsible for completing the fermentation of the reduced sugars. The chosen non-*Saccharomyces* isolates were: COLC27, DESP53 and SOL13 (*L. thermotolerans*); COLR7, FIANO12 and SOUV1 (*Metschnikowia* spp.); CHIAR4, MALV45 and PECO10 (*S. bacillaris*).

Two different media were used for microvinifications: a commercial grape juice from a producer that claims organic production without addition of any antimicrobials, treated only by pasteurization and filtration to eliminate all possible contamination with the spontaneous microbiota; and a fresh grape must that did not receive any thermal treatment or addition of SO_2, kept refrigerated during the transportation to the laboratory until the moment of inoculation. The

concentration of some nutrients, especially nitrogen-based, were significantly different in the two media.

With the data from the weight loss measurement, it was possible to obtain the graphic of the carbon dioxide production over time, represented by gCO_2 produced/100 g of grape juice or must (Figures 6.1 and 6.2).

As expected, the commercial strain of *S. cerevisiae* started the fermentation more vigorously than the non-*Saccharomyces* isolates, but following the sequential inoculation the fermentation went on with a similar profile in all bottles with *S. bacillaris* and *Metschnikowia* spp., while the fermentations with *L. thermotolerans* started to slow down after the first five days. The fermentations in the pasteurized grape juice (Figure 6.1) went on a rather slow speed and took a long time to finish: 44 days for the control with pure *S. cerevisiae* and 49 days for the mixed fermentations with the strains of *Metschnikowia* spp. and *S. bacillaris*. After 70 days, the bottles with isolates of *L. thermotolerans* were still decreasing more than 0.1 g/L per day, but the fermentations were stopped.

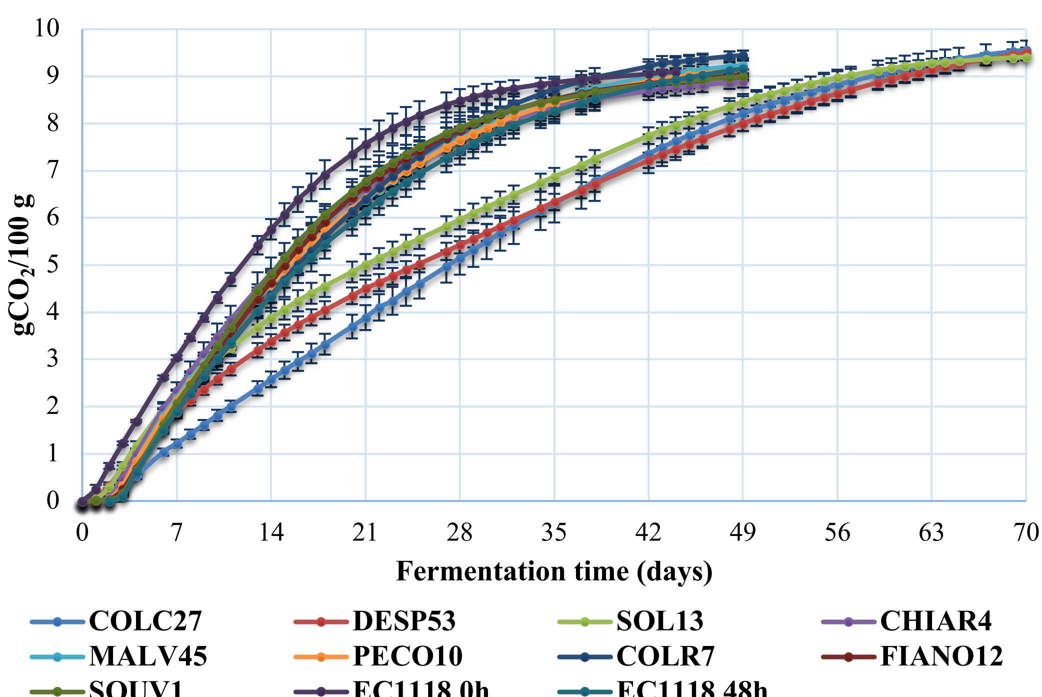

Figure 6.1. Fermentation kinetics of the microvinification trials in pasteurized grape juice.

Fermentations in fresh grape must ran optimally (Figure 6.2), much faster than in the juice, and the pure culture of *S. cerevisiae* finished it within 9 days, while

all the sequential inoculations reached the conclusion after 14 days. There could not be seen significant differences among the isolates of non-*Saccharomyces* in the fermentation kinetics. Nevertheless, in comparison with the control inoculated with single *S. cerevisiae*, it could be noted that the mixed fermentations slowed down the conclusion of the process by a few days.

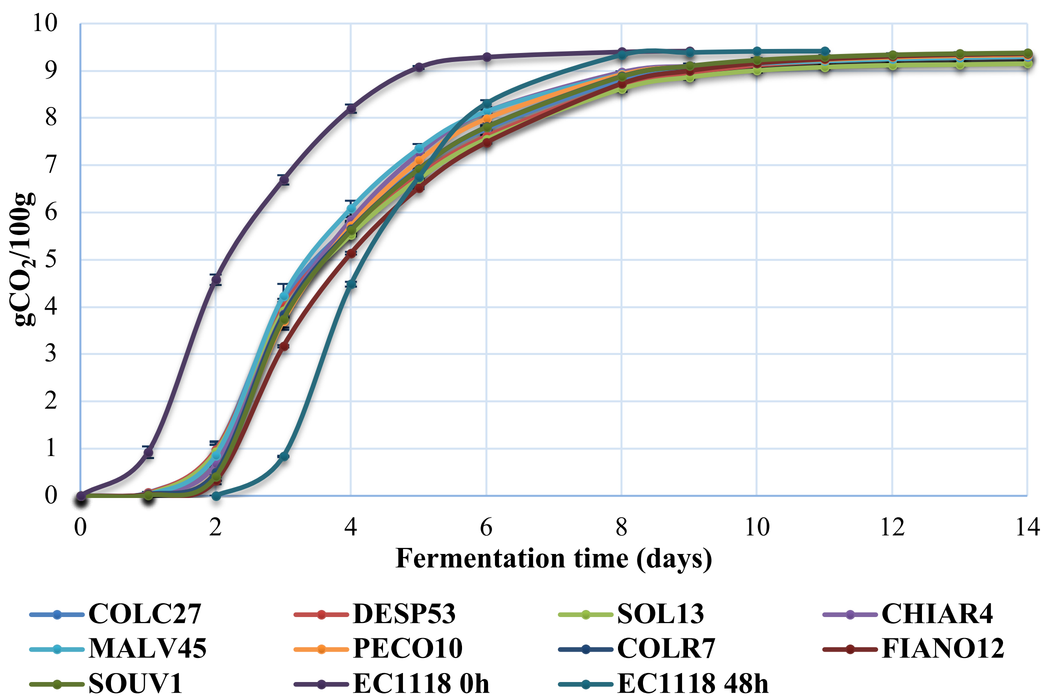

Figure 6.2. Fermentation kinetics of the microvinification trials in natural grape must.

Englezos and co-workers (2018b) found exactly the same results as in the present study, when working with the pair *S. bacillaris*/*S. cerevisiae* in white grape must: 14 days for the conclusion of the mixed fermentations and 9 days for the control single inoculation. Using also the same species and strategy of inoculation, those authors found a similar result in red grape must, where the mixed inoculation took 10 days and the single 7 days (Englezos *et al.*, 2018a); while Lemos Junior and collaborators (2016) verified a delay of four days in the conclusion of the mixed fermentation compared to the single in Incrocio Manzoni grape must (14 and 10 days, respectively). An interval of three days was seen between the conclusion of fermentation in mixed cultures of commercial strains of *M. pulcherrima* and *L. thermotolerans* with *S. cerevisiae* and the control *S. cerevisiae*, in Shiraz wines (Hranilovic *et al.*, 2018). A delay of four days was

observed for sequential inoculation of *L. thermotolerans* and *S. cerevisiae* in Airén wine, where the mixed culture took 14 days to finish and the single *S. cerevisiae* only 10 days (Benito *et al*., 2016). In white variety Emir, Balikci and colleagues (2016) observed that the sequential inoculation of *L. thermotolerans* and *S. cerevisiae* resulted in a delay of two days in the completion of the fermentation when compared to the pure culture of *S. cerevisiae*; the same results observed in Sangiovese and Cabernet Sauvignon (Gobbi *et al*., 2013).

Hence, longer fermentation times observed for mixed inoculations were not surprisingly seen in this study, as they have been long described in literature. Although timely and reliable completion of fermentation are of primary importance in the wine industry, the trend of mixed fermentations considers the advantages of this approach and the use of non-*Saccharomyces* is thought to compensate the slower fermentation with the gains in quality (Hranilovic *et al*., 2018). It is possible to consider the slower fermentation kinetics for the mixed cultures as relevant for a better retention of volatile compounds and a reduction on the demand for energy in the winery to cool down tanks that observe too vigorous fermentations (Medina *et al*., 2013).

From the chemical analysis to characterize also the fermentation media before the inoculation, it was shown that the pasteurized grape juice contains five times less nitrogen available for the nutrition of the yeasts and seven times more sulfite than the grape must. The quantity of sugar available was similar between them. It can be discussed then that possibly the grape juice applied for the first fermentation trial was lacking on essential nutrients, or may contain some compounds derived from the thermal treatment, that could have caused inhibition of the yeast starters inoculated. Specific studies addressing the impact of nitrogen supply and other nutritional compounds in the dynamics of non-*Saccharomyces* and *S. cerevisiae* fermentations are still scarce (Lage *et al*., 2014). All the isolates and the commercial strain struggled to grow in the grape juice and the non-ideal conditions can also have accentuated the competition among them. In the grape must, on the other hand, there was the necessary amount of nutrients to allow the optimal development of the inoculated yeasts and inhibitory compounds were not present in quantities that could have harnessed their growth.

The vigor of the fermentation depends on the behavior of the non-*Saccharomyces*, but most of all on the metabolism of the *S. cerevisiae*

sequentially inoculated and the interactions between them. For this reason, it is important to follow also the cell concentration of both species during the process.

Indeed, some differences could be seen in the microbial populations throughout the process. Figures 6.3 and 6.4 show the concentrations of the non-*Saccharomyces* isolates and the commercial *S. cerevisiae* inoculated at each bottle, represented by the decimal logarithm of the CFU/mL during the fermentation process. The concentration of EC 1118 cells during the control single fermentation is also shown within each graphic to have a comparison of the effect of the interaction in the growth of *S. cerevisiae*.

There can be seen major differences in the behavior of the three non-*Saccharomyces* species and subtle differences among the isolates of the same species. During the fermentation of grape juice (Figure 6.3), the strains of *L. thermotolerans* caused the highest inhibition of the co-inoculated *S. cerevisiae* and DESP53 and SOL13 even dominated the fermentation until around 20 days. DESP53 reached a maximum concentration of 1.4×10^7 CFU/mL after four days of inoculation. COLC27 also inhibited *S. cerevisiae*, but at a lesser extent than the others and was not able to dominate. The three *L. thermotolerans* were detected in the bottles until 35 days after the inoculation.

S. bacillaris showed equivalent concentrations with the sequentially inoculated yeast until about 16 days of fermentation and CHIAR4 and MALV45 were detectable until 29 days, while PECO10 until 35 days. The maximum cell concentration was reached by MALV45 after only two days of inoculation, at 1.3×10^7 CFU/mL. The isolates of *Metschnikowia* spp. could grow well in the first days of fermentation, especially FIANO12, who reached a cell counting of also 1.3×10^7 CFU/mL, four days after the inoculation, similarly to the other non-*Saccharomyces*. However, they were dominated immediately after the sequential inoculation with *S. cerevisiae* and FIANO12 disappeared after around 16 days, while COLR7 survived until 13 days and SOUV1 was already not detectable after only 4 days. The decrease of *Metschnikowia* species in the early days of mixed fermentations due to the low ethanol resistance and sensitivity to the inhibition by other microorganisms was already reported for this genus (Sadoudi *et al.*, 2012; Contreras *et al.*, 2015; González-Royo *et al.*, 2015; Varela *et al.*, 2016; Wang *et al.*, 2016; Barbosa *et al.*, 2018).

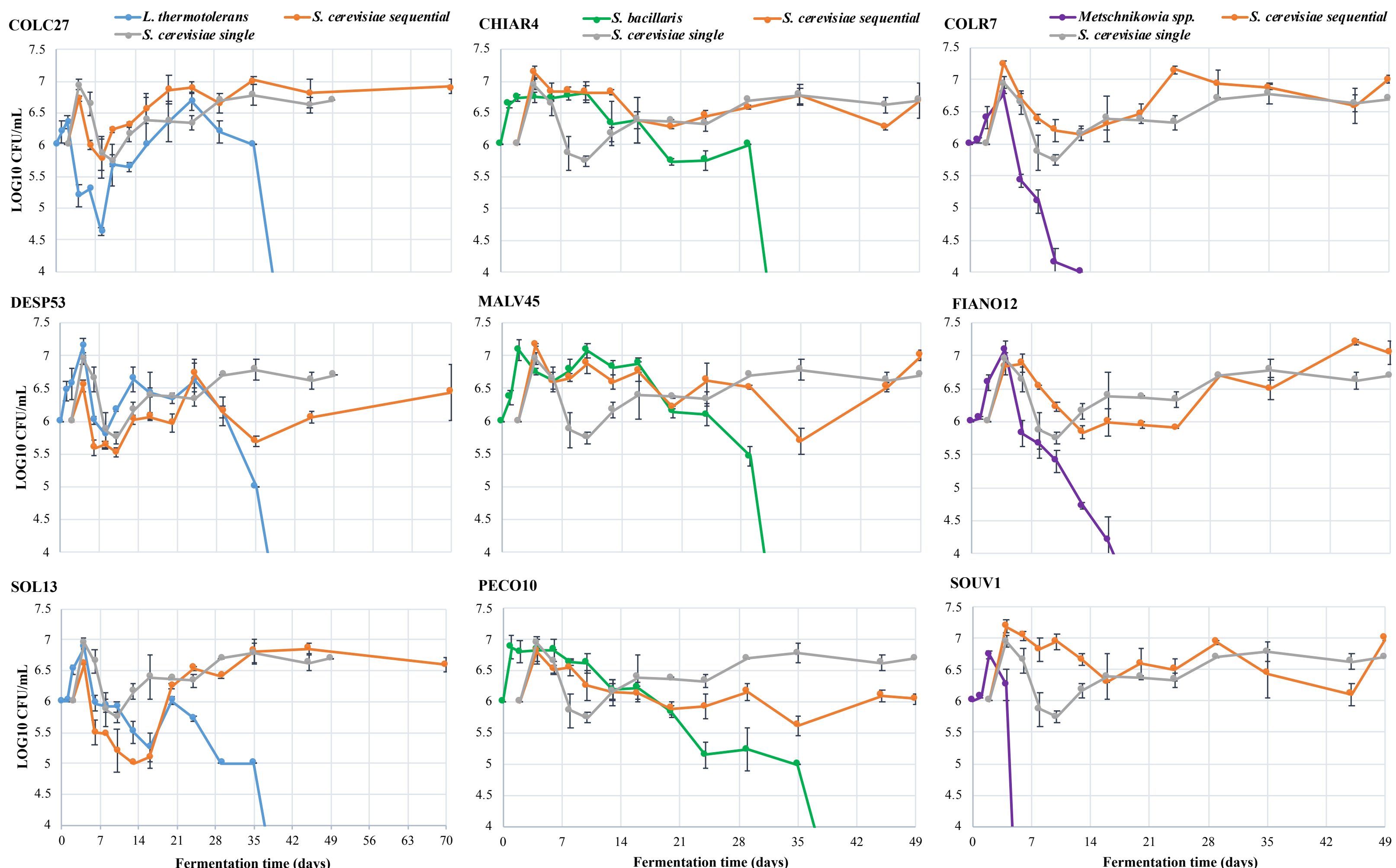

Figure 6.3. Population dynamics during mixed fermentations of pasteurized grape juice.

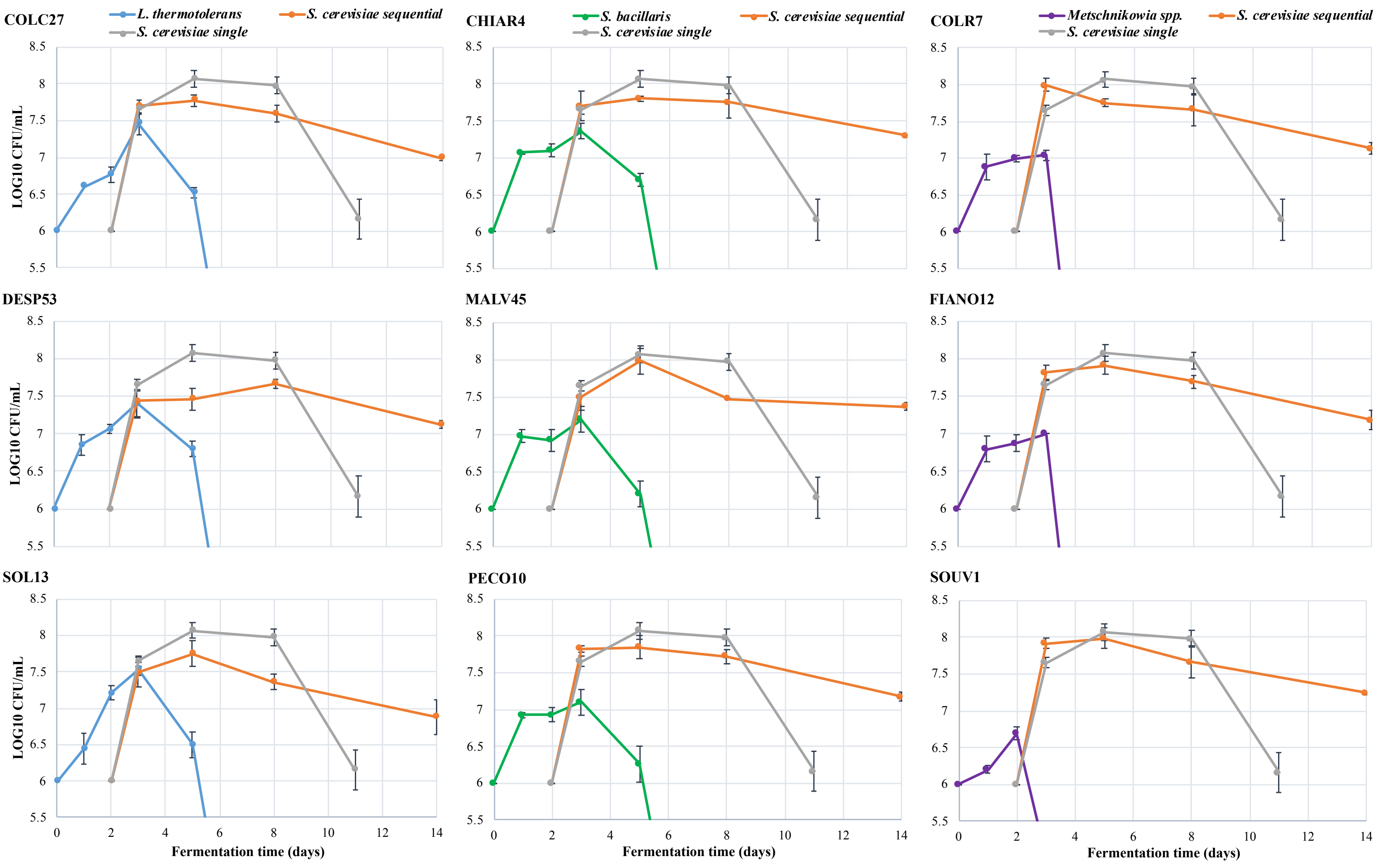

Figure 6.4. Population dynamics during mixed fermentations of natural grape must.

