UNIVERSITY OF

PADOVA **UDINE** **VERONA**

Magister Degree course in

VITICOLTURE, OENOLOGY AND MARKETING

Department of Food Science

Developed for the achievement of

Master of Science (LM - 69)

From vineyard to wine, Lactic Acid Bacteria and yeast identification using molecular methods

Supervisor: Prof. Marisa Manzano

Assistant supervisor: Priya Vizzini

Student: Anabella Varela

ACADEMIC YEAR: 2014-2015

CONTENTS

ABSTRACT

Usually all attempts to characterize the microbial diversity in wine fermentations have employed standard methods of enrichment and isolation to cultivate various microbial constituents before taxonomic identification. This estimation of microbial diversity, in addition to being time-consuming, is often problematic since many microorganisms may not grow on standard laboratory media. Moreover culture-independent molecular methods allow more rapid profiling of complex populations, or quantification of targeted species, thereby enhancing the information available to the winemaker.

The aim of the present study is to describe the yeast and lactic acid bacteria communities found in the vineyard, the winery and the wine, using culture-dependent and culture independent molecular methods. Samples came from botrytized Picolit grapes during 2013 vintage in Corno di Rosazzo, Friuli Venezia Giulia Region, Italy.

On this work, the DNA extracted directly from the must and wine (culture-independent technique) was analyzed by denaturing gradient gel electrophoresis (DGGE), as well as the DNA extracted from isolated colonies that came from the vineyard and winery samples (culture-dependent technique). Both types of DNAs were specifically amplified by PCR using particular groups of universal primers depending on the nature of the sample (bacteria, *Saccharomyces* or non-*Saccharomyces* yeasts).

Saccharomyces sensu stricto yeasts were amplified with ShafGC and Shar specific primers. This yeast was found in the Winery and in the Picolit wine. The DGGE study showed that all samples corresponded to the *Saccharomyces cerevisiae* species, being identical to the commercial yeast used as a starter.

Non-*Saccharomyces* 26S ribosomal rDNA genes were amplified by Nested PCR using the primers NL1-NL4 for step 1 and NL1GC-LS2 for step 2. In the vineyard, the DGGE analysis allowed the identification of several yeasts like *Kloeckera sp., Metchnikowia sp., Pichia sp., Hansenula sp.* and *Schizosaccharomyces sp.* Other than *Saccharomyces cerevisiae,* no other

yeast was found in the wine, indicating the starter's ability to reduce the variability of the yeasts during the fermentation process. *Brettanomyces sp.* and *Candida sp.* were absent in the yeast analyzed. Samples that didn't correspond to a reference strain used were sequenced allowing the identification of *Torulaspora delbrueckii* and *Debaryomyces hansenii*.

Finally, bacteria samples were analyzed by employing gradient gel electrophoresis (DGGE) of polymerase chain reaction (PCR) amplified with 338fGC and P_4V_3 primers. Several microorganisms were found in the vineyard and the winery but only the strains of *Lactobacillus casei/ Lactobacillus paracasei* and *Leuconostoc mesenteroides* were found in the Picolit wine, indicating the selection due to the yeast fermentation process. Only 2 samples of the vineyard and one contact plate of the cellar were identified as *Oenococcus oeni*. Samples that didn't correspond to a reference strain used were sequenced allowing the identification of *Lactobacillus hilgardii, Lactobacillus mali, Pediococcus parvulus.*

In this work we demonstrate that PCR-DGGE is a viable alternative to standard plating methods for a qualitative assessment of the microbial constituents from de vineyard to the wine.

Key words: Picolit wine; lactic acid bacteria and yeast identification; PCR-DGGE.

INTRODUCTION

1. Picolit grape

Picolit is an ancient sweet wine, established during Roman times. It was for several centuries served to the clergy and nobility of northern Italy.

The variety is typical of the north-east part of Italy, a traditional white wine region: Friuli Venezia Giulia. Since 2006 the grape is allowed in the Denominazione di Origine Controllata e Garantita (DOCG) of Colli Orientali del Friuli.

Piccolo means small in Italian and its crop is indeed small, with between 15 and 30 berries per cluster. This is because of a defect in pollination, and the resulting juice is thus very rich in sugars.

Picolit has small to medium sized bunches but relatively loose. The pentagonal leaf is three or four lobed, the berry is small and oval, with a slight point, translucent when ripe; with a firm, resistant skin and abundant bloom that appears a dark gray-yellow. The pips are quite large. The vine is naturally vigorous and must be restrained. Bud break is early, while ripening is fairly late. All these characteristics turn Picolit into a "problem grape," with genetic instability in its area of cultivation and the necessity of finding the perfect site with nutrient poor soils, good exposition and ventilation. It is also very delicate variety, meaning that it must be harvested by hand so the fruit does not get damaged.

The grape is often made in the *passito* style and also late harvest method, allowing for an even greater concentration of sugars. With *passito* wines, the Picolit grapes are normally harvested in mid-October and then dried to raisins on straw mats before pressing. The late harvest styles are picked several weeks later, just before the grapes raisin on the vine. As a consequence, the resulting must may significantly affect the microbial communities, determining yeast

dynamics that are different from ‘normal’ must fermentations (Urso et al., 2008). Low quantities and labour intensive harvesting means Picolit commands high prices.

After grape harvesting, the whole clusters, or selected berries, are subjected to soft-pressing and the juice is cold-decanted. Fermentation is carried out either naturally or with the addition of a starter culture. The culture used for these fermentations should be able to promptly respond to osmotic stress and be able to increase their load immediately after inoculation. After the fermentation, ageing of the wine takes place in barrique (small barrels) for a period of 12-24 months.

As a wine, Picolit displays soft floral aromas with peach and apricot flavors and is generally consumed once the dinner table has been cleared.

2. Microorganisms from vineyard to wine environment

The initial environment that affects the microbial makeup of a wine fermentation is that of the vineyard. Grapes are the primary source of yeasts in wine production, (Prakitchaiwattana et al., 2004; Mills et al., 2008) because they are colonized by yeasts around the grape stomata where small amounts of exudates are secreted. Low numbers of yeasts (10-10^3 cfu/g) are found on unripe grapes, but as the grapes ripen the numbers increase to 10^4-10^6 cfu/g (Jolly et al., 2006). This is due to sugars that leach or diffuse out from inner tissue to the grape skin surfaces, providing nutrition for the yeasts (Jolly et al., 2006). Damaged berries increase the leaching effect. Therefore, the maturity of the grapes and/or the degree of damage to grape berries will largely determine the population numbers (Jolly et al., 2006)

Although *Saccharomyces cerevisiae* and *Saccharomyces bayanus* are widely regarded as the principal yeasts of wine fermentation, they are infrequently isolated from grapes, and there is significant controversy as to their natural origin in wine production (Prakitchaiwattana et al., 2004). Many studies have found that species of *Hanseniaspora* (anamorph *Kloeckera*), *Metschnikowia* and *Candida* are predominant on wine grapes at the time of harvest. Other yeast genera present on berries include: *Cryptococcus, Rhodotorula, Pichia, Zygosaccharomyces* and *Torulopsis*. Also present in the vineyard are numerous other yeasts, some of which have an impact on wine: *Sporobolomyces, Kluyveromyces,* and *Hansenula* (Mills et al., 2008). There is an increasing acceptance that these species, often referred to as indigenous, make important contributions to wine fermentation and character (Prakitchaiwattana et al., 2004).

On the other hand, on damaged berries, *Saccharomyces* is present at significant but low levels (10^5 to 10^6 CFU per berry), compared to total microbial population levels of 10^7 to 10^8 CFU per berry (Mortimer and Polsinelli 1999). In their work, Mortimer and Polsinelli (1999) suggested honey bees, wasps, and fruit flies (*Drosophila*) as likely vectors for carrying and spreading Saccharomyces and other yeasts among damaged grapes.