Looking at the growth kinetics it is possible to suppose that the slower fermentation observed for the mixed fermentations with *L. thermotolerans* could be the result of the reduced growth of *S. cerevisiae* during the first 20 days of interaction with the isolates of that species. Only the successive analysis could show whether this behavior is negative or positive for the outcome of the vinification process.

Although the fermentation in grape must was much faster, the results of the microbial dynamics during the shorter period of the process can be related to the previous results, with *L. thermotolerans* showing slightly higher inhibition of *S. cerevisiae* and *Metschnikowia* spp. being the first to decline facing the rising ethanol concentration (Figure 6.4). Nevertheless, the isolates of *S. bacillaris* and *L. thermotolerans* were immediately overcome by *S. cerevisiae* after the sequential inoculation and could be detected only until the fifth day of fermentation. The same behavior was observed for a sequential inoculation of *L. thermotolerans* with *S. cerevisiae* in Tempranillo, Airén and Riesling wines, where the non-*Saccharomyces* started to decline very fast following the inoculation of *S. cerevisiae* (Benito *et al*., 2015a; 2015b; 2016).

Besides the limitation caused by the rising ethanol concentrations, another possible reason for the loss of viability of the non-*Saccharomyces* in the mixed fermentations could be the decrease of the redox potential in the fermented media, since the alcoholic fermentation creates anaerobic conditions in the bottles (Lage *et al*., 2014). It was shown by Hansen and colleagues (2001) that the availability of oxygen increased the survival time of *Torulaspora delbrueckii* and *L. thermotolerans*. The secretion of antimicrobial peptides by *S. cerevisiae* and cell-cell contact were also already reported as causing inhibition of non-*Saccharomyces* growth (Albergaria *et al*., 2010, Renault *et al.* 2013).

All yeasts, *Saccharomyces* and non-*Saccharomyces*, were able to reach higher cell concentration in the must in comparison with the pasteurized grape juice, reinforcing the suggestion that the composition of the last somehow hindered the yeast metabolism. Three days after the inoculation, the isolates of *L. thermotolerans* ranged from 2.5 to 3.4×10^{7} CFU/mL, *S. bacillaris* between 1.3-2.2×10^{7} CFU/mL, and *Metschnikowia* spp. reached around 1.1×10^{7} CFU/mL

(with the exception of SOUV1, who reached the maximum of 4.9×10^6 CFU/mL in the second day and could not be detected anymore after the third day).

S. cerevisiae was able to reach 1.1×10^8 CFU/mL on the third day in the control single inoculation of grape must, while the maximum concentration achieved by the control in the fermentation in grape juice was 8.7×10^6 CFU/mL, even if the higher value for *S. cerevisiae* in the grape juice was during the mixed fermentation with COLR7, at 1.8×10^7 CFU/mL, clearly showing the influence of the medium in the yeast growth. The same observation was made by a single culture of *S. cerevisiae* in Malvasia Fina and Arinto grape must with two concentrations of YAN, 175 and 387 mg/L, where a higher cell population and higher fermentation rate were found in the must with higher YAN. In the mixed culture, lower cell concentrations and longer fermentation length were registered, under both nitrogen regimes (Lage *et al*., 2014).

However, our findings are not in total agreement with the conclusion of Barbosa and co-workers (2018), who carried out single and mixed fermentations in grape musts from Tinta Roriz grapes, one batch with an initial nitrogen supply of 73.5 mg/L YAN and another supplemented to a concentration of 280 mg/L YAN. They did not detect differences in the growth rates between the two fermentation media, although significant differences were found in the maximum fermentation rate. The process was completed much faster in the must with more YAN, as it was in our case.

6.3.2. Chemical analysis

Even if any of the non-*Saccharomyces* isolates could survive until the end of the fermentations in grape juice and grape must, they were all able to actively grow during the first 48 hours before the sequential inoculation and in some cases also after that. The extent of the impact caused directly by their metabolism and by the interaction with *S. cerevisiae* in the wine composition can be evaluated by the chemical analysis that were carried out. The results for the first fermentation, in the pasteurized grape juice, were summarized in Table 6.1, while the second fermentation is illustrated in Table 6.2.

Table 6.1. Chemical analysis at the end of sequential fermentations in pasteurized grape juice, represented as the mean ± standard deviation of three replicates.

Yeast	Residual Sugars (g/L)	Ethanol (%v/v)	Acetic Acid (g/L)	YAN* (mg/L)	Total Sulfite (mg/L)	Free Sulfite (mg/L)	Acetaldehyde (mg/L)	Glycerol (g/L)	L(+)Lactic Acid (g/L)
Lachancea thermotolerans									
COLC27	5.65±3.19^{bc}	12.15±0.28^{c}	0.53±0.032^{bc}	14.67±0.58^{ab}	57.67±1.53^{a}	6.67±0.58^{bc}	20.0±2.0^{ab}	2.40±0.26^{c}	0.38±0.12^{a}
DESP53	7.17±2.11^{c}	12.03±0.16^{abc}	0.57±0.01^{cd}	14.50±0.71^{ab}	66.33±0.58^{cde}	8.33±0.58^{c}	22.0±1.0^{ab}	3.40±0.26^{d}	0.89±0.15^{a}
SOL13	0.52±0.23^{a}	12.03±0.09^{abc}	0.48±0.012^{b}	15.33±1.15^{ab}	60.67±0.58^{ab}	9.67±0.58^{c}	14.33±0.58^{a}	2.37±0.38^{c}	3.44±0.73^{b}
Starmerella bacillaris									
CHIAR4	1.40±0.54^{ab}	11.52±0.017^{a}	0.57±0.01^{cd}	14.67±1.53^{ab}	64.33±3.06^{bcde}	6.67±2.08^{bc}	24.33±3.21^{bc}	1.05±0.21^{ab}	
MALV45	2.09±0.26^{abc}	12.09±0.025^{abc}	0.64±0.03^{e}	14.67±2.08^{ab}	64.33±0.58^{bcde}	7.33±0.58^{bc}	23.33±3.21^{b}	1.30±0.14^{ab}	
PECO10	1.38±0.2^{ab}	11.87±0.34^{abc}	0.63±0.045^{de}	16.33±2.08^{b}	64.33±2.31^{bcde}	6.67±1.53^{bc}	25.67±4.04^{bc}	1.85±0.21^{bc}	
***Metschnikowia* spp.**									
COLR7	2.10±1.36^{abc}	12.14±0.13^{bc}	0.53±0.0^{bc}	17.50±0.71^{b}	68.50±2.12^{de}	4.50±0.71^{ab}	33.0±1.41^{cd}	3.90±0.0^{d}	
FIANO12	1.75±1.27^{ab}	11.60±0.16^{ab}	0.37±0.014^{a}	14.0±0.0^{ab}	63.0±0.0^{abcd}	4.50±0.71^{ab}	28.0±1.41^{bc}	4.15±0.35^{d}	
SOUV1	1.75±0.5^{ab}	11.56±0.082^{a}	0.37±0.01^{a}	13.67±2.52^{ab}	62.33±2.52^{abc}	6.67±0.58^{bc}	27.0±2.83^{bc}	4.05±0.071^{d}	
Saccharomyces cerevisiae									
EC 1118	0.91±0.76^{a}	11.68±0.063^{abc}	0.56±0.015^{bc}	11.0±0.0^{a}	68.67±3.06^{e}	3.0±1.41^{a}	40.0±4.24^{d}	0.80±0.28^{a}	0.17±0.14^{a}

*YAN = primary amino nitrogen + ammonia.

Different letters in the same column indicate a significant difference in Tukey's HSD test ($p<0.05$).

For the first round of fermentations, in grape juice, it can be seen that only the control inoculation with *S. cerevisiae* and the mixed inoculation with SOL13 and *S. cerevisiae* were able to consume almost all the available sugar, while in the other bottles there were more than 1 g/L of sugar, even after more than 40 days of fermentation. The fermentations with *S. bacillaris* and *Metschnikowia* spp. were considered finished when the weight was constant, but those with *L. thermotolerans* had to be stopped after 70 days, although a slight weight loss was still being registered. Indeed, in the fermentations with COLC27 and DESP53 there were still more than 5 g/L of sugars.

The production of ethanol showed some diverging profiles among the isolates of the same species. Two isolates of *Metschnikowia* spp. (FIANO12 and SOUV1) and one of *S. bacillaris* (CHIAR4) were able to reduce the ethanol content in the mixed fermentations compared to the control, while the six other isolates caused an increase in the production of ethanol. Specially the isolates of *L. thermotolerans* were responsible for a higher yield in the ethanol production, since they consumed less sugars and nevertheless raised the ethanol content by at least 0.35% (v/v).

As regarding the volatile acidity, the strains of *L. thermotolerans* did not caused differences in the mixed fermentations compared to the control, while two *S. bacillaris* generated a higher production of acetic acid and two *Metschnikowia* spp. helped to reduce its level.

The quantity of available nitrogen at the end of fermentations showed that *S. cerevisiae* consumed it more in the single fermentations than in the sequential inoculations, even if the pure fermentation was shorter and some isolates of non-*Saccharomyces* reached higher concentrations of cells during the process than the *S. cerevisiae* in the control. Little is known about the nutritional needs of the non-*Saccharomyces* yeasts, but is possible that they needed less than *S. cerevisiae* and the competition between them may have caused a reduction in the consumption. The starting amount of YAN was rather low (46.5 mg/L), however there was still 30% of that at the end of the process, represented in almost all its totality by the ammonia component, while the PAN fraction was almost completely consumed by all isolates.

All mixed fermentations showed a lower level of SO_2 at the end than the single EC 1118, but significantly only two isolates of *L. thermotolerans* (COLC27

and SOL13) and two of *Metschnikowia* spp. (FIANO12 and SOUV1) could be considered for a reduction in the level of sulfite. For the free fraction of the sulfites, the single fermentation had the lowest value, even having the highest total SO_2, showing that most of it is in the bounded form. The isolates of *L. thermotolerans* had the most free SO_2, followed by *S. bacillaris* and then *Metschnikowia* spp.

The production of acetaldehyde and glycerol were also significantly different in all treatments in comparison with the control. All isolates helped to reduce the level of acetaldehyde, especially those of *L. thermotolerans*. It is interesting to note how the level of acetaldehyde is related to that of free SO_2, because acetaldehyde is one the main responsible for linking to the SO_2 and thus reducing its level. The lower concentrations of acetaldehyde (*L. thermotolerans*) corresponded to the highest level of free SO_2, and the highest acetaldehyde content (EC 1118) was present in the bottles with less free SO_2.

On the other hand, the production of glycerol was higher in all mixed cultures than in the single. The isolates of *Metschnikowia* spp. can be highlighted for an increase of around five times the content of glycerol in wines. An increased production of glycerol is usually linked to increase also in acetic acid production (Prior *et al*., 2000), but exactly the opposite trend was observed among the mixed fermentations with our isolates of non-*Saccharomyces*.

L(+)-Lactic acid (LA) metabolism followed the same trend as the trials shown in Chapter 5, with SOL13 being the highest producer, followed distantly by DESP53 and COLC27. However, the values in the sequential fermentations were significantly lower than those presented by *L. thermotolerans* in the single inoculation trials, possibly due to the competition with *S. cerevisiae* for the available sugar.

The results of all replicates from the ten different combinations of inoculation were summarized in a PCA, except the data of LA production, and are shown in Figure 6.5.

The Principal Component Analysis showed a clear separation of the isolates based on the species and the first and second components were able to explain 49.7% of the variability. Generally speaking, *Metschnikowia* spp. produced more glycerol and acetaldehyde, less ethanol and acetic acid. *L. thermotolerans* isolates did not finish the fermentation, leaving more residual sugars, produced more

ethanol though, and less acetaldehyde. The isolates of *S. bacillaris* were differentiated by the higher production of volatile acidity and reduced production of glycerol. Nevertheless, it is important to highlight that some differences were observed among strains of the same species, more pronounced for *Metschnikowia* spp. and less for *S. bacillaris*, as it was observed in the molecular and physiological characterization described in Chapter 3, when the tested isolates of *Metschnikowia* spp. showed much more intraspecific variability than those of *S. bacillaris*.

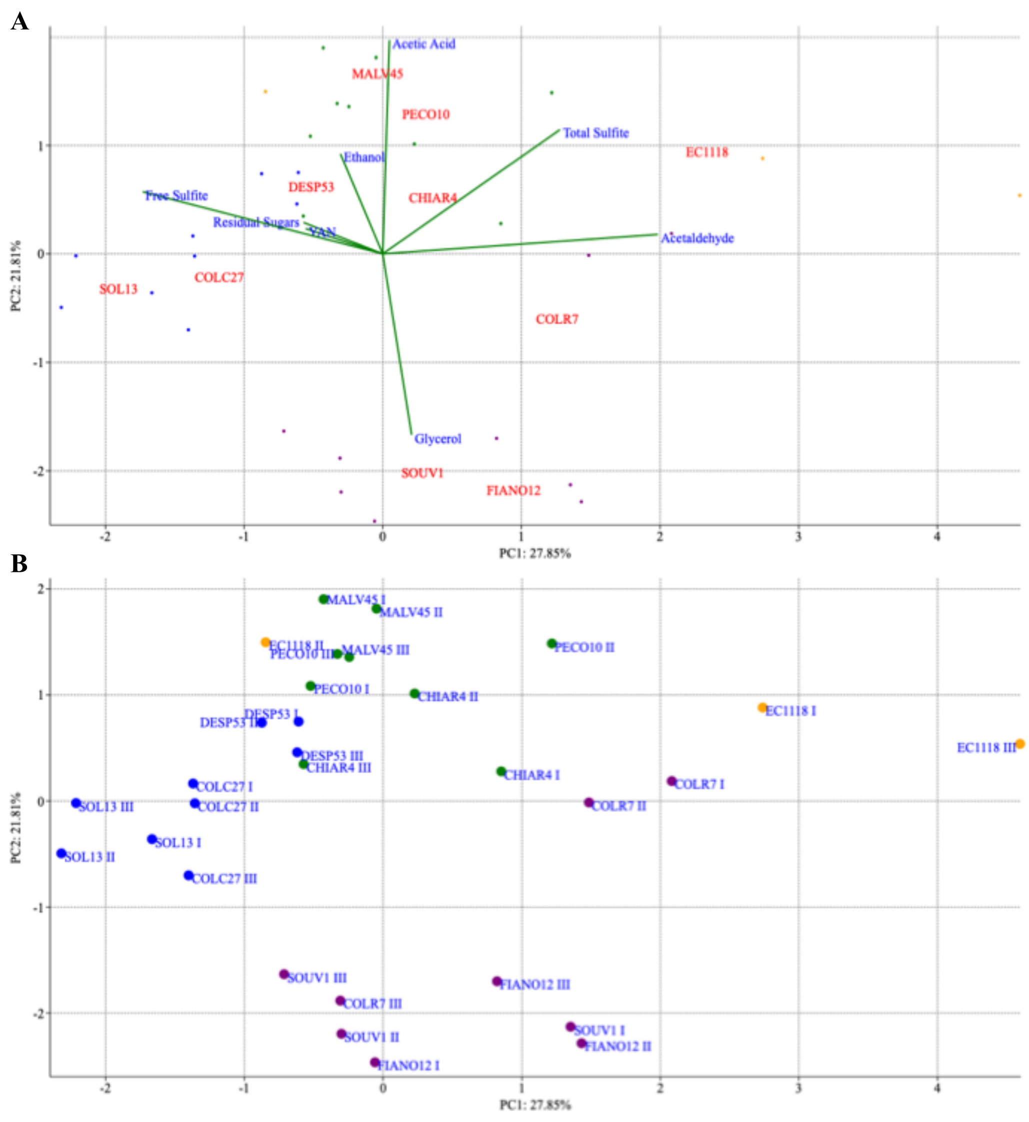

Figure 6.5. [A] Loading plot and [B] Score plot of the Principal Component Analysis of some important oenological parameters obtained after the sequential inoculation of pasteurized grape juice with isolates of non-*Saccharomyces* and *S. cerevisiae*.

In the comparison between the mixed fermentations and the control with single inoculation of *S. cerevisiae*, it could be highlighted the reduction in the levels of acetaldehyde and increase in the production of glycerol, maintaining the values of acetic acid within the acceptable range. Although the fermentations in the pasteurized grape juice did not proceed with optimal kinetics and development of the yeasts, as it was show in the previous graphics (Figure 6.1 and 6.3), it was interesting to emphasize some positive impacts caused by the addition of non-*Saccharomyces* to the fermentation process.

Table 6.2 summarized the results from the second fermentation trial, in Pinot Grigio grape must. Confirming the results already observed in the graphics of the fermentation kinetics and microbial concentrations (Figure 6.2 and 6.4), the chemical analysis also shown that the fermentation in grape must ran in optimal conditions and the medium was more suitable for the yeast development than the grape juice used previously.

All strategies of inoculation reached the conclusion of the fermentation and were able to consume the available sugars almost in their totality. Due to the metabolism of the non-*Saccharomyces* strains, the ethanol content was reduced in all mixed inoculations when compared to the control, and it was more evident for *L. thermotolerans* and *S. bacillaris*. This observation is in accordance with several previous studies of mixed fermentations, who justified the results with the respiratory metabolism of non-*Saccharomyces* strains (Kutyna *et al*., 2010; Contreras *et al*., 2014; Quiros *et al*., 2014; Morales *et al*., 2015, Benito *et al*., 2015b).

The levels of acetic acid produced were much lower than those registered for the fermentation in grape juice, which is an advantage since this by-product could depreciate the wine quality (Barbosa *et al*., 2018). It has been already suggested that acetic acid could be increased in excessively clarified grape musts, due to the removal of essential constituents such as polyphenolic compounds, unsaturated fatty acids or solid particles (Garcia-Moruno *et al*., 1993; Guilloux-Benatier and Feuillat, 1993), and this seem to be the case in our experiments. The strains of non-*Saccharomyces* maintained in the mixed fermentations approximately the same level as that observed for the control, with a slightly increase for SOL13 and the three *S. bacillaris*, however this rise is not excessive and would not reach a threshold that compromise the wine quality.

Table 6.2. Chemical analysis at the end of sequential fermentations of grape must, represented as the mean ± standard deviation of three replicates.

Yeast	Residual Sugars (g/L)	Ethanol (%v/v)	Acetic Acid (g/L)	YAN* (mg/L)	Total Sulfite (mg/L)	Free Sulfite (mg/L)	Acetaldehyde (mg/L)	Glycerol (g/L)	L(+)-Lactic Acid (g/L)
Lachancea thermotolerans									
COLC27	0.09±0.015^{abc}	11.97±0.0092^{bc}	0.19±0.01^{abc}	41.67±0.58^{bc}	49.0±0.0^{b}	<3	40.0±0.0^{cd}	4.67±0.65^{ab}	0.53±0.053^{ab}
DESP53	0.13±0.014^{c}	11.95±0.013^{b}	0.19±0.0058^{ab}	39.67±0.58^{b}	61.0±2.0^{cd}	<3	45.0±0.0^{d}	5.30±0.26^{abc}	1.22±0.24^{b}
SOL13	0.40±0.049^{d}	11.76±0.036^{a}	0.26±0.0071^{de}	48.00±1.41^{d}	65.33±2.08^{d}	<3	56.50±4.95^{e}	5.27±0.55^{abc}	4.42±0.72^{c}
Starmerella bacillaris									
CHIAR4	0.07±0.026^{abc}	11.95±0.011^{b}	0.28±0.01^{e}	30.50±0.71^{a}	41.50±0.71^{a}	<3	31.0±1.41^{ab}	6.30±0.3^{c}	
MALV45	0.06±0.021^{ab}	11.95±0.042^{b}	0.22±0.025^{cd}	32.67±2.08^{a}	38.50±0.71^{a}	<3	28.0±0.0^{a}	5.73±0.32^{bc}	
PECO10	0.07±0.017^{abc}	12.0±0.0047^{bcd}	0.22±0.01^{bcd}	32.33±2.52^{a}	38.0±0.0^{a}	<3	32.0±2.88^{abc}	5.45±0.35^{bc}	
***Metschnikowia* spp.**									
COLR7	0.10±0.0058^{bc}	12.02±0.022^{cd}	0.18±0.015^{a}	45.50±0.71^{cd}	59.0±1.73^{c}	<3	38.0±0.0^{bcd}	4.97±0.42^{abc}	
FIANO12	0.13±0.03^{c}	12.04±0.014^{cd}	0.17±0.0058^{a}	43.0±1.73^{bcd}	60.67±0.58^{cd}	<3	40.67±2.31^{bcd}	5.53±0.57^{bc}	
SOUV1	0.08±0.012^{abc}	12.05±0.03^{de}	0.16±0.01^{a}	47.0±1.41^{d}	58.67±2.08^{c}	<3	41.33±1.53^{d}	4.55±0.21^{ab}	
Saccharomyces cerevisiae									
EC 1118	0.04±0.012^{a}	12.11±0.0091^{e}	0.17±0.0058^{a}	46.0±1.41^{cd}	71.67±1.53^{e}	<3	57.50±2.12^{e}	4.0±0.42^{a}	0.15±0.13^{a}

*YAN = primary amino nitrogen + ammonia.

Different letters in the same column indicate a significant difference in Tukey's HSD test ($p<0.05$).

Testing mixed fermentations of *S. cerevisiae* with six different non-*Saccharomyces* in Sauvignon Blanc, including the three species explored in the present study, Whitener and colleagues (2016) also found that *S. bacillaris* was the highest producer of acetic acid. Nevertheless, other authors already described the effective reduction of volatile acidity level for the pairs *M. pulcherrima/S. cerevisiae* (Comitini *et al*., 2011; Sadoudi *et al*., 2012), *L. thermotolerans/S. cerevisiae* (Gobbi *et al*., 2013; Benito *et al*., 2016) and for S. *bacillaris/S. cerevisiae* (Rantsiou *et al*., 2012).

The three isolates of *S. bacillaris* and one of *L. thermotolerans* (DESP53) needed more nutrients than the others and caused the highest consumption of YAN, significantly higher than the single culture of *S. cerevisiae*, showing that for these treatments some competition for nitrogen compounds could have occurred with *S. cerevisiae*, even though no detrimental effects were seen in the fermentation progress (Figure 6.4). Previous studies have already reported the increase in YAN consumption by mixed fermentation with non-*Saccharomyces* compared to single *S. cerevisiae* (Andorrà *et al*., 2010; Medina *et al*., 2012). Besides high-demanding nitrogen needs, a lower nitrogen release could also be related with lower final YAN levels in the mixed cultures (Benito, 2018).

Comparing with the fermentation in grape juice, the remaining YAN was higher in the fermentation of grape must, however, since the initial level was much higher in grape must, the consumption of YAN was actually almost seven times higher. The quantity of ammonia at the end of fermentations was similar in all fermentations (around 15 mg/L), but the biggest difference is in the primary amino nitrogen. PAN reached zero in the bottles of the first fermentation round, while in the second it was still around 30 mg/L, except for *S. bacillaris*, where it was around 20 mg/L. These results showed how the limitation of PAN could have been one of the main reasons for the abnormally slow fermentations in grape juice compared to the grape must, as it was discussed previously.

The quantity of total SO_2 was significantly lower in all mixed fermentations than in the control, especially for *S. bacillaris*. Other than being directly added to grape must/wine as a preservative during vinification, its presence in wine can be attributed to yeasts, which produce it to varying extents (Jackowetz *et al*., 2010). A possible reason for lower sulfur metabolism could be the lower SO_2 tolerance reported for several strains of non-*Saccharomyces* (Benito Á. *et al*., 2015).

Sulphur dioxide is used as an antimicrobial, antioxidative and anti-enzymatic agent in winemaking (Peynaud, 1984). The total SO_2 consists of bound and free forms, with the former having a weaker antimicrobial function. A number of carbonyl compounds (mainly acetaldehyde, pyruvic acid and a-keto-glutaric acid) can bind with free SO_2 to form a complex compound (bound SO_2). The binding of bisulphite ion by acetaldehyde reduces the availability of free SO_2, resulting in a reduction in the antimicrobial efficacy of SO_2 (Liu and Pilone, 2000). This can explain why SO_2 presence induces the mechanism of resistance of acetaldehyde formation by yeasts (Stratford *et al*., 1987; Pilkington and Rose, 1988). In spite of problems associated with the use of SO_2, it is usually considered difficult to produce good quality wine without it (Somers, 1998). However, it would be possible to reduce the quantity added by the winemaker through the inoculation of yeasts that produce less acetaldehyde and therefore cause less hindrance to the SO_2 effectiveness.

In our fermentations of grape must, it can be supposed that all SO_2 was present in its bounded form, since the quantification of free sulfites resulted lower than the detection limit of the instrument (3 mg/L) for all bottles. This was caused most likely due to the higher quantities of acetaldehyde in all bottles, when compared to the fermentation of grape juice. Nevertheless, almost all non-*Saccharomyces* caused a reduction in the level of acetaldehyde in comparison with single EC 1118, above all *S. bacillaris*, with the *L. thermotolerans* SOL13 as the exception. Moreover, the concentrations of acetaldehyde were directly proportional to the total SO_2 observed. Our non-*Saccharomyces* followed the same behavior described elsewhere, with less acetaldehyde being produced in mixed fermentation than single (Benito *et al*., 2015b; Benito *et al*., 2016). On the contrary of our analysis, Nisiotou and co-workers (2018) found a higher level of total SO_2 and acetaldehyde for the sequential inoculation with *S. bacillaris* than in the control. There are large species and strain differences in the production of this compound, with reported values between 0.5 to 286 mg/L for *S. cerevisiae*, and up to 66 mg/L for non-*Saccharomyces* species, while the olfactory threshold in wines ranges from 100-125 mg/L (Fleet and Heard, 1993; Zoecklein *et al*., 1995; Liu and Pilone, 2000).

The production of glycerol was higher for all treatments in the grape must when compared to grape juice. And in the same way as in the grape juice, in grape

must all mixed inoculations raised the glycerol content with respect to the control. The increase was more evident for the isolates of *S. bacillaris*, who reached the highest level among the non-*Saccharomyces*, in contrast to what was observed in the grape juice, but accordingly to the long reputation of this species as a consistent high glycerol producer (Comitini *et al*., 2011). Glycerol's relevance in the sensorial properties will depend on the style of the wine, generally contributing to smoothness, sweetness and complexity (Ciani and Maccarelli, 1998; Jolly *et al*., 2006).

As regarding LA, all three isolates of *L. thermotolerans* saw an increase in the production in comparison with the grape juice, maintaining again the same relative ranking between them, however the values in the sequential inoculation were still below those reached with *L. thermotolerans* in the single inoculation trials performed before (Chapter 5). *S. cerevisiae*, on the other hand, produced approximately always the same level of LA, much lower than *L. thermotolerans*. The production of LA occurs during the first days of *L. thermotolerans* fermentation, as it was shown in Chapter 5, and is strictly related to its active growth. The exponential decrease in this non-*Saccharomyces* population during sequential and co-inoculations with *S. cerevisiae*, as shown in this study and reported elsewhere, cause a strong decrease in the production of this metabolite. These results confirm the relevance of choosing compatible yeasts strains for mixed cultures and the importance of a inoculation delay for *S. cerevisiae* (Kapsopoulou *et al*, 2007; Benito, 2018). The explanation for the differences observed between the production of LA by the same isolates when in pure cultures or mixed inoculations in grape juice and grape must could be then the different population growth, influenced by the medium composition and the presence of *S. cerevisiae*.

As it has been discussed previously, the biological acidification of wines caused by LA is considered to positively affect their organoleptic properties and microbial stability, being an interesting tool in regions where lack of acidity could be an issue (Jolly *et al*., 2014). In some Italian regions, the necessity to achieve phenolic maturation in some red grape varieties, in order to have more intense and ripe flavor of the wines, and the effects of climate change, result in a general higher pH, one example of context where the use of starter cultures of *L. thermotolerans* could be a solution to the producers' needs (Gobbi *et al*., 2013).

With conditions of mixed inoculation similar to those of this study, other authors found a production of LA of 1.70 g/L (Chen *et al.*, 2018), 3.18 g/L (Benito *et al.*, 2016), 2.75 g/L (Benito *et al.*, 2015a), 0.22 g/L (Benito *et al.*, 2015b), 6.38 g/L at winery scale and 1.55 g/L at lab scale (Gobbi *et al.*, 2013) and 5.13 g/L (Kapsopoulou *et al.*, 2007). This great diversity could be the reflect of strain differences, since LA metabolism can vary greatly among the isolates of *L. thermotolerans*, as discussed in details in Chapter 5.

It should be noted by the higher production of glycerol and lactic acid, and lower production of acetic acid, how the ideal conditions of nutrition in the fermentation medium are critical for the correct development of the yeasts, not only for the faster and more efficient fermentation of sugars, but also from the point of view of the production of those important metabolites.

The results of the chemical analysis in the wines of the second set of fermentation experiments were also organized in a PCA (Figure 6.6), for a better visualization of the effects of the mixed fermentations compared to the control with single starter culture and also the comparison between the nine different non-*Saccharomyces* yeast strains tested.

The PCA showed again a clear separation of the mixed inoculations based on the non-*Saccharomyces* species that were present and the first two components could explain 78% of the variability. It is also interesting to note that the isolates of *Metschnikowia* spp. were positioned closer to the control with single inoculation of EC 1118, while the isolates of *S. bacillaris* seem to produce the wines with more differences. Some dissimilarities were also observed among strains of same species, more markedly for *L. thermotolerans* SOL13.

As for some general observations, *S. bacillaris* strains were responsible for higher production of glycerol and acetic acid, lower production of sulfites and acetaldehyde, and a higher need for nitrogen supply. Those characteristics were more apparent in CHIAR4 than in MALV45 or PECO10.

Recent studies with mixed inoculations of *S. bacillaris* and *S. cerevisiae* in four white (Chardonnay, Muscat, Riesling and Sauvignon Blanc) and four red grape varieties (Cabernet Sauvignon, Merlot, Pinot Noir and Shiraz) have shown a consistent trend in the chemical profile of all the wines, with reduction in ethanol and increase in glycerol, in comparison with the control (Englezos *et al.*, 2018a, 2018b). Also previous studies have already reported the same observations, in

Kotsifali/Mandilari (Cretan grapes; Nisiotou *et al.*, 2018), Incrocio Manzoni (Lemos Junior *et al.*, 2016), Barbera (Englezos *et al.*, 2016), Montepulciano d'Abruzzo (Tofalo *et al.*, 2016), Bovale (Zara *et al.*, 2014), Merlot, Nero d'Avola and Frappato grapes (Giaramida *et al.*, 2013).

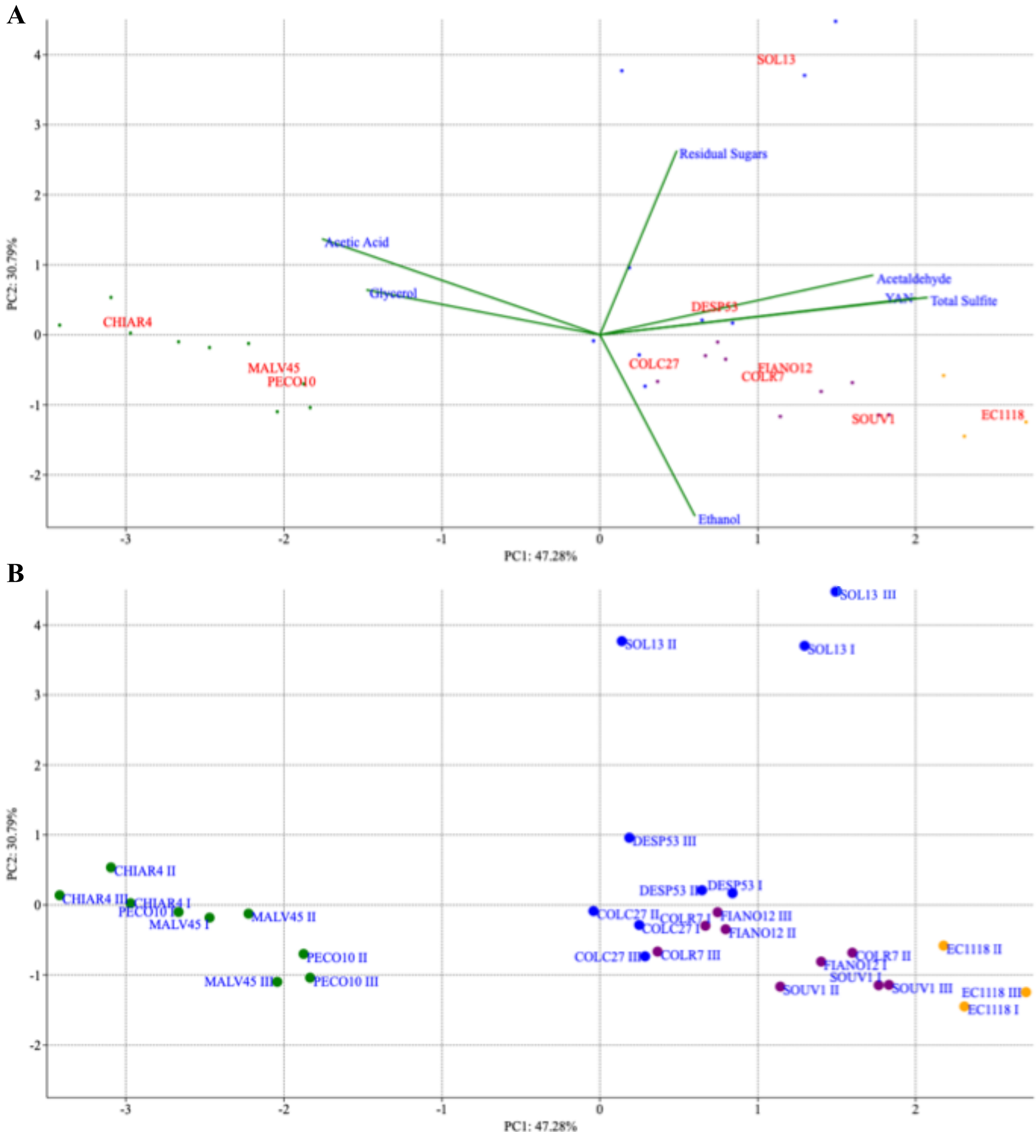

Figure 6.6. [A] Loading plot and [B] Score plot of the Principal Component Analysis of some important oenological parameters obtained after the sequential inoculation of grape must with isolates of non-*Saccharomyces* and *S. cerevisiae*.