A distinct turning point occurs between the vineyard and the winery. As soon as the grapes are handled they become exposed to a new pool of organisms. The transfer of molds, yeasts and bacteria from equipment and surfaces represents the potential introduction of "resident" winery microbes to the grapes and, conversely, new sources of substrate are made available to existing microbes on the grape (Mills et al., 2008). The microbial populations present on equipment surfaces will vary according to the extent of sanitation employed on everything from picking knives, mechanical harvesters and grape bins, to crushers, tanks, hoses and pumps, to the walls and floors. Various species from the genera *Saccharomyces, Candida, Pichia,* and *Brettanomyces* can be associated with winery equipment and surfaces. However, cellar surfaces play a smaller role than grapes as a source of non-*Saccharomyces* yeasts, as *S. cerevisiae* is the predominant yeast inhabiting such surfaces (Jolly et al., 2006)

As a result of grape microflora and "resident" winery microbes, the resultant juice, must and wine are complex microbial ecologies hosting a diverse collection of yeast and bacteria. The specific environmental conditions in the must, i.e. high osmotic pressure (sugar concentration), presence of SO_2, and temperature, all play a role in determining what species can survive and grow. Obviously a major factor affecting microbial composition in wine fermentations is the practice of inoculation with commercial or otherwise selected strains of *S. cerevisiae*. Inoculation can be particularly effective in combination with SO_2 in reducing non-*Saccharomyces* populations and promoting the growth of *S. cerevisiae* (Mills et al., 2008).

2.1. Yeast evolution during fermentation

Quoting Cocolin et al. (2000), yeasts predominate during the alcoholic fermentation. A diverse population of yeasts including species of *Kloeckera*, *Metschnikowia*, *Candida*, *Hanseniaspora* and *Saccharomyces* are often present in the initial stages of most wine fermentations while lesser numbers are found on winery equipment. The non-*Saccharomyces* yeasts typically grow for several days before the fermentation is dominated by one or more *Saccharomyces cerevisiae* strains along with a concurrent increase in ethanol concentration (Table 1).

Nonetheless, several studies have demonstrated that non-*Saccharomyces* yeasts such as *Kloeckera sp., Metschnikowia sp., Candida sp. and Hansenula sp.* are able to persist in wine fermentations, albeit at a lower level than *S. cerevisiae* strains (Cocolin et al., 2000, Rantsiou et al., 2012). The predominance of *S. cerevisiae* in this setting is a likely result of its high ethanol tolerance and also relatively resistance to SO_2 as compared with other yeasts present in the wine environment (Mills et al., 2008). It has also been showed that *K. apiculata* and *C. stellata* have increased tolerance to ethanol at lower temperatures (10–15 °C) (Ciani et al. 2010). Such increases in the ethanol tolerance of non-Saccharomyces yeasts at low temperatures appear to be the major factor that affects their stronger contribution to low-temperature fermentations (Ciani et al. 2010).

It has been shown that the response of *S. cerevisiae* to osmotic stress can result in increased acetic acid contents due to the upregulation of genes encoding for aldehyde dehydrogenases (Rantsiou et al., 2012). Even though the acetic taste in sweet wines is in part masked by the high residual sugars after fermentation, winemakers would like to reduce its content which generally penalizes the final sensory quality of wines, becoming a limit to its commercialization, also in view of international legal limits for the acetic acid (Rantsiou et al., 2012). Mixed *Saccharomyces* and non-*Saccharomyces* fermentation strategy has been used in high sugar musts with the aim of reducing the acetic acid content of the final wine (Rantsiou et al., 2012).

Non-*Saccharomyces* yeasts		
Largely aerobic yeasts	Apiculate yeasts with low fermentative activity	Yeasts with fermentative metabolism
Candida spp.	*Hanseniaspora uvarum*	*Zygosaccharomyces spp*
Pichia spp	*Kloeckera javanica*	*Torulaspora spp*
Hansenula spp	*Kloeckera apis*	*Kluyveromyces marxianus*
Rhodotorula spp		*Metschnikowia pulcherrima*
Debaryomyces sp		
Cryptococcus albidus		

Table 1: Classification of most common non- *Saccharomyces* yeast

2.2. Lactic Acid Bacteria evolution during fermentation

As to bacteria, depending on the acidity and the nutrient, oxygen and ethanol concentrations in the juice or wine, the active bacteria present typically include both Lactic Acid Bacteria (LAB) and Acetic Acid Bacteria (Mills et al., 2008). Lactic acid bacteria are present in all musts grape and wines (Ribéreau- Gayon et al., 2007). Other bacteria such as *Bacilli, Clostridia, Actinomyces* or *Streptomyces* have been identified in the wine environment; however, these represent relatively rare occurrences (Mills et al., 2008).

The LAB involved in wine are comprised of acid and ethanol-tolerant strains primarily from four genera *Lactobacillus, Pediococcus, Leuconostoc,* and *Oenococcus* (formerly *Lc. oenos*) (Mills et al., 2008). These microbes are commonly found on grapes and in the winery environment. Newly fermented wines contain low populations of LAB, usually less than 10^3 CFU per mL, however, damage to the grapes increases this number by several orders of magnitude (Mills et al., 2008). Like table 2 shows, at the same moment in which the lactic population regresses after having reached its maximum value, first the homofermentative *Lactobacillus*, then the heterofermentative Lactobacillus and later the homofermentative Coccus and *L. mesenteroides* disappear from the medium for the benefit of *L. oenos* (Ribéreau- Gayon et al., 2007)

Days	Alcoholic title (% vol.)	*Leuconostoc oenos*	*Leuconostoc mesenteroides*	*Pediococcus damnosus*	*Lactobacillus hilgardii*	*Lactobacillus brevis*	*Lactobacillus plantarum*	*Lactobacillus casei*	Popolazione totale
0	0	nd	$2,9\times10^{2}$	$6,0\times10^{2}$	$1,1\times10^{3}$	nd	$7,5\times10^{1}$	$7,7\times10^{1}$	$2,5\times10^{3}$
3	7	nd	$1,7\times10^{4}$	$3,8\times10^{4}$	$8,0\times10^{4}$	$2,0\times10^{4}$	$2,0\times10^{4}$	$2,0\times10^{4}$	$1,7\times10^{5}$
6	9	nd	$9,6\times10^{4}$	$3,7\times10^{4}$	$4,0\times10^{4}$	$4,5\times10^{3}$	nd	nd	$1,5\times10^{5}$
10	13	$4,2\times10^{3}$	$3,2\times10^{3}$	$4,9\times10^{3}$	$4,4\times10^{3}$	nd	nd	nd	$1,8\times10^{4}$
18	13	$3,4\times10^{6}$	nd	nd	nd	nd	nd	nd	$3,4\times10^{6}$

Table 2: Representation of different LAB during the alcoholic fermentation of Cabernet Sauvignon (Ribéreau- Gayon et al., 2007) nd: not determined, numbers represent the CFU / mL

Four main factors that dictate the extent of LAB growth in wine are pH, temperature, ethanol and antimicrobial additions such as SO_2 or lysozyme. These latter additions purposely reduce LAB concentrations to enable proper growth of *S. cerevisiae* and/or to microbially stabilize the wine (Figure 1) Wine pH also strongly influences which LAB species will be present. Higher pH wines (above pH 3.5) often harbor species of *Lactobacillus* and *Pediococcus*, both during and after fermentation, while lower pH wines (< 3.5) typically only contain *O. oeni* (Mills et al., 2008). Ethanol production from the dominant *S. cerevisiae* population also serves to reduce all LAB populations in the first few weeks of the alcoholic fermentation (Mills et al., 2008). However, as the wine is stored, the ability of ethanol-tolerant LAB to emerge increases. Growth substrates can be available at this stage as a consequence of yeast cell lysis and release of nutrients into the wine (Mills et al., 2008). Regarding temperature, strains of lactic acid bacteria are mesophilic, they multiply between 15 and 45° C, but optimum growth is between 20-37° C. When the alcohol title increases up to 13-14% vol., the optimum growth temperature decreases. The growth rates keep getting slower as temperature goes down, and it becomes almost impossible at 14-15°C. Low temperatures prevent the multiplication of bacteria, but do not eliminate them (Ribéreau- Gayon et al., 2007).

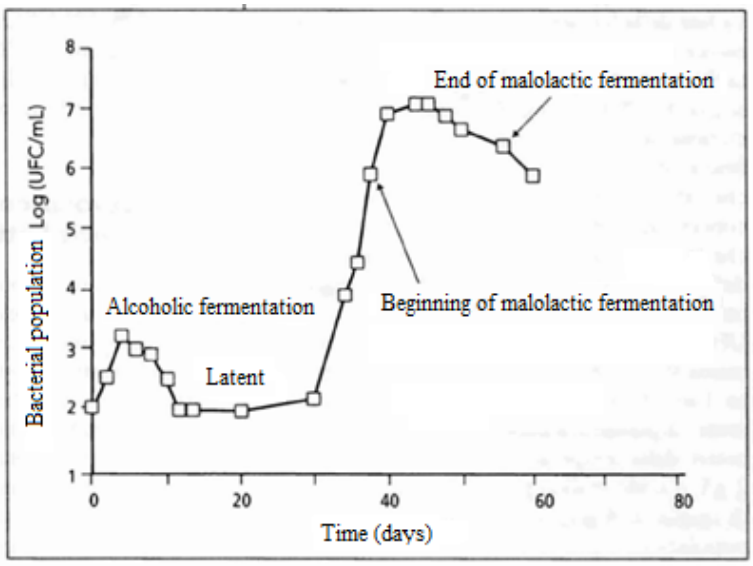


Figure 1: LAB evolution during vinification (Ribéreau-Gayon et al, 2007)

2.3. Yeast and Lactic acid bacteria interaction

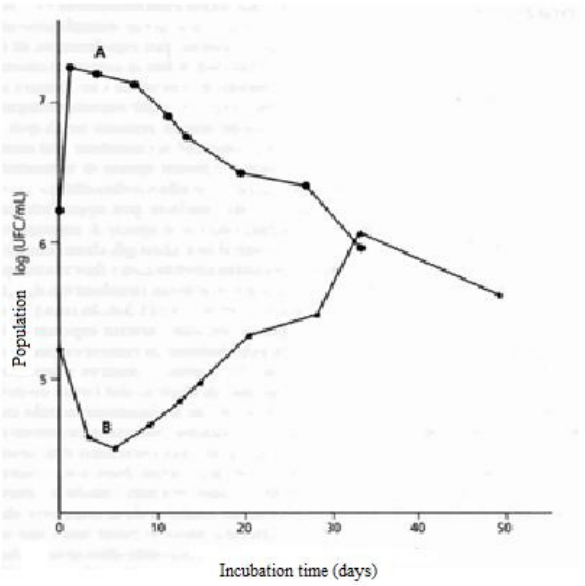


Figure 2: Yeast and LAB evolution (Ribéreau - Gayon et al., 2007) pH 3, 4; Sugar 220g/L. **A**: yeast; **B**: Lactic acid bacteria

Yeasts are well adapted to the growth in grape must. Their multiplication is very fast since the first days of vinification (Figure 2). Even though lactic bacteria also multiply easily, in the must, and without exception, there is predominance of yeasts over bacteria (Ribéreau-Gayon et al, 2007).

In a first stage, which corresponds to the exponential growth of yeasts, the bacteria population diminishes (Ribéreau-Gayon et al., 2007). After a first transitional stage an inverse phenomenon is observed, the decline phase of the yeasts coincides with a phase of fast multiplication of bacteria (Ribéreau-Gayon et al., 2007).

3. Microorganism identification: Traditional Methods Vs Molecular Methods

Efforts to determine the population size and potential impact of different microbes on the winemaking process are critical for the production of a flavorful product. Most approaches to identify and enumerate microbes in wine are determined by culturing homogenates of the product on plates of agar media (enrichment techniques). Yeast colonies are then enumerated, isolated and identified using standard morphological, biochemical and physiological tests, based on culture methods (Deák, 2003). While this technique still remains as the main approach for isolation of microorganism from natural habitats, they are time consuming and can underestimate the size and diversity of a population as sub-lethally injured or viable but non-culturable (VBNC) cells. Therefore, for a better understanding of microbial diversity, other techniques which complements to the traditional method are necessary.

Molecular techniques are based on the extraction and analysis of DNA sequences. These methods are an efficient and fast way for profiling the microbial ecology of habitats. DNA can be either extracted from isolated colonies developed on culture media (culture-dependent techniques) or directly from environmental samples (culture-independent techniques) (Figure 3). This last strategy circumvents the steps of isolation and culturing of microorganism which are known for their selectivity leading to a non-representative view of the extent of microorganism diversity.

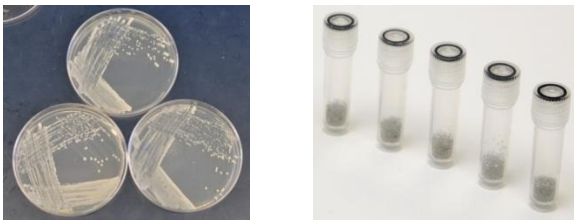


Figure 3: Left: Agar plates for the isolation of microorganism. Right: Screw tubes with glass beads used on this study for the DNA extraction

One important advantage of culture-independent molecular techniques is the ability of detecting viable but not culturable in agar species (Mills et al., 2008). As Prakitchaiwattana et al. (2004) concluded in their study, both methods, traditional and culture-independent molecular techniques, should be used in parallel for profiling the yeast ecology of wine grapes.

4. Polymerase chain reaction

Polymerase chain reaction (PCR) is revolutionary method developed by Kary Mullis in the 1980s that enables researchers to produce millions of copies of a specific DNA sequence in approximately two hours. PCR is based on using the ability of DNA polymerase to synthesize new strand of DNA complementary to the offered template strand. Because DNA polymerase can add a nucleotide only onto a preexisting 3'-OH group, it needs a primer to which it can add the first nucleotide. This requirement makes it possible to delineate a specific region of template sequence that the researcher wants to amplify. At the end of the PCR reaction, the specific sequence will be accumulated in billions of copies (amplicons) as reported in Figure 4.

The conditions of the PCR assay involve many variables:

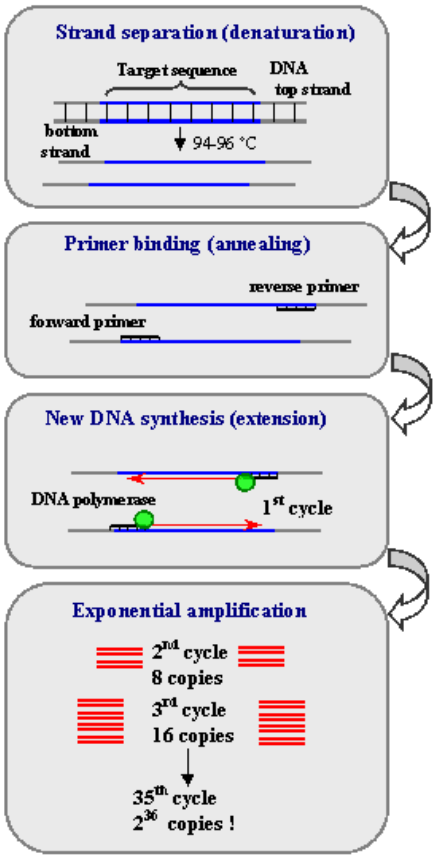


Figure 4: Steps in the PCR cycle

- DNA template: the sample DNA that contains the target sequence. At the beginning of the reaction, high temperature is applied to the original double-stranded DNA molecule to separate the strands from each other.

- DNA polymerase: a type of enzyme that synthesizes new strands of DNA complementary to the target sequence. For this work it was used Taq DNA polymerase (from *Thermus aquaticus*).

- Primers: short pieces of single-stranded DNA complementary to the target sequence. The polymerase begins synthesizing new DNA from the end of the primer.

- Nucleotides (dNTPs or deoxynucleotide triphosphates): single units of the bases A, T, G, and C, which are essentially "building blocks" for new DNA strands.

Detection of any particular species in a mixture will depend on hybridization of the molecules of primer DNA with its template DNA, and the affinity of the primer for homologous sequences in this DNA (Prakitchaiwattana et al., 2004).

Because PCR assays are governed by the principles of enzyme kinetics, it is important to control the initial concentration of template DNA. Excessive template DNA can cause smearing of DNA bands on DGGE gels, as well as inhibition of PCR (Prakitchaiwattana et al., 2004). High populations of yeast cells give too much template DNA and, therefore, dilution is needed to give an acceptable outcome.

The conditions of gel electrophoresis present another suite of variables that can impact on the detection and resolution of DNA bands. Decreasing the pore size of the gels used for DGGE can give an increased resolution and sharper, more intense bands of the DNA amplicons (Prakitchaiwattana et al., 2004).

5. Theoretical aspects of Denaturant Gradient Gel Electrophoresis (DGGE)

Denaturant Gradient Gel Electrophoresis (DGGE) is a molecular fingerprinting method that separates the PCR-generated products on the basis of differences in nucleotide sequence.

Separation of the double strand DNA amplicon is based on the decreased electrophoretic mobility of a partially melted double-stranded DNA molecule in polyacrylamide gels containing a linear gradient of DNA denaturants (a mixture of urea and formamide) (Figure 5).

The melting of DNA fragments proceeds in discrete so-called *melting domains*, stretches of base-pairs with an identical melting temperature (meaning that the melting temperature (*Tm*) of these domains is sequence specific).