For *L. thermotolerans*, SOL13 showed many differences to COLC27 and DESP53. The first had a much lower yield in ethanol production, possibly due to a displacement of the metabolism for a higher production of lactate, acetate and

acetaldehyde. It can be highlighted that isolates of *L. thermotolerans* caused the highest reduction in ethanol content and those of *Metschnikowia* spp. were responsible for the lowest production of volatile acidity and smallest need of nutrients. Interestingly, two recent studies of sequential fermentations with the same commercial strains of *L. thermotolerans* and *M. pulcherrima* in Shiraz (Hranilovic *et al*., 2018) and Riesling (Benito *et al*., 2015b) grape musts have found exactly the same trends as in the present study, such as rising in the glycerol production, reduction in the levels of total SO_2, acetaldehyde (only measured in Riesling) and alcohol and no significant changes in acetic acid.

Isolates of *L. thermotolerans* were recently tested in mixed fermentations of Airén (Benito *et al*., 2016), Emir (Balikci *et al*., 2016), Tempranillo (Benito *et al.*, 2015a), Sangiovese and Cabernet Sauvignon wines (Gobbi *et al*., 2013), always leading to increase in total acidity (due to LA production), and sometimes also rise in glycerol content and reduction in ethanol and acetic acid, usually improving the overall quality.

The study conducted by Barbosa and colleagues (2018) with mixed fermentations of *S. cerevisiae* and *M. pulcherrima* in Tinta Roriz grape must showed the potential of this species to significantly decrease levels of ethanol, volatile acidity and hydrogen sulfide. Co-inoculation of *M. pulcherrima*/*S. cerevisiae* in Merlot grape must showed decrease in ethanol, increase in glycerol and no changes in acetic acid concentrations in comparison to the single *S. cerevisiae* (Varela *et al*., 2017), exactly the same findings as in the present study. In Shiraz and Chardonnay wines, the sequential inoculation of the same species resulted in reduced ethanol and acetic acid, while the concentration of glycerol increased in Chardonnay and decreased in Shiraz (Varela *et al*., 2016). No significant differences were found in Macabeo wines, although a slightly increase in glycerol and volatile acidity and decrease in ethanol were observed in the mixed culture *M. pulcherrima*/*S. cerevisiae* respect to the control (González-Royo *et al*., 2015).

The control fermentation in grape must with a single inoculation of *S. cerevisiae* was used in this study to confirm the potential of the mixed inoculations to reduce the ethanol and acetaldehyde content of the wines, as well as increasing the glycerol and LA levels (in the case of *L. thermotolerans*), without harming the optimal progress and conclusion of the fermentation process.

6.3.3. Aromatic profile

The previous sections discussed the fermentation from the process point of view and the physicochemical attributes of the wines. It has been shown that the mixed fermentations caused significant impact in the wines, even considering the limited growth of the non-*Saccharomyces*. Besides the fermentation kinetics and chemical analysis, it is fundamental during the selection of yeasts for commercial winemaking to determine the influence in the flavor and aroma (Varela *et al.*, 2017). Doing so, further effects in the complexity and quality of the wines could be viewed from the perspective of the so-called 'bouquet', represented by the collection of aromatic compounds. Rossouw and Bauer (2016) described a noteworthy impact in the final aroma profile, regardless of the non-*Saccharomyces* strains inability to dominate numerically throughout the entire fermentation. They confirmed that the metabolic impact of these yeasts during the early stages of fermentation is sufficient to trigger significant changes to the final balance of volatile alcohols and esters produced.

The evaluation of wines through GC-MS at the end of fermentation of the Pinot Grigio grape musts allowed the identification and quantification of 37 volatile compounds, separated in the following classes: esters (9), fatty acids (3), alcohols (10), carbonyl compounds (4), volatile phenols (2) and terpenes (9). All of them are represented in Table 6.3, although the discussion was focused on the molecules with significant differences and concentrations above their specific odor threshold. It should nonetheless be noted that even small differences in the concentrations of some compounds could change the perception and preference of the wines on sensory evaluations (Jemec and Raspor, 2005; Jolly *et al.*, 2006).

Most of these compounds were a result of the fermentation process, and their different concentrations could be mainly attributed to the dominant yeast species and fermentation conditions (Padilla *et al.*, 2016). It is usually considered that the differential synthesis of aromatic compounds may occur because non-*Saccharomyces* species vary from *S. cerevisiae* in the distribution of metabolic flux during fermentation and therefore differ in ethanol production, biomass synthesis, and by-product formation (González *et al.*, 2018), and moreover the biosynthesis is strain-dependent (Escribano-Viana *et al.*, 2018).

Table 6.3. Volatile aromatic compounds (µg/L) found after the fermentation of grape must with non-*Saccharomyces* and *S. cerevisiae* strains.

Compounds	*Lachancea thermotolerans*			*Starmerella bacillaris*			*Metschnikowia* spp.			*S. cerevisiae*		
	COLC27	DESP53	SOL13	CHIAR4	MALV45	PECO10	COLR7	FIANO12	SOUV1	EC 1118	OT^{a}	OD^{b}
Alcohols												
(E)-3-Hexen-1-ol	95.10 ± 0.47^{ab}	86.17 ± 1.75^{a}	86.75 ± 2.05^{a}	120.65 ± 1.62^{e}	120.28 ± 2.53^{de}	112.43 ± 1.47^{cde}	104.91 ± 0.10^{bc}	113.48 ± 8.38^{cde}	107.88 ± 2.41^{cd}	88.82 ± 2.86^{a}	400$^{(1)}$	fruity, fresh grass
(Z)-3-Hexen-1-ol	21.89 ± 0.45^{abc}	21.12 ± 0.39^{ab}	20.29 ± 0.33^{a}	22.80 ± 0.08^{c}	25.74 ± 0.15^{de}	24.65 ± 0.45^{d}	27.29 ± 0.86^{e}	26.82 ± 0.42^{e}	25.61 ± 0.40^{de}	22.63 ± 0.17^{bc}	400$^{(1)}$	green, cut grass
1-Butanol	93.63 ± 3.33^{a}	98.01 ± 2.84^{a}	129.75 ± 13.44^{c}	98.57 ± 1.80^{ab}	130.61 ± 0.31^{c}	101.27 ± 7.68^{ab}	92.35 ± 1.50^{a}	96.78 ± 5.78^{a}	122.36 ± 4.29^{bc}	89.88 ± 7.46^{a}	$1.5\times10^{5}$$^{(2)}$	medicinal, green herb
1-Pentanol	2.93 ± 0.22^{a}	12.36 ± 4.41^{abc}	20.82 ± 0.59^{cde}	10.33 ± 0.08^{ab}	21.52 ± 3.38^{cde}	16.69 ± 1.27^{bcd}	32.24 ± 2.71^{f}	25.09 ± 0.45^{def}	21.06 ± 0.07^{cde}	28.63 ± 5.03^{ef}	80000$^{(3)}$	fruity, balsamic
2-Butanol	9941.70 ± 401.23^{b}	8995.33 ± 301.38^{b}	9285.42 ± 407.04^{b}	9064.47 ± 243.75^{b}	13384.76 ± 24.69^{c}	10492.58 ± 565.06^{b}	10334.54 ± 97.06^{b}	11024.36 ± 1422.51^{b}	10583.36 ± 44.57^{b}	4858.74 ± 286.26^{a}	50000$^{(2)}$	fruity, sweet apricot, medicinal
3-Methyl-1-butanol	**254422.15± 2845.45^{cd}**	**227364.88± 2333.71^{bc}**	**227210.61± 54.86^{bc}**	**172329.86± 6818.57^{a}**	**257291.80± 14164.68^{de}**	**209675.95± 3030.04^{b}**	**254886.12± 3587.07^{cd}**	**255136.52± 8730.85^{cde}**	**283691.01± 12722.62^{e}**	**244818.76± 2190.56^{cd}**	60000$^{(2)}$	whiskey, malt, cheese, sweet, herbaceous
Benzyl alcohol	132.59 ± 1.00^{a}	575.61 ± 34.54^{d}	252.29 ± 12.88^{bc}	290.34 ± 14.71^{c}	279.16 ± 64.93^{c}	292.83 ± 0.80^{c}	139.28 ± 10.57^{a}	136.34 ± 1.07^{a}	162.90 ± 0.81^{ab}	118.74 ± 1.22^{a}	$2\times10^{5}$$^{(4)}$	floral, leaf
β-Phenylethyl alcohol	11569.19 ± 453.33^{ab}	11800.04 ± 92.06^{ab}	10824.10 ± 854.41^{a}	**18479.77 ± 2525.66^{de}**	13475.91 ± 343.10^{abc}	**14681.71 ± 1474.27^{abcd}**	**15093.69 ± 425.27^{bcd}**	**17527.70 ± 308.79^{cde}**	**15686.64 ± 291.85^{bcd}**	**21434.73 ± 1086.26^{e}**	14000$^{(5)}$	rose, honey, spice, lilac, pollen
n-Hexanol	**1127.13 ± 13.58^{de}**	**1125.00 ± 12.81^{de}**	**1163.33 ± 2.75^{de}**	924.13 ± 12.33^{b}	950.11 ± 39.73^{bc}	944.75 ± 6.65^{b}	768.44 ± 12.13^{a}	907.36 ± 0.12^{b}	1057.54 ± 27.43^{cd}	**1181.91 ± 69.13^{e}**	1100$^{(2)}$	herbaceous, grass, woody, toasty, dry
Vanillyl alcohol	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	n.d.	creamy, vanilla, coconut
Σ Alcohols	280198.69± 8038.67^{cde}	249613.43± 1426.79^{bc}	250882.87± 1402.38^{bcd}	201305.80± 9642.62^{a}	286783.36± 16062.46^{e}	242130.62± 1219.72^{b}	281150.30± 3476.61^{cde}	284885.02± 10067.01^{de}	291697.62± 14902.03^{e}	274722.41± 2064.48^{bcde}		

Compounds	*Lachancea thermotolerans*			*Starmerella bacillaris*			*Metschnikowia* spp.			*S. cerevisiae*		
	COLC27	DESP53	SOL13	CHIAR4	MALV45	PECO10	COLR7	FIANO12	SOUV1	EC 1118	OT[a]	OD[b]
Carbonyl compounds												
α-Ionone	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	3[6]	violet
β-Damascenone	**2.15 ± 0.04^{b}**	**1.59 ± 0.11^{ab}**	**1.27 ± 0.04^{ab}**	**6.07 ± 0.26^{c}**	**1.88 ± 0.06^{ab}**	**1.00 ± 0.00^{a}**	**5.05 ± 0.16^{c}**	**1.53 ± 0.11^{ab}**	**5.28 ± 0.81^{c}**	**5.70 ± 0.24^{c}**	0.05[1]	baked and ripe fruit, apple, rose, honey
β-Ionone	1.19 ± 0.01^{ab}	1.37 ± 0.04^{abc}	1.56 ± 0.11^{c}	1.45 ± 0.09^{bc}	2.07 ± 0.16^{d}	1.22 ± 0.04^{ab}	1.17 ± 0.01^{a}	1.22 ± 0.01^{ab}	1.17 ± 0.01^{a}	1.17 ± 0.01^{a}	5[2]	balsamic, rose, violet
Furfural	0.56 ± 0.01^{a}	0.51 ± 0.04^{a}	0.58 ± 0.03^{a}	0.50 ± 0.00^{a}	0.54 ± 0.01^{a}	0.53 ± 0.01^{a}	0.57 ± 0.03^{a}	0.53 ± 0.01^{a}	0.53 ± 0.02^{a}	0.54 ± 0.02^{a}	14100[5]	sweet, bread, almond, floral
Σ Carbonyl compounds	4.04 ± 0.10^{ab}	3.61 ± 0.31^{a}	3.62 ± 0.11^{a}	7.95 ± 0.45^{e}	5.00 ± 0.06^{bc}	3.14 ± 0.07^{a}	6.79 ± 0.13^{d}	3.28 ± 0.12^{a}	5.97 ± 0.59^{cd}	7.01 ± 0.23^{de}		
Fatty acids												
3-Methylbutanoic acid	**184.49 ± 18.99^{b}**	**124.37 ± 3.24^{a}**	**100.85 ± 6.34^{a}**	**133.27 ± 12.01^{a}**	**122.24 ± 1.59^{a}**	**129.27 ± 9.94^{a}**	**305.65 ± 10.71^{c}**	**141.59 ± 26.32^{ab}**	**349.00 ± 8.28^{c}**	**308.24 ± 6.50^{c}**	33[5]	rancid, cheese, sweaty
Hexanoic acid	**1946.31 ± 265.93^{ab}**	**2163.68 ± 70.03^{ab}**	**1453.16 ± 1.49^{a}**	**2010.02 ± 130.28^{ab}**	**1612.45 ± 9.52^{ab}**	**2708.90 ± 227.27^{b}**	**6839.13 ± 184.94^{d}**	**3983.88 ± 691.20^{c}**	**7078.33 ± 341.74^{d}**	**3870.24 ± 87.36^{c}**	420[5]	cheese, sweaty, fatty
Octanoic acid	**4954.37 ± 134.43^{ab}**	**4635.20 ± 213.89^{a}**	**3993.93 ± 288.13^{a}**	**6712.78 ± 733.64^{c}**	**4342.94 ± 208.43^{a}**	**6167.82 ± 352.05^{bc}**	**10687.32 ± 86.06^{e}**	**11111.26 ± 358.16^{e}**	**11678.76 ± 256.02^{e}**	**8648.53 ± 323.71^{d}**	500[5]	fatty, rancid, cheese
Σ Fatty acids	7527.85 ± 568.73^{bc}	6810.36 ± 121.03^{ab}	5553.73 ± 304.17^{a}	9118.53 ± 480.73^{c}	6502.50 ± 11.14^{ab}	9013.73 ± 103.87^{c}	17812.07 ± 288.60^{f}	15274.78 ± 305.54^{e}	18534.28 ± 886.10^{f}	12827.00 ± 404.57^{d}		
Esters												
Ethyl 2-methylbutyrate	0.04 ± 0.01^{ab}	0.05 ± 0.01^{ab}	0.05 ± 0.01^{ab}	0.07 ± 0.00^{ab}	0.06 ± 0.01^{ab}	0.05 ± 0.01^{ab}	0.04 ± 0.01^{ab}	0.03 ± 0.01^{a}	0.04 ± 0.01^{ab}	0.09 ± 0.02^{b}	18[5]	strawberry, fruity, anise
Ethyl 3-methylbutyrate	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	3[1]	strawberry, fruity, pineapple