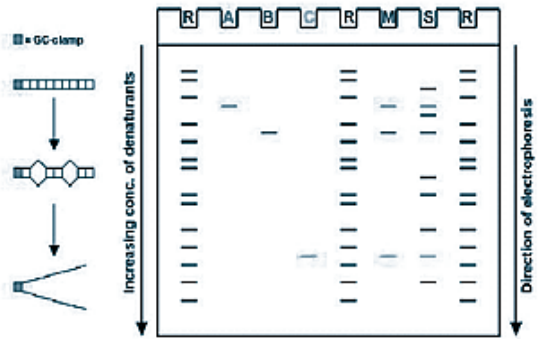


Figure 5: The diagram demonstrates how samples from different microbial communities can be compared. R: reference pattern, A: organism 1, B: organism 2, C: organism 3, M: mix of organism 1, 2 and 3, S: unknown sample

Once a domain with the lowest melting temperature reaches its melting temperature at a particular position in the denaturing gradient gel, a transition of a helical to a partially melted molecule occurs, creating branched molecules, and migration of the molecule will practically halt. Sequence variation within such domains causes the melting temperatures to differ, and molecules with different sequences will stop migrating at different positions in the gel, resulting in a pattern of bands (Muyzer & Smalla, 1997).

When using DGGE (Figure 6), it is essential for an optimal resolution the attachment of a GC-rich sequence, a so called GC-clamp, to one side of the DNA fragment. This clamp is added to the 5´-end of one of the PCR primers, coamplified and thus introduced into the amplified DNA fragments. This GC-rich sequence acts as a high melting domain preventing the two

DNA strands from complete dissociation into single strands. The length of the GC-clamp can vary between 30 and 50 nucleotides.

We can identify unknown microorganism by comparing them with reference strains, because same sequences reach the same position in the gel. Finally, all those bands that remained unidentified can be isolated by excised bands from the gels and sequenced to give species identity.

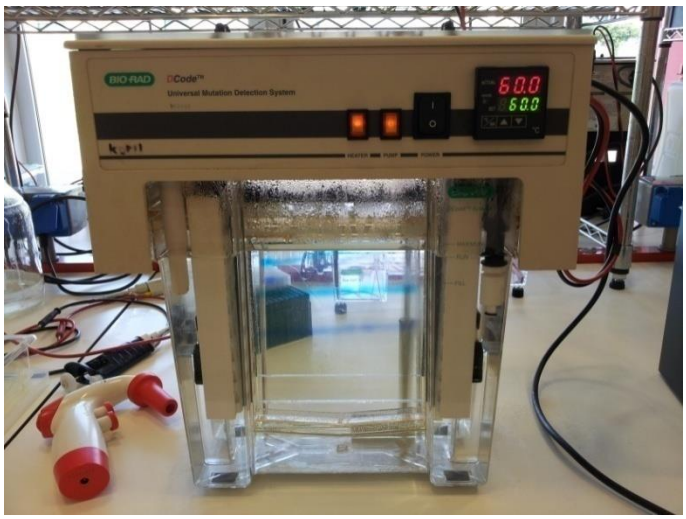


Figure 6: Electrophoretic apparatus used for DGGE during this study.

MATERIALS AND METHODS

1. Samples and Sampling procedures

1.1. Vineyard

On December 2013, representative samples were taken from different points of the vineyard. Samples of leaves (up and downhill), bunches (uphill and downhill), bark (uphill and downhill) and dry berries of Picolit grape were put on sterile bags separately and stored in a fridge. At the beginning of this work, said samples were already processed in physiologic solution and stored at -80°C, ready to be streaked in Malt Agar plate for its later DNA extraction.

1.2. Winery

Sampling was performed on December the 6th 2013. It was done by 3 seconds contacts of the target surface with 15 mL volume of WL and WL Differential Agar (Oxoid, Milan, Italy) contact plates.

List of the points of sample:

CP1	Main door
CP2	Barriche cellar door
CP3	Pallet used for drying of Picolit grapes
CP4	Wall above the barriche cellar door
CP5	Tube holder
CP6	Inside surface of a clean empty tank
CP7	Inside surface of a clean tank with Picolit 2012 wine
CP8	Instalation of the cooling system
CP9	Top of the tank with Picolit 2012 wine
PW	Picolit 2013 wine tank

Contact Plates collected in the winery were incubated at 30°C for 3 days for WL and 7 days for WLD Nutrient Agar plates. Yeast colonies were visually screened and one colony of each present morphology group was picked from plates and subsequently planted on Malt Agar

(Oxoid, Milan, Italy) in order to obtain pure colonies. After incubation at 30°C for 3 days the developed culture was transferred in 1 mL of Malt broth (Oxoid, Milan, Italy) and kept at 30°C for 3 days. Successively 0.5 mL of glycerol (Sigma-Aldrich, Germany) was added and isolates were stored at -80 °C.

1.3. Zymaflore® ST Active dry yeast

Active dry yeast, produced by Laffort (France), is a pure *Saccharomyces cerevisiae* culture selected for the production of sweet wines as well as for dry wines intended for aging.

On 2013 a 10 g sample of this commercial yeast used as a starter at the winery subject of this study was taken in sterile plastic tube and stored at a dry, cool place until DNA extraction. The DNA was already extracted at the beginning of this work, which is why we proceeded to work with this DNA directly.

1.4. Must and wine:

Direct DNA from the must and wine samples had already been extracted at the beginning of this work, which is why we proceeded to work with this DNA directly. Depending in the different times of sampling there were three types of samples:

- Non-inoculated must
- Wine at the end of fermentation (one hour after inoculation with *Pied de cuvee* method)
- Wine before aging in barrique (after last racking and before aging in barrique)

These samples came from botrytized grapes of Picolit during 2013 vintage in Corno di Rosazzo, Friuli Venezia Giulia Region, Italy.

Also samples of must and wine were taken on the same year in 50 mL sterile tubes. These samples were identified, isolated and stored at -80°C. In order to evaluate the presence of Lactic Acid Bacteria (LAB) aliquots of 1 mL of must and wine were transferred into plates

and included in a medium of MRS agar (Oxoid, Milan Italy) for Lactic acid bacteria. They were supplemented with antifungal/antimold agent Delvocid (DSM Food Specialties, Netherlands). Plates were incubated at 30°C for 5 days under microaerophilic conditions. Bacteria colonies were enumerated, isolated and subsequently struck onto MRS agar medium in order to obtain pure colony and incubated at 30°C for 3 days. Isolated bacteria were subjected to both a Gram staining (Gram stain kit, MCC Corp, Torrance, California) and a catalase test (10% v/v hydrogen peroxide). Only catalase negative and Gram positive bacteria were transferred in 1 mL of MRS broth, and kept at 30°C for 3 days. Successively 0.5 mL of glycerol (Sigma-Aldrich, Germany) was added and isolates were stored at -80°C. Afterwards, DNA from each sample was extracted following the phenol chloroform isoamyl protocol described later on this work.

Must and End of fermentation Picolit wine isolates used:

Must	End Fermentation
M-MRS 4	E-MRS 2
M-MRS 6	E-MRS 3
M-MRS 7	E-MRS 4
	E-MRS 6
	E-MRS 7

Yeast and bacteria standardized strains used on the present work:

Saccharomyces strains	Non-*Saccharomyces* strains	Bacteria Strains
Saccharomyces cerevisae ATCC[a] 51	*Brettanomyces bruxellensis* DSMZ[c] 70726	*Lactobacillus casei* TMW 1.1259
Saccharomyces bayanus DBVPG[b] 6171	*Candida etanolica* UCD[d] 7	*Lactobacillus paracasei* 1811
Saccharomyces paradoxus DBVPG[b] 6411	*Hansenula uvarum* UCD[d] 6717	*Lactobacillus sakei* DSMZ[c] 6333
	Metchnikowia pulcherrima DSMZ[c] 70336	*Lactobacillus plantarum* LACpla22
	Schizosaccharomyces ludwigii DSMZ[c] 70550	*Lactococcus lactis* DIAL[e] 296-2
	Pichia membranifaciens UCD[d] 22	*Leuconostoc mesenteroides* DIAL[e]
	Kloeckera apiculata UCD[d] 646	*Oenococcus oeni* OV111

[a]ATCC American Type Culture Collection, Manassas, VA, USA

[b]DBVPG Dipartimento Biologia Vegetale, Perugia, Italy

[c]DSMZ Deutsche Sammlung von Mikroorganism und Zellkulturen GmbH, Braunschweigh, Germany

[d]UCD Department of Viticulture and Enology, Davis, CA, USA

[e]DIAL Dipartimento di Science degli Alimenti, Udine, Italy

2. Revitalization of vineyard and winery samples

From all the samples collected in the vineyard, one replica was randomly chosen for every isolated colony. A total of 75 vineyard samples were isolated. The following are the samples that were chosen for analysis.