Compounds	*Lachancea thermotolerans*			*Starmerella bacillaris*			*Metschnikowia* spp.			*S. cerevisiae*		
	COLC27	DESP53	SOL13	CHIAR4	MALV45	PECO10	COLR7	FIANO12	SOUV1	EC 1118	OT[a]	OD[b]
Ethyl butyrate	**374.42 ± 3.15^{cde}**	**385.27 ± 3.42^{de}**	**308.31 ± 8.42^{b}**	**352.39 ± 5.94^{c}**	**383.09 ± 10.84^{de}**	**371.07 ± 3.66^{cd}**	**400.17 ± 2.77^{ef}**	**388.39 ± 3.53^{de}**	**419.77 ± 8.92^{f}**	**238.05 ± 13.02^{a}**	20[(1)]	apple, fruity, sweet, strawberry, banana
Ethyl hexanoate	**966.88 ± 30.85^{bc}**	**927.22 ± 25.16^{b}**	**704.78 ± 30.36^{a}**	**746.39 ± 20.90^{a}**	**875.72 ± 25.94^{b}**	**883.55 ± 2.93^{b}**	**1036.95 ± 3.85^{c}**	**1064.46 ± 20.97^{cd}**	**1145.24 ± 15.32^{d}**	**655.72 ± 46.05^{a}**	14[(5)]	strawberry, anise, green apple, brandy
Ethyl octanoate	**989.54 ± 32.33^{bcd}**	**996.32 ± 9.84^{bcd}**	**770.24 ± 20.17^{a}**	**845.01 ± 19.38^{abc}**	**1059.93 ± 12.48^{cd}**	**962.99 ± 36.43^{abc}**	**1421.66 ± 92.49^{f}**	**1348.63 ± 30.30^{ef}**	**1187.91 ± 124.95^{de}**	**838.45 ± 42.83^{ab}**	5[(5)]	fatty, sweet, floral, pear, pineapple
Ethyl decanoate	**230.47 ± 14.45^{ab}**	**201.05 ± 14.64^{a}**	**213.09 ± 9.21^{ab}**	**298.87 ± 11.60^{ab}**	**221.29 ± 9.16^{ab}**	**390.81 ± 6.36^{ab}**	**1121.66 ± 144.91^{c}**	**400.47 ± 16.28^{b}**	**1258.55 ± 35.84^{c}**	**320.05 ± 0.18^{ab}**	200[(5)]	waxy, fruity, apple, grape
Ethyl lactate	1203.14 ± 11.24^{f}	1626.40 ± 57.31^{g}	6341.63 ± 792.84^{h}	439.03 ± 28.62^{b}	674.97 ± 14.91^{de}	552.68 ± 1.61^{bc}	650.08 ± 4.67^{cd}	650.26 ± 44.31^{cd}	786.05 ± 43.70^{e}	150.76 ± 6.02^{a}	1.5×10^{5}[(2)]	fruity, buttery
Isoamyl acetate	**1485.60 ± 39.07^{d}**	**1362.07 ± 3.20^{cd}**	**1027.73 ± 4.78^{b}**	**632.67 ± 11.17^{a}**	**950.13 ± 9.57^{b}**	**700.76 ± 14.30^{a}**	**1261.35 ± 31.94^{c}**	**1237.73 ± 0.88^{c}**	**1390.06 ± 26.36^{d}**	**1072.24 ± 82.74^{b}**	160[(2)]	banana, fruity, sweet
n-Hexyl acetate	14.15 ± 0.06^{de}	10.76 ± 0.07^{bc}	9.43 ± 0.44^{abc}	6.87 ± 0.13^{a}	7.92 ± 1.51^{ab}	8.93 ± 0.03^{ab}	9.66 ± 0.33^{abc}	12.59 ± 0.74^{cd}	16.33 ± 0.35^{e}	21.73 ± 1.77^{f}	670[(2)]	apple, cherry, pear, floral
Σ Esters	5217.82 ± 119.00^{c}	5528.98 ± 108.06^{c}	9284.71 ± 600.99^{e}	3423.16 ± 76.25^{a}	4100.13 ± 143.73^{ab}	3978.39 ± 56.51^{ab}	5729.34 ± 238.32^{cd}	4998.43 ± 115.00^{bc}	6649.10 ± 492.87^{d}	3231.21 ± 243.68^{a}		
Volatile phenols												
Vanillin	3.75 ± 0.05^{ab}	4.04 ± 0.08^{ab}	3.61 ± 0.36^{ab}	3.15 ± 0.08^{a}	5.47 ± 0.26^{cd}	4.61 ± 0.44^{bc}	7.97 ± 0.47^{e}	4.84 ± 0.21^{bcd}	9.74 ± 0.60^{f}	5.92 ± 0.23^{d}	60[(7)]	vanilla, powder, dry grass, woody
2-6-Dimethoxy-phenol	5.26 ± 0.11^{bcd}	5.44 ± 0.16^{cd}	3.84 ± 0.40^{abc}	4.75 ± 0.55^{abcd}	4.94 ± 0.09^{bcd}	6.01 ± 1.01^{d}	3.70 ± 0.19^{ab}	13.39 ± 0.06^{e}	3.20 ± 0.09^{a}	13.35 ± 0.48^{e}	570[(8)]	medicinal, smoky
Σ Volatile phenols	9.23 ± 0.16^{abc}	9.02 ± 0.07^{abc}	7.45 ± 0.76^{a}	7.90 ± 0.47^{ab}	10.13 ± 0.03^{bc}	13.12 ± 1.01^{d}	11.36 ± 0.71^{cd}	18.23 ± 0.15^{e}	12.93 ± 0.51^{d}	20.42 ± 1.36^{e}		

Compounds	*Lachancea thermotolerans*			*Starmerella bacillaris*			*Metschnikowia* spp.			*S. cerevisiae*		
	COLC27	**DESP53**	**SOL13**	**CHIAR4**	**MALV45**	**PECO10**	**COLR7**	**FIANO12**	**SOUV1**	**EC 1118**	**OT[a]**	**OD[b]**
Terpenes												
(E)-Linalool oxide	1.65 ± 0.02^{abcd}	1.32 ± 0.02^{ab}	1.20 ± 0.07^{a}	2.11 ± 0.13^{d}	1.55 ± 0.08^{abcd}	1.49 ± 0.15^{abc}	1.43 ± 0.01^{abc}	1.84 ± 0.21^{bcd}	2.02 ± 0.04^{cd}	1.55 ± 0.37^{abcd}	6 (9)	rose, camphor, floral green
(Z)-Linalool oxide	0.44 ± 0.06^{ab}	0.93 ± 0.01^{d}	0.43 ± 0.01^{ab}	0.41 ± 0.00^{ab}	0.38 ± 0.06^{ab}	0.33 ± 0.04^{a}	0.63 ± 0.08^{c}	0.53 ± 0.01^{bc}	0.36 ± 0.01^{ab}	0.30 ± 0.07^{a}	6 (9)	rose, camphor, fruity
α-Ionol	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.05 (10)	raspberry, violet
α-Terpineol	1.67 ± 0.30^{d}	28.00 ± 1.34^{e}	1.05 ± 0.10^{b}	1.57 ± 0.10^{cd}	0.94 ± 0.04^{ab}	0.73 ± 0.01^{ab}	0.98 ± 0.05^{ab}	1.10 ± 0.04^{bc}	0.95 ± 0.07^{ab}	0.53 ± 0.11^{a}	250 (5)	lilac, floral, sweet
β-Citronellol	6.64 ± 0.20^{d}	1.58 ± 0.07^{a}	1.85 ± 0.06^{a}	6.19 ± 0.69^{cd}	5.16 ± 0.09^{bc}	4.40 ± 0.21^{b}	6.27 ± 0.15^{cd}	8.85 ± 0.24^{e}	5.84 ± 0.35^{cd}	10.89 ± 0.46^{f}	100 (1)	green, fruity
Geraniol	3.51 ± 0.13^{bc}	3.37 ± 0.21^{abc}	0.72 ± 0.23^{a}	4.86 ± 0.41^{c}	2.07 ± 0.89^{ab}	12.46 ± 0.54^{d}	16.50 ± 0.12^{e}	13.82 ± 0.01^{de}	12.10 ± 1.26^{d}	11.15 ± 1.38^{d}	30 (1)	floral, green , fruity
Linalool	2.08 ± 0.27^{b}	2.08 ± 0.06^{b}	1.75 ± 0.07^{b}	2.07 ± 0.12^{b}	1.93 ± 0.24^{b}	0.85 ± 0.05^{a}	1.69 ± 0.03^{b}	0.99 ± 0.08^{a}	1.69 ± 0.20^{b}	1.84 ± 0.02^{b}	25 (5)	flower, lavender
Nerol	1.16 ± 0.01^{a}	1.47 ± 0.04^{ab}	3.68 ± 0.11^{d}	1.91 ± 0.06^{b}	3.96 ± 0.32^{d}	2.88 ± 0.16^{c}	1.13 ± 0.01^{a}	1.60 ± 0.04^{ab}	1.14 ± 0.13^{a}	1.48 ± 0.13^{ab}	400 (11)	vegetable, sweet, fruity
Terpinen-4-ol	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	130 (12)	dust, nutmeg
Σ Terpenes	17.16 ± 0.15^{b}	38.40 ± 1.68^{d}	10.42 ± 0.44^{a}	18.45 ± 0.23^{b}	18.09 ± 0.16^{b}	21.24 ± 0.21^{b}	30.03 ± 0.05^{c}	30.78 ± 3.63^{c}	22.32 ± 0.86^{b}	27.95 ± 0.25^{c}		

Mean ± standard deviation values of three independent replicates are indicated. <LOQ = below the Limit of Quantification.

Different letters in each row indicate significant difference according to Tukey's HSD test ($p<0.05$). Values in bold are above the OT.

[a] OT (Odor Threshold): The reference (μg/L) is given between parentheses: (1) Guth, 1997; (2) Peinado *et al*., 2004; (3) Li, 2006; (4) Gómez-Míguez *et al*., 2007; (5) Ferreira *et al*., 2000; (6) Burdock, 2010; (7) Culleré *et al*., 2004; (8) Parker *et al*., 2012; (9) Joshi and Gulati, 2015; (10) Zhang *et al*., 2013; (11) Marais, 1983; (12) Pino and Mesa, 2006; n.d. = not determined.

[b] OD (Odor Descriptor).

The impact of the mixed fermentations in the aroma of wines depends also on the composition of the initial grape must, including its nutrient and nitrogen availability. This means that it may be very difficult to determine and predict the particular impact of an individual species of inoculated starter, although certain compounds could show some reproducibility (Rossouw and Bauer, 2016). In our case, the aromatic profile was only evaluated in the wines at the end of fermentation of the natural grape must, since the previous analysis have shown that the yeast growth and fermentation performances were seriously compromised in the bottles with the pasteurized grape juice. Moreover, the treatments applied to the grape juice aiming the microbial stability of the product could have caused also depletion of important aroma precursors, in the same way that have diminished the nutrient availability. Nevertheless, the volatile compounds were quantified for the control fermentation in grape juice, and the comparison with the control in the fermented grape must showed much lower concentrations in the first case and confirmed that it would not be worthwhile to analyze the other bottles of fermented grape juice (data not shown).

The most abundant group in all wines was the higher alcohols, followed by fatty acids and esters. From the 37 molecules analyzed, five of them resulted below the limit of quantification, namely: vanillyl alcohol, α-ionone, ethyl 3-methylbutyrate, α-ionol and terpinen-4-ol. Considering the statistical analysis of the 32 detectable compounds, only furfural was not produced in significant different quantities among the diverse inoculation strategies. For what concern the aromatic threshold, 12 volatile compounds were present in at least one of the treatments with concentrations above the limit described in literature (indicated below Table 6.3 for each compound) for the perception of the aroma by human nose.

The compounds above the odor threshold were: three higher alcohols (3-methyl-1-butanol, β-phenylethyl alcohol and n-hexanol), one ketone (β-damascenone), three fatty acids (3-methylbutanoic, hexanoic and octanoic acids) and five esters (ethyl butyrate, ethyl hexanoate, ethyl octanoate, ethyl decanoate and isoamyl acetate). All of them were produced with significant differences among the sequential inoculations and the control with single *S. cerevisiae*, sometimes with increase and sometimes decrease.

The highest increase was observed for the esters, responsible for pleasant fruity and floral aromas. Especially the ethyl esters were increased due to the non-*Saccharomyces* action, even though in literature they were already reported as much lower producers than *S. cerevisiae* (Rojas *et al*., 2003). The accumulation of esters depends on the balance between the ester-producing metabolism and the activity of esterase enzymes, present in both *S. cerevisiae* and non-*Saccharomyces* yeasts (Ubeda-Iranzo *et al*., 1998; Swiegers and Pretorius, 2005). Even small changes in the concentration of these molecules could be perceived in the sensory evaluation of the wines (Englezos *et al*., 2018a).

The sum of the compounds separated by classes resulted most of the time in higher values for *Metschnikowia* spp. than the other non-*Saccharomyces* and the single inoculum, especially for the fatty acids and higher alcohols. The control with single inoculation of *S. cerevisiae* was responsible for producing more carbonyl compounds and volatile phenols, than the mixed cultures.

Higher alcohols are the largest group of aromatic compounds and are believed to contribute to the wine complexity in concentrations below 300 mg/L, while above 400 mg/L they could have a negative effect (Amerine *et al*., 1980; Rapp and Mandery, 1986). In the present study, any of the fermentations produced an overall amount of higher alcohols superior to 300 mg/L, and the lowest producer was CHIAR4 with approximately 200 mg/L. All *Metschnikowia* spp. isolates, one *S. bacillaris* (MALV45) and one *L. thermotolerans* (COLC27) caused an increase compared to *S. cerevisiae*, even though it was not significant. It was already reported high production of higher alcohols for strains of *M. pulcherrima*, *L. thermotolerans* and *S. bacillaris* (Padilla *et al*., 2016; Escribano-Viana *et al*., 2018).

Nevertheless, for two of the higher alcohols that reached concentrations above the odor threshold in the single inoculum (β-phenylethyl alcohol and n-hexanol), it was observed a general reduction in the concentration for the mixed inoculations, significant in most cases. For β-phenylethyl alcohol, an essential aromatic alcohol for the overall flavor quality and contributing to pleasant floral aromas, it was reported in literature the opposite of the present observations, with higher productions by the pairs *L. thermotolerans*/*S. cerevisiae* and *M. pulcherrima*/*S. cerevisiae* (Comitini *et al*., 2011; González *et al*., 2018). Our

isolates of *L. thermotolerans* and one *S. bacillaris* (MALV45) caused a decrease in β-phenylethyl alcohol to levels below the odor threshold, while for n-hexanol, that could be negative by imparting vegetable and herbaceous notes, only the isolates of *L. thermotolerans* did not result in decrease below the threshold.

The other alcohol produced above the sensory threshold, 3-methyl-1-butanol (isoamyl alcohol), which could negatively contribute with herbaceous notes, was the main component of the higher alcohols group for all treatments, and the concentration varied among the fermentations. It increased significantly only for SOUV1 and decreased for two *S. bacillaris*.

Fatty acids are believed to negatively impact the wine bouquet with fatty and rancid descriptors and the three compounds quantified in this study surpassed the sensory threshold. All strains of *L. thermotolerans* and *S. bacillaris* were able to significantly reduce the content of all fatty acids, but the three *Metschnikowia* spp. caused an increase for hexanoic and butanoic acid. For 3-methylbutanoic acid (isovaleric acid), considered a wine fault in too high concentrations, one isolate of *Metschnikowia* spp. (FIANO12) was also able to significantly decrease, and the other two isolates maintained statistically the same concentration as the single inoculum. Nevertheless, it is considered that a concentration of fatty acids above 20 mg/L would impact negatively the wine quality (Ribéreau-Gayon *et al*., 2006), what did not happen for any of the wines in the present research.

The mechanisms that lead to the formation of ethyl esters from the corresponding fatty acids are still unexplored, but it has been shown that the concentration of the fatty acid precursor modulates the ethyl ester production (Saerens *et al*., 2008). And considering the reverse reaction, the fatty acids could contribute to the aromatic equilibrium of wine by counteracting the hydrolysis of their respective esters (Swiegers *et al*., 2005). Indeed, the higher concentration for fatty acids in the present fermentations with the *Metschnikowia* spp. isolates were accompanied by correspondent higher levels of the ethyl esters.

The carbonylic compound β-damascenone has a very low odor threshold and thus was above this value in all wines, possibly contributing with floral and fruity aromas. Only for CHIAR4 the absolute quantity was higher than the control, but not statistically significant, while for six of the other non-*Saccharomyces* a significant decrease was observed.

Regarding the volatile phenols, their presence is described as always undesirable, due to the possibility of masking the fruity notes of white wines, even at low concentrations below the odor threshold (Padilla *et al*., 2016). All of our isolates caused a reduction in the total amount of volatile phenols (not significant only for FIANO12), represented mainly by 2-6-dimethoxy-phenol (Syringol), responsible for medicinal and smoky aromas, even though they were all far below the perception threshold. Padilla and colleagues (2016) reported the lower production of volatile phenols by *M. pulcherrima*, but in our study this was more evident for *Lachancea* and *Starmerella*.

Terpenes add positively to the wine aroma and are released when glucosidases free bound glycosylated precursors (Carrau *et al*., 2005). *M. pulcherrima* strains were already reported to increase the concentrations of α-terpineol, geraniol and nerol after single inoculation, due to their β-glucosidase activities (Rodríguez *et al*., 2010). However, during mixed fermentations the nerol and geraniol decreased significantly, and only α-terpineol was still high, probably due to the ability of *S. cerevisiae* to convert nerol and geraniol into α-terpineol (Di Stefano *et al*., 1992; Mateo and Jiménez, 2000). Indeed, in our fermentations all the mixed inoculations produced higher α-terpineol than *S. cerevisiae* alone (even if only for FIANO12, CHIAR4 and the three *L. thermotolerans* it was significant), but only four of them increased also geraniol (just COLR7 significantly) and five produced higher nerol (three significant differences). *L. thermotolerans* and *S. bacillaris* isolates were also highlighted as terpene producers with high β-glucosidase activities (Cordero-Bueso *et al*., 2012; Whitener *et al*., 2016), even though this was observed in the previous characterization of our isolates only for *Metschnikowia* spp.

In a sequential inoculation of *M. pulcherrima* and *S. cerevisiae* in Viura-Malvasía white must, Tronchoni and co-workers (2018) have also verified, in accordance with us, a decrease in the production of β-damascenone and 2-6-dimethoxy-phenol by the mixed culture, and increased concentrations for 3-methyl-1-butanol. On the other hand, divergences with the present study were found for n-hexanol, esters and fatty acids, with a lower production by mixed cultures in their observations; and β-phenylethyl alcohol, which was lower in the control single inoculation. In Merlot wines, an overall increase in the esters and

higher alcohols by the mixed inoculation with *M. pulcherrima* was in accordance with our findings (Varela *et al*., 2017).

Also with mixed inoculation of *M. pulcherrima* and *S. cerevisiae*, Varela and colleagues (2016) found increased levels for ethyl butyrate and ethyl hexanoate and decreased ethyl octanoate, ethyl decanoate and isoamyl acetate, resulting in no significant differences in the total esters content compared to the single inoculation, in Chardonnay grape must. The concentrations of 3-methyl-1-butanol and β-phenylethyl alcohol were higher in the single inoculum, but no differences were seen in total higher alcohols. Regarding fatty acids, 3-methylbutanoic and hexanoic acids increased, while octanoic acid decreased in the mixed inoculation, although again when considering the total concentration, no changes were observed for fatty acids. In the same study, but with Shiraz grape must, the authors found results more similar to ours than with Chardonnay (Varela *et al*., 2016). Considering the same esters that we quantified, they all increased in the mixed inoculation compared to the single and thus the total esters content was significantly higher for *M. pulcherrima*/*S. cerevisiae* mixed culture. The content of fatty acids was also in accordance with us, showing higher values for the mixed culture. The only difference to our study was for the higher alcohols, where they found increased β-phenylethyl alcohol and decreased 3-methyl-1-butanol, the opposite of the present study.