Streaked samples Vineyard

LEAVES

UPHILL	DOWNHILL
LU-WL-1	LD-WL-1
LU-WL-2	LD-WL-2
LU-WL-3	LD-WL-3
LU-WL-4	LD-WL-4
LU-WLD-1	LD-WL-5
LU-WLD-2	LD-WL-6
LU-WLD-3	LD-WL-7
LU-WLD-4	LD-WLD-1
	LD-WLD-2
	LD-WLD-3
	LD-WLD-4
	LD-WLD-5
	LD-WLD-6

Dry Berries

B-WL 3
B-WL 4
B-WL 5
B-WL 6
B-WLD 1
B-WLD 2
B-MRS

BUNCHES

UPHILL	DOWNHILL
BU-WL-1	BD-WL-1
BU-WL-2	BD-WL-2
BU-WL-3	BD-WL-3
BU-WL-4	BD-WL-4
BU-WL-5	BD-WL-5
BU-WL-6	BD-WL-6
BU-WL-7	BD-WL-7
BU-WLD-1	BD-WL-8
BU-WLD-2	BD-WLD-1
BU-WLD-3	BD-WLD-2
BU-WLD-4	BD-WLD-3
BU-WLD-5	BD-WLD-4
BU-MRS 2	BD-WLD-5
	BD-WLD-6
	BD-MRS-3

BARK

UPHILL	DOWNHILL
BRU-WL-1	BRD-WL-1
BRU-WL-2	BRD-WL-2
BRU-WL-3	BRD-WL-3
BRU-WL-4	BRD-WL-4
BRU-WL-5	BRD-WLD-1
BRU-WLD-1	BRD-WLD-2
BRU-WLD-2	BRD-WLD-3
BRU-WLD-3	BRD-WLD-4
BRU-WLD-4	BRD-WLD-5
BRU MRS-2	

In the case of the winery's contact plates, all the samples were taken out of the fridge at -80°, given that each one of them came from a different colony. The following is the list of all 58 samples from the winery with which the work was done.

Contact Plate Isolates

CP1	
CP1-WL1 BHI	CP1-PCA 1MB
CP1-WL2 MB	CP1-PCA 2BHI
CP1-WLD1	CP1-PCA 3MB
CP1-WLD2 BHI	CP1-PCA 4BHI
	CP1-PCA 5BHI

CP2	
CP2-WLD1 MB	CP2-PCA 2MB
CP2-PCA 1BHI	CP2-PCA 3BHI

CP3	
CP3-WL1 MB	CP3-PCA 1BHI
CP3-WL2 MB	CP3-PCA 2BHI
CP3-WLD1 MB	CP3-PCA 3BHI

CP4	
CP4-WLD 1BHI	CP4-PCA BHI1
CP4-WLD 2MB	CP4-PCA BHI2
	CP4-PCA BHI3

CP5			
CP5-WL MB1	CP5-WL MB2	CP5-WL MB3	CP5-WLD BHI6
CP5-WLD BHI1	CP5-WLD MB3	CP5-WLD MB4	CP5-WLD BHI7
CP5-PCA BHI1	CP5-PCA BHI2	CP5-WLD BHI5	CP5-WLD BHI8

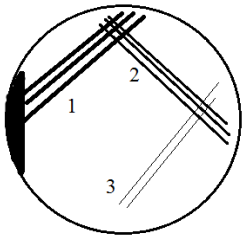
CP6	
CP6-WL MB1	
CP6-WLD-BHI 1	CP6-WLD-BHI 2
CP6-PCA BHI 1	CP6-PCA BHI 2

CP7
CP7-WLD BHI1
CP7- PCA BHI 1
CP7- PCA BHI 2

CP8	
CP8-WL 1 MB	CP8-WL 2 MB
CP8-WLD BHI 1	CP8-WL 3 MA
CP8-PCA BHI 1	CP8-PCA BHI 2
	CP8-PCA BHI 3

CP9	
CP9 WL MB1	CP9 WL MB2
CP9 WLD BHI 1	CP9 WLD MB 2
CP9-PCA BHI 1	CP9-PCA BHI 2
	CP9-PCA MB 3

Said samples were taken out of the fridge at -80°C. Once unfrozen, a 50 µL sample was taken with the micropipette, having the precaution of homogenizing the medium correctly before its withdrawal. Said sample was put in a Malt Agar Plate margin (Oxoid, Milan, Italy). In case the sample had a potential contamination this sample was spread on the surface of the agar with successive and overlapping streaks using a flamed and cooled loop at each step. Figure below illustrates the technique.



Streaked plates were put into incubation at 37°C until there was a grown and isolated colony to extract the DNA.

3. Morphologic classification and identification:

Before its extraction, the colonies were classified according to their morphology and observed through the optical microscope to separate the bacteria from the yeast.

In order to be able to differentiate the potentially *Saccharomyces sensu stricto* yeasts from the non-*Saccharomyces* yeasts, all yeasts were planted in a WL Nutrient Agar (Oxoid, Milan, Italy). After incubation at 25°C for 4-5 days, colonies potentially referring to the genus *Saccharomyces* can be distinguish in base of their color (appear as white) and morphology .

By doing this classification we were able to proceed with a different PCR protocol for the following 3 sample groups: bacteria, *Saccharomyces* and non-*Saccharomyces.*

Regarding bacteria samples, we took only one sample of each kind of morphology to reduce the number of samples to be analyzed.

LIST OF BACTERIA DNA ANALYSED

LEAVES	
UPHILL	**Morfology**
LU-WL-3	Dry Light yellow
LU-WL-4	Glossy Light yellow
LU-WLD-2	Yellow mucous
LU-WLD-3	White glossy
DOWNHILL	
LD-WL-5 a	White very small
LD-WLD-4	White mucous
LD-WLD-5	Dry white
BUNCHES	
UPHILL	
BU-WL-3	Translucent mucous
BU-MRS 2	Small white
DOWNHILL	
BD-WLD-3	Glossy creamy yellow
BD-WLD-4	White Translucent isolated colony
BD-WLD-5	Small ochre (coccus)
BD- MRS 3	Small white
BARK	
UPHILL	
BRU-WLD-1 2	Translucent white with halo
BRU-WLD-2 1	Very Small glossy white
BRU-WLD-4	Small white (coccus)
BRU-MRS 2	Small yellow
DOWNHILL	
BRD-WL-4	White with irregular and expansive halo
BRD-WLD-4	White with ridges

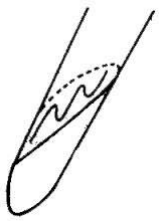
Table 3.a: Morphology classification of bacteria samples used on this study

Contact Plate Isolates			
CP1	**Morfology**		**Morfology**
CP1-WL1 BHI	Glossy creamy white	**CP3**	
CP1-WLD2 BHI	Small dark green	CP3-PCA 2BHI	Small fluorescent yellow
CP1-PCA 4BHI	Very small ochre (bacilo)	CP3-PCA 3BHI	Fluorescent yellow
CP1-PCA 5BHI	Mottled opache white	**CP4**	
CP2		CP4-PCA BHI3	Cotton like white. It expands
CP2-PCA 1BHI	Mucous creamy orange		
CP2-PCA 3BHI	Glossy light pink		
CP5		**CP6**	
CP5-WLD BHI1	White big with green edges	CP6-PCA BHI 1	Creamy white
CP5-WLD BHI6	Big dark green		
CP8			
CP8-WLD BHI 1	Light green		
CP9			
CP9-PCA BHI 2	White with an expansive and irregular halo		

Table 3.b: Morphology classification of bacteria samples coming from Contact plates isolates used on this study

3.1. Storage of pure colonies

Out of precaution, and in case it's necessary once more to resort to the isolated colonies from which the DNA was extracted, said colonies were put in a slant for its proper storage for the duration of this study. The bacteria were put in a PCA Agar medium (Oxoid, Milan, Italy), and the yeasts in Malt Broth Agar (Oxoid, Milan, Italy). Always operating in a sterile environment, the metallic loop was put in the fire, and after cooling it down, a little bit of bacterial patina was taken from a plate. Afterwards, the slant was opened and flamed. The colony was transferred starting at the bottom of the test tube and moving the loop with zig-zag movements all the way out, being careful not to penetrate the terrain.