In a series of fermentations with different grape varieties, Englezos and colleagues (2018a, 2018b) tested the effects of mixed fermentations with *S. bacillaris* and *S. cerevisiae* in the chemical and aromatic composition of wines. Many differences were found with the present study, suggesting a strong influence of the grape variety and the strains used. Considering the total higher alcohols, in almost all grape varieties the same result was observed as our isolates MALV45 and PECO10 in Pinot Grigio, with no significant change to the single inoculation, while the lowering of higher alcohols in Pinot Noir was in accordance with our strain CHIAR4, and their mixed fermentation in Sauvignon Blanc was the only case of increase in this group of compounds. Regarding the alcohols above the odor threshold, 3-methyl-1-butanol, β-phenylethyl alcohol and n-hexanol, our strains reduced or maintained their levels, the same result in Riesling, Sauvignon Blanc, Cabernet Sauvignon, Pinot Noir, Merlot and Shiraz

for 3-methyl-1-butanol; in Chardonnay, Muscat, Pinot Noir, Merlot and Shiraz for β-phenylethyl alcohol; and all red varieties for n-hexanol. For the fatty acids, our results are in accordance with all white and red varieties studied by these authors, where an overall reduction in the content was observed. Looking at the quantified esters, different considerations have to made for the ethyl and acetate esters. Our strains were able to increase the first (except ethyl decanoate) and decreased the second. The same observations were made only in Sauvignon Blanc for the ethyl esters and in all white and red varieties for the acetate esters. Our three strains decreased the total content of terpenes, interestingly the same observation made for the white wine varieties, while the decrease or no change in β-damascenone was unanimous between our strains in Pinot Grigio and all varieties examined by Englezos and colleagues (2018a, 2018b).

In a co-inoculation of *S. cerevisiae* and *S. bacillaris* in Montepulciano d'Abruzzo grape must (Tofalo *et al*., 2016), the total production of esters and fatty acids was higher for the mixed fermentation, in accordance with the present study only for the esters. Also in agreement with our findings, the co-culture produced less higher alcohols. When analyzing the single components, both studies found increase in 1-butanol, 3-hexen-1-ol and ethyl butanoate, and decrease in β-phenylethyl alcohol for the mixed fermentation.

Different strategies of inoculation were carried out by Balikci and co-workers (2016) to evaluate mixed fermentations of *L. thermotolerans* and *S. cerevisiae* in Emir grape must. The sequential inoculation of *S. cerevisiae* 48 hours after the inoculation of *L. thermotolerans* resulted in a reduction in the levels of all esters and higher alcohols (except n-propanol). In the present study, the strain SOL13 behaved similarly with the mentioned investigation, while the strain DESP53 agreed in the reduction of higher alcohols but caused an increase in the esters, and COLC27 achieved the total opposite result, increasing the higher alcohols and esters, even though the differences were not significant in some cases.

In Airén wines (Benito *et al*., 2016), again the results of the mixed fermentation with *L. thermotolerans* were more similar to our strain SOL13, with decreased values or no significant changes for some fatty acids, higher alcohols and esters. The main differences to the present study is that our COLC27 and DESP53 increased some esters, and all three isolates decreased the content of β-

phenylethyl alcohol, while their sequential inoculation caused a significant increase. It is noteworthy to point out that these authors found an increase of two times for the concentration of ethyl lactate, while in the present study the observed increases ranged from 10 to 40 times (SOL13) for the mixed inoculations compared to the single. The findings of Gobbi and colleagues (2013) in white grape must were very similar to those described by Benito *et al*. (2016).

Three recent studies have compared the aromatic profile of sequential fermentations with non-*Saccharomyces*/*S. cerevisiae* yeasts and included strains of *L. thermotolerans* and *M. pulcherrima*: in Tempranillo grape must (Escribano-Viana *et al*., 2018); Shiraz (Hranilovic *et al*., 2018); and Riesling (Benito *et al*., 2015b). The first used indigenous strains, while the others applied the same commercial strains. Considering the main volatile compounds and those above the odor threshold in the present study, surprisingly noteworthy disparities between the different investigations were observed. For the higher alcohols, the most similar to our study was Tempranillo, with *Metschnikowia* spp. being the highest producer. In Shiraz, *L. thermotolerans* was consistently the highest producer of 3-methyl-1-butanol, β-phenylethyl alcohol and n-hexanol, while in *Riesling* the top producer was the single culture of *S. cerevisiae*. Considering the esters, *L. thermotolerans* was able to produce more than *M. pulcherrima* in both Riesling and Shiraz, but in Riesling the production of *L. thermotolerans* was also higher than the single inoculum, while in Shiraz there was no difference between *L. thermotoleran*s and *S. cerevisiae*. In our case there was no difference between the non-*Saccharomyces*, but they were both higher than the control. In Tempranillo the acetate esters were quantified and resulted lower in *L. thermotolerans*. Confirming the observation of the present study, also in Tempranillo and Riesling wines the production of ethyl lactate was much higher in the mixed fermentation containing *L. thermotolerans*. Regarding the fatty acids, the fermentations behaved similarly according to the type of must. While in white varieties, Riesling and Pinot Grigio of the present study, the strains of *Metschnikowia* spp. resulted in more fatty acids, in red cultivars, Shiraz and Tempranillo, the control was responsible for the highest content of these compounds, and in all cases the least producer was *L. thermotolerans*. Terpenes and β-damascenone were not quantified in Tempranillo, but different profiles were present in the other wines.

Metschnikowia spp. was the top producer of terpenes in our Pinot Grigio and produced more β-damascenone in Riesling, while *L. thermotolerans* the most terpenes and β-damascenone in Shiraz and more terpenes in Riesling.

The concentrations of the 32 compounds that were present in quantities significantly different were used for the Principal Component Analysis (Figure 6.7), considered separately for the three replicates of each of the ten diverse treatments, i.e. sequential inoculation of nine different non-*Saccharomyces* yeasts and *S. cerevisiae* and the control with the commercial *S. cerevisiae* strain EC 1118 alone.

This PCA clearly showed one more time the relevant impact of the mixed starters in the properties of the wine, as the PCA with the physicochemical parameters (Figure 6.6). For the aromatic profile it is possible to consider that the mixed fermentations were placed even farther from the control in comparison with the previous PCA. Moreover, the replicates of the same inoculation were positioned very close and grouped relatively next to the yeast strains of the same species, but with significant differences between species. The isolates of *L. thermotolerans* and *S. bacillaris* are closer among them than with *Metschnikowia* spp. The replicates are indicated by the name of the strain followed by a Roman numeral, and the species have different colors, where *L. thermotolerans* is blue, *S. bacillaris* is green, *Metschnikowia* spp. is purple and the control is orange. These observations gave a very strong evidence that the differences in the volatile compounds produced were the result of the presence of the non-*Saccharomyces* yeasts inoculated at the beginning of the fermentation, and not caused by the spontaneous microbiota that could have had an influence.

The first and second components that are represented in the graphic explained 43.5% of the variance, and the third component explained additional 11.29%. The PC1 was more positively influenced by the fatty acids and respective ethyl esters, while PC2 was more correlated with the higher alcohols and volatile phenols. In general, the isolates of *Metschnikowia* spp. produced the highest quantities of volatile aroma compounds and contributed more to the aromatic complexity of the wines, confirming the trend observed in the physiological characterization of Chapter 3, when only the isolates of *Metschnikowia* spp. showed positive results

for the tested enzymatic activities, that are believed to be responsible for the release of aromas.

Figure 6.7. [A] Loading plot and [B] Score plot of the Principal Component Analysis of some volatile compounds with significant statistical difference between the sequential inoculations of grape must with isolates of non-*Saccharomyces* and *S. cerevisiae*.

In order to better visualize the effects of the non-*Saccharomyces* isolates, it was calculated the increase or decrease in the production of each of the volatile compounds for each mixed inoculation in relation to the pure culture of EC 1118. The results were transformed in a heat-map and organized following the UPGMA cluster (Figure 6.8).

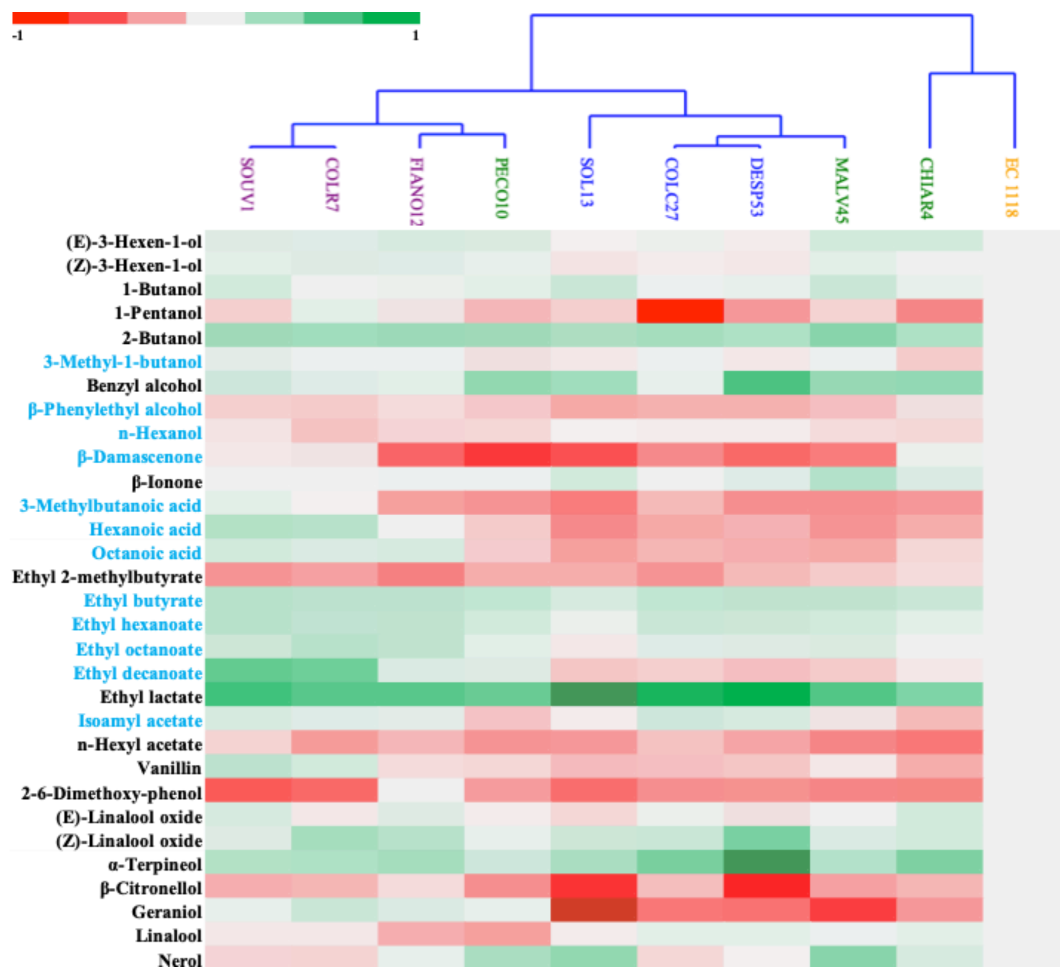

Figure 6.8. Cluster and heat-map based on the similarities between the non-*Saccharomyces* isolates sequentially inoculated with *S. cerevisiae* in the increased or decreased production of volatile aroma compounds, in comparison with the control single inoculation. Concentrations above the odor threshold are highlighted in blue.

As some general trends, it was possible to observe again that *Metschnikowia* spp. strains were responsible for the highest gain in the aromatic complexity by producing more compounds with increased concentrations respect to the single inoculation, with a special highlight to the esters with concentrations above the aromatic threshold. Only the strains of *Metschnikowia* spp. provoked an increase in the production of fatty acids. Comparing the three strains, SOUV1 and COLR7 behaved very alike while FIANO12 was slightly different. This is especially true for the higher production of 3-methylbutanoic acid, hexanoic acid, β-damascenone, ethyl decanoate, vanillin and linalool by the first two, while the last produced more 2-6-dimethoxy-phenol, being also the only non-*Saccharomyces* to do not cause a decrease in this compound compared to the control. The

differences between SOUV1 and COLR7 were increased 1-butanol and 3-methyl-1-butanol for the first and increased (Z)-linalool oxide and geraniol for the second.

Some important differences were seen between the three strains of *L. thermotolerans*. DESP53 produced very much higher concentrations of benzyl alcohol, (Z)-linalool oxide and α-terpineol, not only compared with the other two strains of the same species but among all ten different fermentations. SOL13 increased 1-butanol and nerol, besides ethyl lactate already discussed before, although the production of the other esters was lower and a noticeable decrease in geraniol was observed. COLC27 caused the highest decrease in 1-pentanol, but showed more 3-methylbutanoic acid, n-hexyl acetate and β-citronellol than the other two *L. thermotolerans*, since they caused a prominent decrease in these compounds.

Considering the aromatic compounds separately, it can be seen that *S. bacillaris* strains gave more positive contributions than *L. thermotolerans*, although the overall complexity was similar between them and below *Metschnikowia* spp. The production of nerol, benzyl alcohol and (E)-3-hexen-1-ol could be highlighted as higher for *S. bacillaris* than the others. MALV45 produced more higher alcohols and esters, PECO10 more fatty acid and terpenes and CHIAR4 had more carbonyl compounds. It could be highlighted the increase of 1-butanol, 2-butanol, 3-methyl-1-butanol, isoamyl acetate and nerol by MALV45, while β-phenylethyl alcohol and β-damascenone were the biggest contributions of CHIAR4; and PECO10 gave more geraniol and ethyl decanoate.

In an investigation with six different species of non-*Saccharomyces* yeasts, including *L. thermotolerans*, *M. pulcherrima* and *S. bacillaris* (Whitener *et al*., 2016), it was concluded that each of the co-fermentations gave a unique sensory and metabolic profile and that *S. bacillaris* gave the most remarkable and distinct aroma profile. Their study greatly increased the understanding of these yeasts' metabolism and contribution to wine aroma, but reported as an obstacle the strong dependence on the wine matrix that was used (Sauvignon Blanc grape must).

Another challenge in the study of the aromatic composition is the interaction between the components of the mixed culture. Data in literature are not always consistent for flavor metabolites produced during the mixed fermentations in

comparisons with the single cultures of each strain (Fleet, 2008). Howell and colleagues (2006) concluded that wines made with mixed yeast cultures have a different volatile aroma profile than the blend of wines made with the strains separately, confirming thus the influence of the interaction in the individual metabolisms. So, it is very complex to understand to what extent the differences in the aroma profiles observed here are due to the metabolism of the different non-*Saccharomyces* isolates tested and how much of that could be explained by the modulation of activities of the specific *S. cerevisiae* strain used in this study because of the yeast-yeast interaction.

6.4. CONCLUSIONS

Even if any of the non-*Saccharomyces* isolates was able to survive past halfway of the fermentation process, their impact in the wine quality was clearly acknowledged in all subsequent analysis. Taking into account the parameters analyzed during this study, the tested strains of alternative yeasts showed an adequate interaction with *S. cerevisiae* during sequential inoculation and caused positive effects in the physicochemical and aromatic profile of the wines.

The initial composition of the grape must had a clear effect in the development of the yeasts, seriously compromised in the pasteurized grape juice, where the fermentation took around 50 days to finish, impractical for real processes. The fermentation ran optimally in the natural grape must of Pinot Grigio grapes. This difference is very likely due to the initial nitrogen availability, much lower in the grape juice.

The musts inoculated with a commercial *S. cerevisiae* 48 hours after the initial inoculation of the non-*Saccharomyces* isolates took five days more to finish in comparison with the control initially inoculated with EC 1118, probably because the development of the non-*Saccharomyces* somehow slowed down the normal growth of *S. cerevisiae*, but not to an extent that could harm the satisfactory completion of the fermentative process.

Isolates of *Metschnikowia* spp. were immediately suppressed after the sequential inoculation, while those of *S. bacillaris* and *L. thermotolerans* were able to survive longer, probably related to the ethanol tolerance characteristic of

each species. No differences were observed in fermentation kinetics between the nine strains of the three species.

The strains of *L. thermotolerans* caused the highest decrease in the ethanol content and produced high quantities of L(+)-Lactic acid. The *S. bacillaris* yeasts were responsible for the highest production of glycerol, but also resulted in the maximum levels of acetic acid. Conversely, they showed the lowest production of SO_2 and acetaldehyde. Mixed fermentations with the *Metschnikowia* spp. had the smallest content of acetic acid and the smallest consumption of YAN, probably due to the limited growth of this species in comparison with the others.

In comparison with the single inoculation with EC 1118, all sequential inoculations successfully reduced the ethanol content, decreasing also the levels of SO_2 and acetaldehyde. An overall interesting increase of glycerol was observed but also of detrimental acetic acid, although this last was not significant in some cases and, even when significant, not to levels that could generate concerns about the wine quality.

Regarding the aromatic profile, all non-*Saccharomyces* strains positively influenced the final outcome, increasing the complexity. All of them increased the concentration of esters, compounds usually regarded as responsible for pleasant fruity and floral aromas. They also caused the reduction of volatile phenols, which on the contrary are mainly responsible for negative attributes.

Comparing the three non-*Saccharomyces* species, mixed fermentations with *Metschnikowia* spp. produced the highest quantities of esters, higher alcohols and terpenes, but also presented more fatty acids. The *L. thermotolerans* produced less fatty acids and volatile phenols, showing also an elevated content of esters, mainly driven by the very high production of ethyl lactate. Isolates of *S. bacillaris* were those generating the least increase in aromatic complexity.

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Chapter 7

Concluding remarks

CHAPTER 7. CONCLUDING REMARKS

7.1. GENERAL DISCUSSION

Wine, the result of the very straightforward process of crushing grapes and letting the sugar to be naturally converted into alcohol and carbon dioxide, is part of humans' diet since thousands of years ago, and probably any other food or beverage has been the subject of so many research and discussions. Alexander Fleming, the bacteriologist who discovered Penicillin in 1928, was credited for saying that "Penicillin cures, but wine makes people happy". Louis Pasteur, the pioneer in studies that clarified the role of yeast in winemaking about 160 years ago (Whitener *et al*., 2016), once quoted that "Wine is the most healthful and most hygienic of beverages". Throughout millennia, wine has not only captured the attention and imagination of poets and philosophers (Burlingame, 2008), but also scientists had turned their eyes to the wonders of this gift that nature had given us.

The scientific community found in wine an absolutely worthy subject for investigations, very often justifying the research as means to improve its quality and the process of making it. From the grapevine to the bottle, passing through all the chemical and biological steps, almost every aspect of winemaking has been meticulously described and subjected to all kinds of scientific method experiments. Nevertheless, it seems that there are still much space for new studies, as in almost all universities and scientific institutes located in the wine-producing regions of the world it is possible to locate a research group dedicated to oenology and/or viticulture.

Obviously, wine research does not only exist because of the fondness of scientists. The interest for those studies comes from governments and players of the wine industry, who are willing to invest their capital on that, since wine business actually moves huge amounts of money worldwide. The most recent Statistical Report on World Vitiviniculture of the International Organisation of Vine and Wine (OIV) has shown that in 2017, among other data, 7.5 millions of hectares was the global area under vines, 73.3 millions of tons of grapes were grown, 248 millions of hectoliters of wine were produced (279 millions of

hectoliters is the estimated production for 2018) and 244 millions of hectoliters were consumed in the world, all of this making up an international trade in value of about 30.4 billion EUR (OIV, 2018). This colossal market is mainly driven by the consumer's preferences, but the reduction of production costs is also an important factor to be considered in the balance. The research aiming to obtain wines with higher overall quality is as important as the improvements to reach more efficient, reliable and cost-effective production process. With all that said, the time, human force and funds deposited in oenological research are very justified due to the socioeconomic importance of this product for the markets and regions were is being produced.

It could look simple for the least watchful minds, but the process of growing grapes, the fermentation in the winery and the chemical composition inside a bottle are actually so complex that their study validate the achievement of Doctor of Philosophy titles all over the world, just like this one. Studying wine involves not only chemistry and biology, but also history, geography, philosophy, art, culture, economy, law, tourism, and so on. Even medicine and lifestyle, as countless papers were written about the effects of wine consumption for human health. And to whom it may concern, this paper was partially written under the effects of wine. Hot topics in the wine science include now also subjects of major relevance for human nature, such as geopolitics and climate change.

Coming back to the focus of the present PhD research, it was planned with the ultimate goal of improving the overall wine quality, including aspects of typicity and complexity. In order to achieve superior features in the chemical composition, physical structure and organoleptic perception, the optimized use of the microbial resources was proposed. For a long time, the alcoholic fermentation of grape musts has been considered a one man show played by the yeast *Saccharomyces cerevisiae*. Even when the real complexity of the process was acknowledged, the myriad of other bacteria and yeast species inherently present on the grapes surface were relegated and sometimes considered as a risk of spoilage.

The dominance of *S. cerevisiae* happens naturally due to the outstanding adaptation of this species to the fermenting must and wine environment, which are usually too harsh for the others. But even their role during the early stages of fermentation has been limited by sanitizing actions and the inoculation of a huge

concentration of cells from a pure culture of a very carefully selected strain of *S. cerevisiae* with very predictable and desirable outcomes. The inoculation of the so-called "starter cultures" is one of the cornerstones of modern winemaking. Notwithstanding, the never ending quest for improved quality and the growing appeal that the concepts of *terroir* and typicity have with the consumers have led to a new trend in oenology, which is the better exploitation of the microbial diversity that has always been there.

Some brave winemakers are trying to achieve that by using the spontaneous fermentation, but the majority do not want to take the risks of ending up with stuck fermentations or spoiled products and are not willing to give up the safety that decades of perfectioning the inoculation approach have given us. The most satisfactory alternative to mimic the complexity of spontaneous fermentation without losing the control is the use of mixed fermentation, i.e. the inoculation of starter culture from two or more different species. Several studies have proven that the species present at the beginning of the fermentation have some particular and different metabolism than *S. cerevisiae*, and many of these could actually be used in favor of wine properties.

This group of alternative yeasts comprises about a dozen of species and they have been simply called "non-*Saccharomyces*". It has been a while now since they came to the spotlight, but the number of commercially available products (Supplementary Table S.1) are still far below that of *S. cerevisiae*. The industrial application of the new generation of starter cultures should keep rising in the next years, following the trend of the ever-growing number of laboratory studies. The massive inter- and intraspecific diversity present among the non-*Saccharomyces* is the fuel for new researches, and the body of knowledge that is being accumulated about their metabolic characteristics and effects in wine will definitely help to pave the way for the more rational and common application. The studies are also important to comprehend the interactions between the components of the mixed starter and with the abiotic factors that make up the wine environment. For some very complete and recent reviews in all aspects mentioned, see Petruzzi *et al*. (2017), Padilla *et al.* (2016) and Mateo and Maicas (2016).

Therefore, the first task to be accomplished was the establishment of a culture collection containing a high number of yeast isolates belonging to an elevated number of different species, in order to have sufficient material to work with (**Chapter 2**). The project started with the gathering of a lot of samples coming from diverse regions of Italy. Our goal was to have a robust diversity, and not to reflect any specific *terroir*. For that reason, samples of grapes or freshly pressed musts from many different cultivars, climates and topographical conditions were collected. It was important to maintain the integrity of the berries and transport the musts refrigerated to the laboratory in order to have more prevalence of non-*Saccharomyces*, as *S. cerevisiae* was not the target. We decided to include additional samples of honey and overripe or dried fruits due to the possibility of finding interesting species for oenology also in these other habitats, having in common the high concentration of sugar.

More than 400 isolates were obtained and the identification through a primary morphological analysis followed by robust molecular techniques allowed us to name more than 20 different species, some familiar and others strange to the wine sector. The first aim was then clearly achieved and the yeast collection will be maintained at the Food Microbiology Laboratory of the University of Verona, considering that yeasts have an enormous potential for many other technological applications beyond winemaking and could possibly be useful for future projects.

Due to the logistic and structural impossibility of characterization of all isolates, this number of 400 was reduced four times, following the decision to focus on three species that are already known and applied in oenology, besides the fact that they were those found in higher quantity and higher distribution among the samples. *Lachancea thermotolerans*, *Starmerella bacillaris* and *Metschnikowia pulcherrima* have a good reputation for winemaking and presented an elevated diversity in the present isolation procedure, fundamental pre-requisites for a meaningful screening. Nevertheless, in reality it was not possible to associate unambiguously any isolate to the species *Metschnikowia pulcherrima*, due to limitations in the sequencing approach that is standard for yeasts and worked fairly well with all other isolates. This aspect was explored a little bit deeper and nonetheless it was decided to use all the isolates assigned to the genus *Metschnikowia*.

The total of about one hundred yeasts from these three species were submitted to an extensive phenotypic and genotypic characterization (**Chapter 3**). The protocols for genotyping were satisfactory and have shown some variability among the isolates of the same species at strain level. It was not possible to draw any conclusions about the relations between the genotypic characterization and the geographical origin or grape variety from which the isolates were obtained. Many factors could be involved in the microbiota diversity present in each sample, such as climate, soil, vineyard management and arrangement, health condition and stage of ripeness of the fruits at the moment of harvest, grape variety, vineyard surroundings. Nevertheless, it has been proven that the isolation from many different samples and locations contributed to the strain diversity that was found, allowing for the progression of the project and broadening the possibilities of finding interesting phenotypes.

The first stage of the selection was based on traditional oenological parameters. Stress tolerance assays aimed to check the compatibility of the isolates with some challenging components that could be present in fermenting musts, what is crucial for the implantation of inoculated cultures. It would be more interesting to have strains that could resist better and survive longer during the fermentation, having more time to express their peculiarities and hopefully giving more significant inputs to the final wine.

The evaluation of potential enzymatic activities had the goal to compare the strains' likelihood to contribute or damage the aromatic profile of wines and technological steps of the winemaking process. Finally, lab scale fermentations with single inoculation were prepared to see the direct impact in some measurable chemical parameters.

This large characterization allowed us to see some important differences between the species, and also between the strains of the same species. The different species have diverse weaknesses and strengths, where the goal of an efficient selection program is to find the best strains within determined species that are able to maximize the advantages and minimize the disadvantages. The goal of the winemaker is then to find among this group of the best strains the one most suitable for the style and quality of wine that is sought to be made. It has to be considered that balance is one the most appreciated qualities in a good quality

wine and it is not possible to have only one strategy of fermentation that fits all expectations.

In our characterization, strains of *Metschnikowia* spp. had the highest potential to modulate the wine aroma by the production of enzymes and to reduce the content of gluconic acid that could be present in unhealthy grapes, but had also the highest limitation to survive under increasing alcohol concentrations. On the other hand, the isolates of *S. bacillaris* showed the highest tolerance to ethanol and the most accentuated production of glycerol, but the high production of also acetic acid could demand some extra attention. Cultures of *L. thermotolerans* confirmed its renowned potential for the acidification of musts and moreover this feature presented the most elevated diversity among strains of all characters taken into consideration in the present study. They could also help to reduce the volatile acidity and had the highest fermentation vigor, but care should be taken to do not negatively interact with *S. cerevisiae* and cause stuck fermentations.

A further characterization was then carried out with some strains of *L. thermotolerans* and *S. bacillaris*, focusing in another application for the non-*Saccharomyces* that is ever gaining more attention from researchers, but is still struggling to become a case of commercial success. Following restrictions to the use of chemical pesticides due to environmental and human health issues and the rapid emergence of resistance phenotypes in the most common plant pathogens, the proposal of biological control gained force. Yeasts offer a lot of desirable skills and have what it takes to become very effective biological control agents (BCA). However, only a few products reached the market and even less are still available, although this potential has been studied for the past 30 years. Nevertheless, a very limited number of studies worried about the fate that the BCA could possibly have after the crushing of the grapes into must. This represents an important gap, since the high concentrations with which the BCA would be spread on the grapes could easily mean that they might persist until the beginning of the fermentation and then actively join the process.

Our strains of *S. bacillaris* and *L. thermotolerans*, especially the first species, showed very promising antagonistic activity versus *B. cinerea* in both *in vitro* and *in vivo* trials (**Chapter 4**). Both species were the subject of very few studies in literature on this regard. Considering the impressive curriculum that they have

already delivered for the use as oenological starters, a new integrated vitivinicultural strategy could be proposed for the exploitation of both potentials, where the non-*Saccharomyces* could be applied on the vineyard to colonize the grape surface, in order to counteract the development of gray rot and afterwards initiate the fermentation process, giving its positive contribution to the wine properties. Some relevant virulence factors were also tested to guarantee the safety utilization of such yeasts without risks to human health.

The most special trait of *L. thermotolerans* received then more attention, since very little is available in literature about the molecular basis of the lactic acid metabolism in this yeast species and the enormous variability among the strains inspires in-depth investigation (**Chapter 5**). It has been shown through molecular techniques that different strains have different sequences for the gene transcribing to lactate dehydrogenase (LDH), the putative enzyme responsible for the final conversion in the metabolism to lactic acid, but strains with similar phenotypes share the same sequence. Moreover, analysis of expression with two representative strains have shown that the highest production of lactate was associated with a higher expression of those genes. These findings could help the elucidation of an important metabolic pathway and be useful in the development of specific markers for the screening of high lactic acid producers.

Taking into consideration the most interesting profiles from the oenological perspective, but also keeping some diversity, three strains from each of the three groups characterized were chosen for the mixed inoculation trials (**Chapter 6**). Even if some non-*Saccharomyces* could be used alone for applications where less alcohol is involved, such as sweet wines or other fermented beverages with less initial sugar, it has been said that their utilization for most of wines would require the presence of *S. cerevisiae* to reach the complete dryness. The microvinifications with the nine chosen strains were carried out using two different grape juices. The first, a commercial grape juice that has been subjected to pasteurization and filtration in order to limit the development of the indigenous microbiota and avoid spontaneous fermentation; and secondly, a natural must of freshly pressed Pinot Grigio grapes without any addition of sulfites or other antimicrobial treatments.

The fermentation in grape juice should allow the development of the inoculated non-*Saccharomyces* without any competition from the natural microbiota. However, it was verified that the treatments to which the grape juice was submitted caused the depletion of important nutrients and possibly the emergence of inhibitory substances, which caused an impaired growth of the yeasts, both non-*Saccharomyces* and the commercial *S. cerevisiae* inoculated to carry out the fermentation to completion. The fermentation took much longer than firstly anticipated and the stress was probably the cause for the increased production of acetic acid. Nevertheless, some important features of the mixed fermentations with non-*Saccharomyces* were confirmed, such as the increase of glycerol and decrease of acetaldehyde. In this first fermentation, the strains of *Metschnikowia* spp., especially FIANO12 and SOUV1, can be highlighted as the most promising.

The second round of microfermentations, in the fresh grape must, was more useful for the evaluation of the non-*Saccharomyces* in the sense that the development was ideal, and even if a natural population of yeasts was present in the beginning, the inoculated strains were able to exert their impact in the final wine, since significant differences were seen between the control with single inoculation and the mixed cultures and moreover between the diverse nine treatments, thus confirming that the differences were due to the inoculated non-*Saccharomyces*. Some important features of the tested species were reinforced and significant differences were accomplished.

In general, strains of *L. thermotolerans* had the highest potential to reduce the ethanol content and they produced relevant amounts of lactic acid. In the aromatic profile, some general trends for the *L. thermotolerans* strains were the diminution of volatile phenols and fatty acids compared to all other treatments, a good production of esters above the control fermentation and equivalent levels of higher alcohols. Noticeable differences were observed between the three strains in the chemical analysis. SOL13 had the highest potential of all strains to reduce the concentration of ethanol in wine, most likely due to the deviation of the metabolic pathway to the remarkable high production of lactic acid, and additionally also a high production of glycerol was observed. However, this strain was also the responsible for the highest production of acetic acid and acetaldehyde. In the

aromatic profile, SOL13 produced the highest level of esters, mainly due to the much higher production of ethyl lactate, very likely related to the production of LA, while for almost all the other volatile compounds the levels for SOL13 were lower than the other strains.

COLC27 and DESP53 had very similar profiles in the chemical parameters and also aromatic profile, but important divergences could be pointed. DESP53 was probably the best among the *L. thermotolerans* strains, because of the considerable production of glycerol and lactic acid coupled with the reduction in ethanol and no changes in the acetic acid when compared to the single *S. cerevisiae*, even if between COLC27 and DESP53 no significant differences were detected. These findings repeated the ones observed in the single inoculations of the *L. thermotolerans* strains. DESP53 has also the advantage of having produced less H_2S in the test on Biggy agar and reached higher cellular concentrations in the first days of the fermentation. In the aromas, DESP53 had lower levels of 3-methyl-1-butanol and fatty acids, molecules that could be negative for the flavor, equivalent levels of interesting esters and more terpenes than COLC27, notably α-terpineol, and benzyl alcohol, contributors to floral aromas.

For the *S. bacillaris* strains, the highest potential to increase glycerol and reduce acetaldehyde and total SO_2 was found, among all species. However, these strains also caused the highest increase in acetic acid. Although the concentrations are still far below the accepted limit in wine and seem to do not represent a problem for the Pinot Grigio wine produced, it could require some attention for other varieties. Another important consideration to be made is the highest consumption of YAN by these non-*Saccharomyces* yeasts, which did not cause any difficulty in the progress of the fermentation analyzed, but again could represent a risk for other musts with a lower nutrient availability.

As it was the case in the previous characterization, the isolates of *S. bacillaris* showed a lower strain variability, even if it can be noticed that CHIAR4 produced more glycerol and also more acetic acid than the other two, probably due to its highest fermentation vigor. In the quantification of volatile compounds, it was shown that *S. bacillaris* was responsible for the lower concentrations for most of the compounds, in comparison with the other non-*Saccharomyces* and also with the control. Nevertheless, more differences were observed among the isolates than

in the quantification of the chemical parameters. Levels of esters and terpenes were similar for all three strains, but CHIAR4 produced less 3-methyl-1-butanol (herbaceous) and more β-phenylethyl alcohol (floral) and β-damascenone (fruity), while MALV45 less fatty acids (rancid, sweaty) and more some esters (fruity), even if the total amount was not significantly different. Due to the narrow range of variation among *S. bacillaris*, seems that all three isolates would have similar potential to the utilization, considering the drawbacks discussed above.

The strains of *Metschnikowia* spp. were those able to survive the shortest time in the mixed fermentations, being more susceptible to inhibition by the presence of *S. cerevisiae* and/or the escalation of the ethanol concentration, and SOUV1 lived less than the other two strains. This reduced growth is probably correlated with the lowest consumption of YAN, so representing the lowest risk to cause stuck fermentations with *S. cerevisiae* sequentially inoculated. Nonetheless, it was evident that, even with the reduced time, they were able to have remarkable impact in the wine. They produced the lowest quantity of acetic acid among the non-*Saccharomyces* and did not cause any change in the concentration observed for the single control. They caused the more discreet reduction in ethanol content and the increase of glycerol was significantly higher than the single inoculum.

The ethanol tolerance of *Metschnikowia* spp. was already the lowest in the characterization tests and for this reason the isolates were not evaluated in pure culture fermentations. But they showed the highest enzymatic activity with potential to positively impact the aromatic profile. This was confirmed by the determination of the volatile profile of wines, where *Metschnikowia* spp. strains gave the highest contributions to the aromatic complexity. They produced higher levels of higher alcohols and esters (not counting ethyl lactate, which was produced in much higher extent by *L. thermotolerans* due to LA metabolism), but also bigger quantities of fatty acids. FIANO12 produced less fatty acids and more β-phenylethyl alcohol than the other two, but also less esters, less β-damascenone and more 2-6-dimethoxy-phenol (medicinal, smoky). FIANO12 and SOUV1 showed lower production of H_2S than COLR7, but this last had the highest proteolytic activity.

To sum up, all three species of non-*Saccharomyces* evaluated in this study showed an interesting prospective to positively impact the overall wine quality,

with possible applications depending on the conditions of the must to be inoculated and the desired style and quality of wine that would be obtained. All steps were fundamental during the screening program, with the huge diversity found during the isolation allowing for the achievement of an encouraging pool of diverse oenological features, which were confirmed during the microvinification trials with a sequential inoculation approach. The wines obtained were all significantly different than the single inoculation of *S. cerevisiae* and the improvements observed justify the investment in the alternative yeasts.