After 4-5 days in incubation at 30°C, the slant was seal with parafilm and stored in the fridge.

4. DNA extraction

4.1. DNA extraction from isolated colonies

Isolates were subjected to DNA extraction according to the methods described by Manzano et al. (2004).

Pure colonies grown on plates were resuspended in 300 µL of breaking buffer (Triton 2%, Sodium Dodecyl Sulphate 1%, NaCl 100 mM, Trizma HCl 10 mM, EDTA 1 mM (pH 8)) in screwcup tubes containing 0.3 g of glass bits with diameter of 0.5 mm for yeast and 0.1 mm for bacteria.

Only for bacteria lysozyme (50 mg/mL; Sygma-Aldrich, Germany) was added for the lysis of bacterial cells, and incubated at 30°C for 30 minutes.

Tubes were supplemented with 300 µL of phenol-chloroform-isoamilalcohol (25: 24: 1, pH 8; Sygma-Aldrich, Germany) unshaken 3 times with vortex for 1 minute, with 1 minute break each. 300 µL of TE buffer (10 mM Trizma base, 1 mM EDTA, pH 8) was supplemented in each tube and tubes were centrifuged for 10 minutes at 13400 rpm. The aqueous phase was collected and precipitated with 1 mL of ice-cold absolute ethanol. Tubes were centrifuged then for 10 minutes at 13400 rpm. Consecutively ethanol was discarded from the tubes and 1 mL of Ethanol 70% was added and centrifuged again for 10 minutes at 13400 rpm. Finally ethanol was discarded and DNA was dehydrated by leaving the open tubes at 37°C overnight.

The day after 50 µL of DNase-free sterile water and 1 µL of DNase-free RNAse (Roche Diagnostics, Milan, Italy) were added into tubes containing dehydrated DNA to digest RNA with incubation at 37°C for 1 h.

4.2. DNA extraction from must and wine samples:

Direct DNA from Picolit must and wine was already available at the beginning of this study.

For its extraction 45 mL tubes with must, wine at the end of fermentation and wine before aging samples were centrifuged for 10 min at 6000 X g at 4°C; the supernatants were discharged, and the pellets were resuspended in washing solution, and DNA was extracted using the VINEO-Extract DNA Kit (Bio-Rad, Milan, Italy) according to the manufacturer's instructions.

5. DNA Standardization

The extracted DNA with a concentration higher than 250 ng / mL was standardized to obtain a final concentration of 250 ng / mL. NanoDrop 2000c (Thermo Scientific, Wilmington, USA) was used to evaluate the initial concentration. Subsequently DNA was stored at -20°C until further analysis.

6. Molecular analysis

6.1. PCR-DGGE protocol for Lactic Acid Bacteria

Samples subjected to this protocol were:

- DNA extracted from colonies classified as bacteria from the vineyard, winery, must and wine
- DNA from reference strains

These samples were amplified using the forward primer BA338fGC (5´-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCACTCCTACGGGAGGCAGCAG-3´) that complements a region conserved among members of the domain Bacteria, and the reverse primer P_4V_3 (5'-ATCTACGCATTTCACCGCTAC-3´) amplifying a 342 bp section of bacterial 16S rDNA genes.

The amplification was carried out in a 50 µL PCR mixture as follows: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mM $MgCL_2$, dATP, dTTP, dCTP, dGTP 200 µM each, 0.2 µM each primer, 1 U GoTaq DNA polymerase (Promega, Madison, USA), and 250ng of total DNA. The PCR program was: 2 min at 95 °C, followed by 30 cycles of 30s at 95°C, 30s at 60°C, and 30s at 72°C, followed by final extension at 72°C for 5 min (C1000^{TM} Touch Thermal Cycler, Bio-Rad, USA)(Figure 7). 5 µl of each amplicon were detected by 1.5% agarose gel electrophoresis (Agarose for routine use, Sigma-Aldrich, Germany) in 0.5X TBE (2mM EDTA, 80mM Tris-acetate, pH 8.0) using 0.5 µg/mL ethidium bromide and compared with 100bp DNA Molecular Weight Marker (Promega, Madison, USA). The amplified products were visualized under UV light and consecutively subjected to DGGE.

Figure 7: BIO RAD C1000TM Touch Thermal Cycler used in the making of this work for the amplification of samples

Denaturing Gradient Gel Electrophoresis

Analysis of amplicons by DGGE (Denaturing Gradient Gel Electrophoresis) was performed with the Dcode Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA). PCR samples were applied directly onto 8% (w/v) polyacrylamide gels in a running buffer containing 40 mM Tris-acetate, 2 mM $Na_2EDTA.H_2O$, pH 8.5 (TAE) and a denaturing gradient from 30 to 60% of urea and formamide (100% refers to a 7 M urea and 40% w/v formamide solution). The electrophoresis was performed at a constant voltage of 130 V for 3.5 h with a constant temperature of 60°C. After electrophoresis the gels were stained for 30 min in 1.25 X TAE containing 0.5 µg/mL ethidium bromide, rinsed, visualized under UV light, and photographed under UV transillumination using the GeneSnap Syngene Software (Cambridge, UK).

6.2. PCR-DGGE protocol for *Saccharomyces sensu stricto* strains

Samples subjected to this protocol were:

- DNA from reference strains
- DNA extracted from potentially *Saccharomyces sensu stricto* colonies from the vineyard, winery, must and wine

These samples were amplified using the forward primer Schaf (5'-GTAGTGAGTGATACTCTT-3') with a "GC clamp" at the 5' end of the primer (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACCGCGCG-3') and the reverse primer Schar (5'-AGAACATGTTGCCTAGAC-3'). Schaf primer anneals from 18 to 38 bp and Schar primer from 210 to 229 bp giving a fragment length of 211 bp (Figure8) (Manzano et al., 2005).

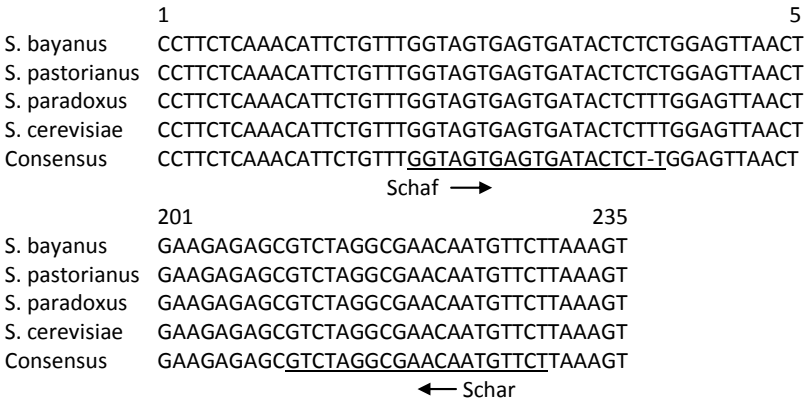


Figure 8: Alignment of ITS2 regions from *S. cerevisiae* (z95931), *S. bayanus* (z95946), *S. pastorianus* (z75732), and *S. paradoxus* (AJ229059). Primer sequences are underlined.

The amplification was carried out in a 50 µL PCR mixture as follows: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCL_2$, dATP, dTTP, dCTP, dGTP 400 µM each, 0.2 µM each primer, AmpliTaq DNA polymerase (Applied Biosystems®, New York, USA)1.25 U, and 250 ng DNA. The PCR program was: 5 min at 95 °C, followed by 35 cycles of 60s at 95°C, 45s at 47°C, and 45s at 72°C, final extension at 72°C for 7 min ($C1000^{TM}$ Touch Thermal Cycler, Bio-Rad, USA). 5 µl of each amplified product were detected by 1.5% agarose gel electrophoresis (Agarose for routine use, Sigma-Aldrich, Germany) in 0.5X TBE (2mM EDTA, 80mM Tris-acetate, pH 8.0) using 0.5 µg/mL ethidium bromide and compared with 100bp DNA Molecular Weight Marker (Promega, Madison, USA). The amplified products were visualized under UV light and consecutively subjected to DGGE.

Denaturing Gradient Gel Electrophoresis

The Dcode Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA) was used to analyze by DGGE (Denaturing Gradient Gel Electrophoresis) the PCR products obtained. Electrophoresis was performed in a 0.8 mm polyacrylamide gel 8% [w/v] (acrylamide-bisacrylamide 37.5:1) in TAE 1.25X buffer containing a 30 to 40% urea-formamide denaturing gradient(100% corresponded to 7 mol l^{-1} urea and 40% [w/v] formamide), increasing in the direction of the electrophoretic run (Manzano et al. 2002). Gels were subjected to 120 V for 4.5h at 59°C stained using 0.5 µg/mL ethidium bromide for 30 min, rinsed, visualized under UV light and captured using the GeneSnap Syngene Software (Cambridge, UK).

6.3. Nested PCR-DGGE protocol for Non-*Saccharomyces* yeasts

Non *Saccharomyces* yeasts were identified by two step amplification and DGGE analysis. Samples subjected to this protocol were:

- DNA extracted from yeast isolates not belonging to *Saccharomyces sensu stricto* group from the vineyard, winery, must and wine
- DNA of samples that didn't anneal in the PCR for *Saccharomyces sensu stricto* protocol
- DNA for reference strains

PCR Step 1

For this first step of the Nested PCR reaction, samples were amplified using the forward primer NL-1 (5'-GCA TAT CAA TAA GCG GAG-GAA AAG-3') and NL-4 (5'-GGT CCG TGT TTC AAG ACGG-3'), which amplify the divergent D1/D2 domains of the large ribosomal subunit (LSU) rDNA (Manzano et al. 2011).

PCR was performed in a final volume of 50 µl containing 10 mM Tris-HCl, 50 mM KCl, 1.5 Mm $MgCl_2$, 0.2mM each dATP, dCTP, dGTP and dTTP, 0.2 mM of the primers, 1.25U GoTaq-DNA Polymerase (Promega, Madison, USA) and 250 ng of the extracted DNA. The reactions were run for 30 cycles: denaturation was at 95°C for 60 s, annealing at 48°C for 45 s and extension at 72°C for 60 s. An initial 5 min denaturation at 95°C and a final 7 min extension at 72°C were used in a Thermal Cycler (C1000^{TM} Touch Thermal Cycler, Bio-Rad, USA). 5 µl of each amplified product were detected by 1.5% agarose gel electrophoresis (Agarose for routine use, Sigma-Aldrich, Germany) in 0.5X TBE (2mM EDTA, 80mM Tris-acetate, pH 8.0) using 0.5 µg/ml ethidium bromide and compared with 100bp DNA Molecular Weight Marker (Promega, Madison, USA). The amplified products were visualized under UV light and consecutively subjected to the second step of the Nested PCR.

PCR Step 2

One microliter of first step PCR were subjected to a second set of 35 cycle PCR using the forward primer NL1GC, 5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCC ATA TCA ATA AGC GGA GGA AAA G-3' (the GC clamp sequence is underlined) and a reverse primer LS2, 5'-ATT CCC AAA CAA CTC GAC TC-3' (corresponding to nucleotide positions 266 to 285 on the *S. cerevisiae* 26S rDNA gene (GenBank accession number M19229)) that amplified approximately 250 nucleotides within the original amplicon of the 5'- end region of the 26S rDNA gene (Cocolin et al., 2000;Iacumin et al., 2009).

This reaction was performed in a final volume of 50 µL containing 10 mM Tris-HCl, 50 mM KCl, 1.5 Mm $MgCl_2$, 0.2mM each dATP, dCTP, dGTP and dTTP, 0.2 mM of the primers, 1.25 U GoTaq-DNA Polymerase (Promega, Madison, USA) and 250 ng of the extracted DNA. The reactions were run for 35 cycles: denaturation was at 95°C for 60 s, annealing at 52°C for 60 s and extension at 72°C for 60 s. An initial 5 min denaturation at 95°C and a final 7 min extension at 72°C were used in a Thermal Cycler (C1000^{TM} Touch Thermal Cycler, Bio-Rad, USA). 5 µl of each amplified product were detected by 1.5% agarose gel electrophoresis (Agarose for routine use, Sigma-Aldrich, Germany) in 0.5X TBE (2mM EDTA, 80mM Tris-acetate, pH 8.0) using 0.5 µg/mL ethidium bromide and compared with 100bp DNA Molecular Weight Marker (Promega, Madison, USA). The amplified products were visualized under UV light and consecutively subjected to DGGE.

Denaturing Gradient Gel Electrophoresis

For PCR product sequence separation, DGGE (Denaturing Gradient Gel Electrophoresis) analysis was performed as proposed by Cocolin et al. (2000) with the Dcode Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA). PCR samples were applied directly onto 8% (w/v) polyacrylamide gels in a running buffer containing 40 mM Tris-acetate, 2 mM $Na_2EDTA.H_2O$, pH 8.5 (TAE) and a denaturing gradient from 30 to 50% of urea and formamide (100% refers to a 7 M urea and 40% [w/v] formamide solution). The electrophoresis was performed at a constant voltage of 120 V for 4 h with a constant

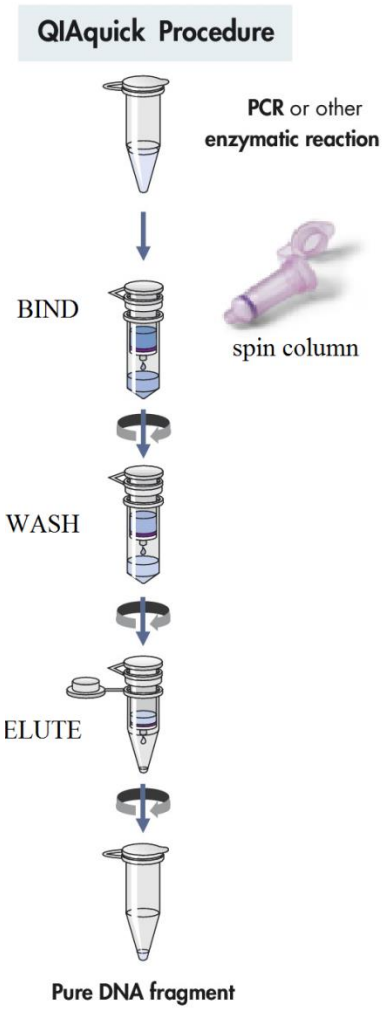
temperature of 60°C. After electrophoresis the gels were strained in 1.25 X TAE containing 0.5 µg/mL ethidium bromide for 40 min, visualized under UV light, and photographed under UV transillumination using the GeneSnap Syngene Software (Cambridge, UK).

7. Sequencing

For all the samples that could not be identified through the method described previously, we proceeded with a specific purification protocol of the amplified, in order to have it later sent to MWG-BIOTECH (Germany) for its sequencing.

To that end, the amplicons that couldn't be identified were re-amplified with the same proportions of buffer, primers, Taq and Cl_2Mg for the preparation of the PCR mix but with the precaution of obtaining a final volume of 100 µL of PCR product.

5 µL of each amplicon were detected by 2% agarose gel electrophoresis (Agarose for routine use, Sigma-Aldrich, Germany) in 0.5X TBE (2mM EDTA, 80mM Tris-acetate, pH 8.0) using 3 µL of loading buffer and 0.5 µg/ mL ethidium bromide and compared with 3 µL 100bp DNA Molecular Weight Marker (Promega, Madison, USA). The amplified products were visualized under UV light and consecutively subjected to purification.



For the purification of the amplicon, the QIAquick PCR Purification Kit was used. The QIAquick system use a simple bind-wash-elute procedure with spin columns to remove primers, nucleotides, enzymes, mineral oil, salts, and other impurities from DNA samples. First the DNA adsorbs to the selective silica-gel membrane. Impurities are efficiently washed away, and the pure DNA fragment is eluted with water.

Following the instructions, in a 1.5 mL eppendorf, 500 µL of binding buffer is added directly to 100 µL of PCR sample, and then the mixture is applied to the QIAquick spin column and centrifuged at 60 sec at 13000rpm. The

filtering was eliminated and 750 µL of ethanol-containing Buffer PE were added in the column. Later, it was centrifuged at 60 sec at 13000rpm. The filtering was eliminated again and it was centrifuged 60 sec at 13000rpm in order to eliminate any residual Buffer PE, which may interfere with subsequent enzymatic reactions.

Finally, DNA was eluted with water. The spin column was put in a 1.5 mL eppendorf. 30 µL of sterile H2O were added in the membrane of the spin column. We waited 5 minutes, and later centrifuged 60 sec at 13000rpm. Finally, the spin column was thrown away because the elute is now found in the eppendorf.

7.1. Issuing of the samples

In order to issue the purified samples for sequencing, the MWG-BIOTECH guidelines were followed:

The samples were dried through the concentrator. Afterwards, they were resuspended with 16 µL of sterilized water. 1 µL was used to make the reading in the NanoDrop. For a product length between 150-300bp we need a sample concentration of 2 ng / µL. 15 µL of purified sample were placed in a 1.5 mL safe-lock eppendorf.