7.2. CONCLUSIONS

I. Grape and grape musts are a sure reservoir for a large number of different yeast species;

II. Different samples can have very different microbial populations, and multiple factors could be held responsible for the variety;

III. Morphological description coupled with RAPD-PCR (primer M13) and ITS sequencing are powerful tools for the discrimination of most of the culturable species encountered in the wine environment;

IV. SAU-PCR can be successfully used to differentiate strains within the species *L. thermotolerans* and *S. bacillaris*, while RAPD-PCR (primer M13) and Rep-PCR (primer $GTG_{(5)}$) offer a sufficient discrimination power for *Metschnikowia* spp. strains;

V. The different species respond differently to diverse stress factors that could be present on grapes and grape musts, and some characteristics are also highly strain-dependent;

VI. *Metschnikowia* spp. is less tolerant to alcohol but more capable of growing utilizing gluconic acid as carbon source;

VII. *S. bacillaris* show a lower strain variability, except for the ethanol tolerance, which is generally higher than the other species tested;

VIII. The tested isolates of *L. thermotolerans* and *S. bacillaris* do not present any of the enzymatic activities taken in consideration, while *Metschnikowia* spp. have protease, esterase and β-glucosidase activities widespread in the isolates;

IX. Production of hydrogen sulfide is highly variable among isolates of *Metschnikowia* spp. and *L. thermotolerans*, but equal for all *S. bacillaris*;

X. In single inoculation trials, isolates of *S. bacillaris* produce more glycerol and acetic acid, while *L. thermotolerans* more L(+)-lactic acid and ethanol;

XI. Isolates of *L. thermotolerans* have a higher fermentation performance with overall higher consumption of reduced sugars, but *S. bacillaris* have a remarkable fructophilic character;

XII. Isolates of *S. bacillaris* did not possess the evaluated virulence factors associated with human pathogenicity, while *L. thermotolerans* isolates showed invasive growth and formation of pseudohyphae, although any of the strains had all virulence factors together;

XIII. All tested strains were effective against *B. cinerea* during both *in vitro* and *in vivo* assays, greatly reducing the radial mycelial growth in agar plates and the decay on infected grape berries;

XIV. Production of L(+)-lactic acid is greatly variable among isolates of *L. thermotolerans* and there are a suggestion that the differences could be explained by mutations of the genes codifying for the enzyme lactate dehydrogenase;

XV. The composition of the grape must, most likely the availability of nitrogen nutrients, have a determinant effect in the yeast growth and fermentative metabolism;

XVI. Non-*Saccharomyces* yeasts inoculated at high concentrations are able to initiate the fermentation of grape musts and actively grow during the first days and, even if they start to decrease before the middle of the process and disappear prematurely before the completion of the sugar conversion, have a significant impact in the wine properties;

XVII. The strategy of mixed fermentation with non-*Saccharomyces* yeasts sequentially inoculated with *S. cerevisiae* is a reliable tool to positively modulate some chemical parameters such as ethanol, glycerol, acetaldehyde, total sulfite, acetic acid and lactic acid contents in wine;

XVIII. The participation of non-*Saccharomyces* yeasts also causes an overall improvement on the wine aromatic complexity, especially considering the

increase in concentrations of important esters that greatly contribute to pleasant floral and fruity aromas;

XIX. The effects of non-*Saccharomyces* starter cultures are highly species- and strain-dependent.

7.3. FUTURE WORK

From the collection of around 400 hundred yeasts established in the first stages of the present project, about one quarter were selected for the following characterization steps, from three chosen genera. Other species found after the identification have potential oenological interest and could be tested for future applications, such as *Torulaspora delbrueckii*, *Hanseniaspora vineae*, *Meyerozyma caribbica* and *Zygosaccharomyces bailii*. They could be genotypically and phenotypically characterized and tested in fermentation trials, the same way as it was done for *L. thermotolerans*, *S. bacillaris* and *Metschnikowia* spp.

Aiming for the use of some isolates as BCA in vineyard to protect the grapevines, it would be necessary to run new tests for the optimization of the product, such as an adhesion method to facilitate the implantation, rational application based on the mechanism of antagonism, adaptation to adverse environmental factors, scaling up of the production process.

The study of the lactic acid metabolism could be expanded to more isolates of *L. thermotolerans*, obtained from different regions worldwide, in order to validate the findings with a broader range of strains. It could be expected that even more possibilities of sequence, arrangement and number of copies for the LDH gene would be observed, giving stronger indications of the correlations of this activity with mutations within the species. With more data it could be interesting to draw some connections between the phenotype, genotype and sample of origin, trying to better understand the evolution and selection pressures that could have led to the differentiation.

Before becoming commercially available products, further tests are needed for the strains characterized in this study. Different combinations of strains, composition of grape musts and strategies of inoculation (time and load) could

result in changes of the outcomes. Some strains could develop better in one condition rather than another. The earlier inoculation of *S. cerevisiae* could minimize the possible problems caused by *S. bacillaris* and still keep its benefits, while a later inoculation could give *Metschnikowia* spp. more time to develop and contribute even more to the aroma. The scaling up of the process until the winery scale is also necessary to corroborate that the positive impact of the non-*Saccharomyces* will not be surpassed by eventual complications.

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APPENDIX

Supplementary Table S. 1. List of non-*Saccharomyces* starter cultures currently available in the market (Petruzzi *et al*., 2017, updated).

Species	Commercial Product	Oenological Features	Distributor
Lachancea thermotolerans	LEVULIA® Alcomeno	High increase of total acidity and a decrease of the alcohol content, bringing wine freshness and balance to the mouth	AEB Group
Metschnikowia pulcherrima	FERMOL Pulkerrima Select	The co-culture is characterized by a higher quantity of superior alcohols, ethyl esters, acetates and terpenes, increasing the complexity and intensity of the aromatic profile	AEB Group
Metschnikowia pulcherrima	PRIMAFLORA® VB BIO	Ability to quickly establish itself in the must, limiting in this way the development of unwanted microorganisms, such as *Brettanomyces*, apiculate yeasts, lactic bacteria	AEB Group
Metschnikowia pulcherrima* and *Saccharomyces cerevisiae	PRIMAFLORA® VR BIO	Limits the combination of SO_2 at the end of fermentation and prevents the selection of SO_2-resistant strains. It contributes to preserving the purity of expression of the territory	AEB Group
Metschnikowia pulcherrima	LEVULIA® Pulcherrima	Increases the aromatic complexity and reduces alcohol content. Used to obtain white and rosé wines more aromatic, complex, fruity	AEB Group
Torulaspora delbrueckii	Viniferm NS TD	Increases the wine's complexity and aromatic spectrum. Releases significant amounts of mannoproteins, adding roundness and volume	Agrovin
Lachancea thermotolerans	Viniflora® Concerto™	Produces lactic acid, giving roundness and balanced acidity to wines in warm regions	Chr. Hansen
Lachancea thermotolerans* and *S. cerevisiae	Viniflora® Rhythm™	Excellent choice for fruit forward red wines and produces round, rich flavors with notes of blackberry and dark fruit. Produces low levels of SO_2	Chr. Hansen
Lachancea thermotolerans*, *Torulaspora delbrueckii* and *S. cerevisiae	Viniflora® Melody™	Increases wine complexity, gives tropical fruitiness and an overall aromatic intensity, combined with a round, balanced mouthfeel	Chr. Hansen
Lachancea	Viniflora®	Produces wines characterized by a round and	Chr. Hansen

Species	Commercial Product	Oenological Features	Distributor
thermotolerans*, *Torulaspora delbrueckii* and *S. cerevisiae	Harmony™	rich flavor, with notes of black fruit. Excellent choice for white or red wines that demand subtle but multi-dimensional differences	
Pichia kluyveri	Viniflora® FrootZen™	Enhances varietal aromas, and thiols aromas	Chr. Hansen
Torulaspora delbrueckii	Viniflora® Prelude™	Increases body, soft structure	Chr. Hansen
Torulaspora delbrueckii* and *S. cerevisiae	Oenoferm® wild & pure	Creamy texture with a pleasant and lasting mouthfeel	Erbslöh
Torulaspora delbrueckii	Zymaflore® Alpha$^{TD\ n.Sacc.}$	Makes wines of high organoleptic complexity	Laffort
Torulaspora delbrueckii* and *Metschnikowia pulcherrima	Zymaflore® Égide^{TDMP}	Harvest bioprotection of grapes and juices, as an SO_2 reduction strategy	Laffort
Metschnikowia pulcherrima	Flavia™	Enhances varietal aromas, terpenes and thiols aromas	Lallemand
Torulaspora delbrueckii	Biodiva™	Enhances aroma and mouthfeel complexity in white and red wines	Lallemand
Torulaspora delbrueckii* and *S. cerevisiae	LEVEL2 TD™	Promotes aromatic intensity, complexity and mouthfeel in white wines	Lallemand
Metschnikowia fructicola	Gaia™	Dominates the must during cold soak to offer a natural protection against spoilage organisms, allowing to reduce the SO_2 at crushing	Perdomini
Schizosaccharomyces pombe	ProMalic®	Allows maloalcoholic deacidification	Proenol
Pichia kluyveri	WLP605	Produces rose petal and floral aromas, contributing to overall bouquet of wine	Vintner's Harvest
Torulaspora delbrueckii	WLP603	Provides aromatic complexity and a fresh fruit characteristic. Produces low volatile acids, volatile phenols, and ethyl acetate	Vintner's Harvest

Supplementary Table S. 2. List of yeast isolates used for the genotypic and phenotypic characterization.

Isolate	Municipality (Province)	Grape Variety (*or Fruit)
Lachancea fermentati		
LS16	Massa Marittima (GR)	Sangiovese
Lachancea kluyveri		
FIANO22	Gravina di Puglia (BA)	Fiano
Lachancea thermotolerans		
COLC11	Colognola ai Colli (VR)	Pinot Grigio
COLC27	Colognola ai Colli (VR)	Pinot Grigio
DESP53	Fossalta di Piave (TV)	Marzemino
FIANO43	Gravina di Puglia (BA)	Fiano
FIANO63	Gravina di Puglia (BA)	Fiano
GLERA15	Refrontolo (TV)	Glera
LS15	Massa Marittima (GR)	Alicante
LT15	Vigolzone (PC)	Malvasia Candia
LT3	Vigolzone (PC)	Malvasia Candia
MALV13	Vigolzone (PC)	Malvasia Candia
MALV17	Vigolzone (PC)	Malvasia Candia
SOL13	Grumes (TN)	Solaris
***Metschnikowia* spp.**		
ALIC2	Massa Marittima (GR)	Alicante
ALIC3	Massa Marittima (GR)	Alicante
BONAR3	Vigolzone (PC)	Bonarda
CLINT4	Trevignano (TV)	Clinton
COLR1	Colognola ai Colli (VR)	Pinot Grigio
COLR3	Colognola ai Colli (VR)	Pinot Grigio
COLR4	Colognola ai Colli (VR)	Pinot Grigio
COLR5	Colognola ai Colli (VR)	Pinot Grigio
COLR6	Colognola ai Colli (VR)	Pinot Grigio
COLR7	Colognola ai Colli (VR)	Pinot Grigio
COLT3	Colognola ai Colli (VR)	Corvina
CONT1	Montecchio Maggiore (VI)	Pinot Grigio
CONT2	Montecchio Maggiore (VI)	Pinot Grigio
CONT4	Montecchio Maggiore (VI)	Pinot Grigio
CORV2	Lazise (VR)	Corvina
DESP65	Fossalta di Piave (TV)	Marzemino
F20	Trevignano (TV)	*Fig
F26	Bardolino (VR)	*Fig
FIANO12	Gravina di Puglia (BA)	Fiano

Isolate	Municipality (Province)	Grape Variety (*or Fruit)
FIANO15	Gravina di Puglia (BA)	Fiano
FIANO23	Gravina di Puglia (BA)	Fiano
FIANO32	Gravina di Puglia (BA)	Fiano
FIANO33	Gravina di Puglia (BA)	Fiano
FIANO41	Gravina di Puglia (BA)	Fiano
FIANO44	Gravina di Puglia (BA)	Fiano
FIANO51	Gravina di Puglia (BA)	Fiano
GLERA2	Valdobbiadene (TV)	Glera
GLERA6	Valdobbiadene (TV)	Glera
MALV3	Frascati (RM)	Malvasia
MALV5	Frascati (RM)	Malvasia Puntinata
MASSI4	Verona (VR)	Table Grape
MERLOT1	Ozzano Emilia (BO)	Merlot
P3	Trevignano (TV)	*Plum
PINOTG1	Montecchio Maggiore (VI)	Pinot Grigio
PINOTG22	Montecchio Maggiore (VI)	Pinot Grigio
RECIOTO1	Tregnago (VR)	Corvina
RECIOTO4	Tregnago (VR)	Corvina
RECIOTO5	Tregnago (VR)	Corvina
SANGIO2	Ozzano Emilia (BO)	Sangiovese
SOUV1	Grumes (TN)	Souvignier Gris
SOUV3	Grumes (TN)	Souvignier Gris
UVATAV4	Trevignano (TV)	Table Grape
VIGN1	Massa Marittima (GR)	Viognier
VIGN2	Massa Marittima (GR)	Viognier
Starmerella bacillaris		
ARMANI3	Dolcè (VR)	Pinot Grigio
ARMANI4	Dolcè (VR)	Pinot Grigio
CHIAR4	Lazise (VR)	Corvina
CHIAR7	Lazise (VR)	Corvina
COLC20	Colognola ai Colli (VR)	Pinot Grigio
COLC34	Colognola ai Colli (VR)	Pinot Grigio
CORV5	Tregnago (VR)	Corvina
CORVINONE3	Tregnago (VR)	Corvinone
CTP63	Tollo (CH)	Pecorino
DESP81	Fossalta di Piave (TV)	Marzemino
F1	Bardolino (VR)	*Fig
GLERA8	Refrontolo (TV)	Glera

Isolate	Municipality (Province)	Grape Variety (*or Fruit)
GLERA10	Refrontolo (TV)	Glera
GLERA12	Refrontolo (TV)	Glera
MAAS3	Montagna (BZ)	Moscato Giallo
MALV10	Vigolzone (PC)	Malvasia Candia
MALV20	Frascati (RM)	Malvasia Candia
MALV36	Frascati (RM)	Malvasia Puntinata
MALV43	Vigolzone (PC)	Malvasia Candia
MALV45	Vigolzone (PC)	Malvasia Candia
MARZEMINO2	Refrontolo (TV)	Marzemino
MARZEMINO3	Refrontolo (TV)	Marzemino
MERLOT3	Ozzano Emilia (BO)	Merlot
MO2	Bardolino (VR)	*Blackberry
PECO4	Crecchio (CH)	Pecorino
PECO6	Crecchio (CH)	Pecorino
PECO8	Lanciano (CH)	Pecorino
PECO10	Ortona (CH)	Pecorino
PECO16	Lanciano (CH)	Pecorino
PECO20	Torrevecchia (CH)	Pecorino
PECO22	Paglieta (CH)	Pecorino
PECO24	Rocca San Giovanni (CH)	Pecorino
PECO26	Tollo (CH)	Pecorino
PECO29	Tollo (CH)	Pecorino
PINOTG3	Montecchio Maggiore (VI)	Pinot Grigio
PINOTG11	Montecchio Maggiore (VI)	Pinot Grigio
PINOTG15	Montecchio Maggiore (VI)	Pinot Grigio
PINOTG21	Montecchio Maggiore (VI)	Pinot Grigio
PINOTG24	Montecchio Maggiore (VI)	Pinot Grigio
PS11	Grumes (TN)	Solaris
RECIOTO8	Tregnago (VR)	Corvina
RONDINELLA2	Tregnago (VR)	Rondinella
RONDINELLA3	Tregnago (VR)	Rondinella
RONDINELLA5	Tregnago (VR)	Rondinella
SOL16	Grumes (TN)	Solaris
ST24	Refrontolo (TV)	Glera

ACKNOWLEDGEMENTS

First of all, I would like to thank Prof.ssa Sandra Torriani, who has been the best tutor I could possibly ask for. She made me feel warmly welcome since the very first day in Italy and her support, knowledge, enthusiasm, kindness and trust were fundamental to make the best out of this project.

A very special thanks to Giada, whose contributions are spread all over the thesis. Not only her hard, dedicated and precise work, but also the friendship, were very highly appreciated.

To all the colleagues of the Laboratory of Food Microbiology, especially Dr. Wilson Junior, Dr. Veronica and Prof.ssa Giovanna Felis, for all the collaboration during the work. Thanks also to the friendly faces in the other laboratories of the corridor.

To all the staff of the Laboratory of Oenological Chemistry for the valuable support, especially Giovanni, also for many nice moments.

To the Institute of Wine Biotechnology and Stellenbosch University, principally Prof.ssa Maret du Toit, and to all the incredible people that made my time in South Africa much better than ever imagined. The impressions of this experience enriched not only my scientific career, but my whole life.

To all the students who shared with me the journey in the laboratory as their co-supervisor, giving me the opportunity to learn always more.

To the many friends who make life much happier, either in Brasil or all over the world, because home is wherever I'm with you. And particularly to Igor, without whom I might not even have applied for a PhD in Verona.

Much gratitude for the PhD School and the University of Verona for the structure and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the scholarship, which made all of this possible. This research was also funded by the University of Verona and the company Bioenologia 2.0 S.r.l. (Oderzo, Italy) in the framework of Joint Projects 2015 and 2016. Thanks to Dr. Gianni Conte for kindly providing us the grape must.

Finally, thanks to the most important people, my wonderful family, who have always been by my side no matter the ocean between us, who have always respected and supported all of my choices, even when we don't talk about and even when that meant to stay away from them for three years.

“and only because
he had no place he could stay in
without getting tired of it and because
there was nowhere to go but everywhere,
keep rolling under the stars”
Jack Kerouac