Also, the primer used in the amplification of each sample was prepared and issued: we took 20 µL of primer with a concentration of 10 pmol/µl (10 µM) and added it in an eppendorf containing 80 µL H2O.

Template and primer tubes were labeled with the Eurofins Genomics Prepaid Barcode. This way, each sample with its Primer was sent to MNG- BIOTECH (Germany) after having filled the samples issuing form.

RESULTS

1. Bacteria differentiation

Bacteria samples were amplified using the universal primers BA338fGC and P4V3. Isolates from the vineyard, winery, must and end of fermentation as well as wine direct DNA extraction gave the expected amplicon of 342bp as reported in Figure 9.

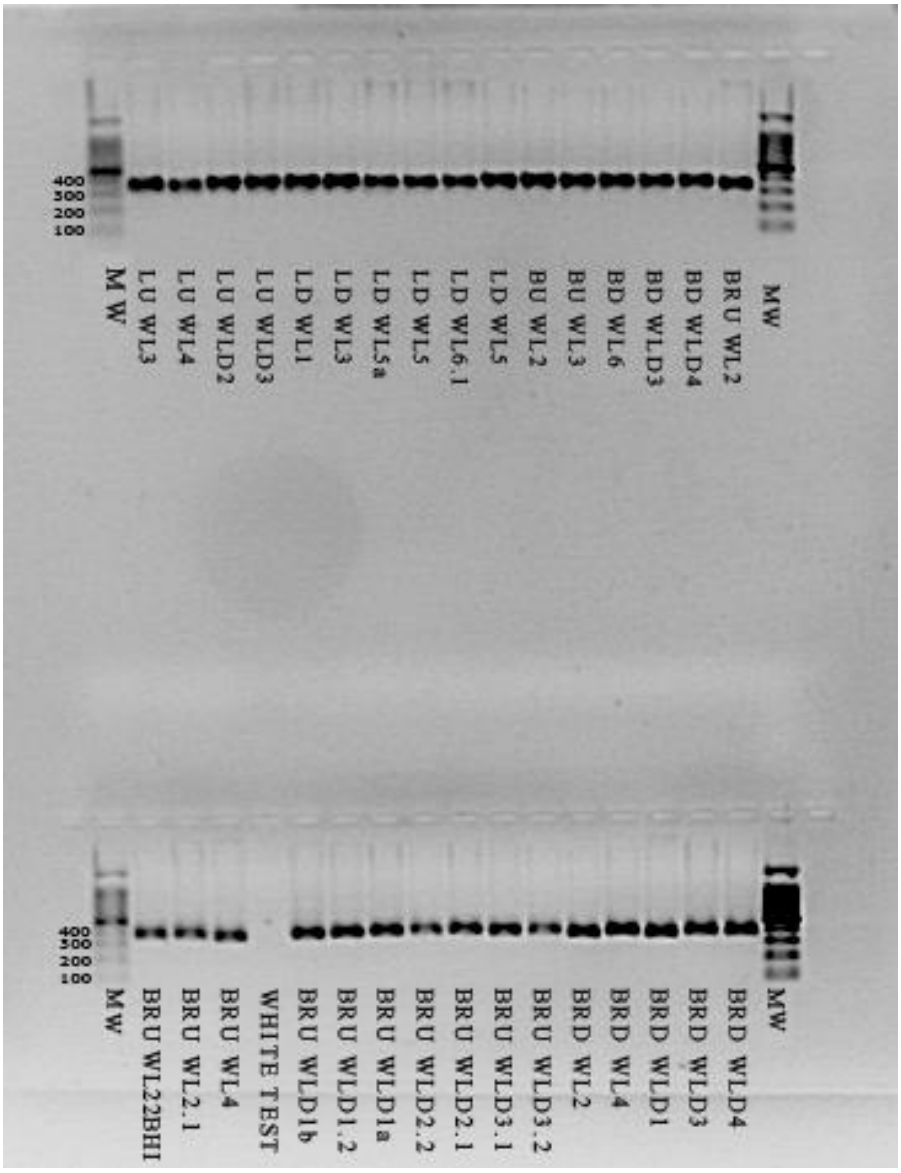


Figure 9: Amplicons obtained of bacteria samples using the forward primer BA338fCG and reverse primer P_4V_3.

All 40 PCR products were analyzed with DGGE. In each DGGE amplicons with same band profile were regrouped and one amplicon of each group was used in a final DGGE next to the reference strains (Figure 10).

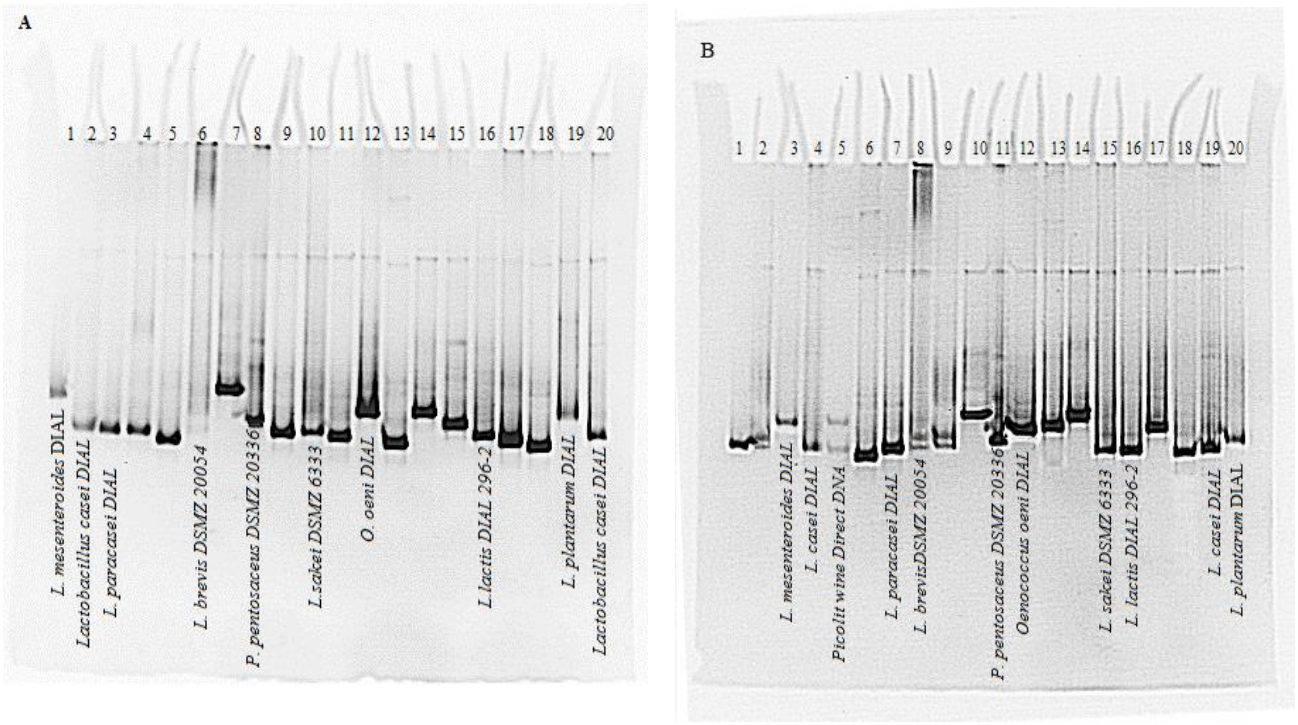


Figure 10: DGGE profile of reference strains next to isolated samples. **A**: line 4 and 18, Leaves uphill; line 5 and 7, Leaves downhill; line 9 and 11, Bark isolates; line 13, Contact plate 3; Line 14, Contact plate 4; Line 15, Contact plate 5; and Line 17, Contact plate 8. **B**: Line1 and 10, end of fermentation isolates; line 2, 6 and9, Winery Contact plate isolates; Line 13 and 17, Bunch isolates; Line 14 and 18, Bark isolates; line 5, Picolit wine direct DNA extraction

As reported in Figure 11, this study showed that 38.5% of the isolated samples were *Lactobacillus casei / Lactobacillus paracasei.* Their presence was identified as much in the must as it was in the wine. They came mainly from the bark, leaves and bunches taken from the vineyard, since only tree samples taken from the winery, Contact plate 1, 8 and 9, were identified as *L. casei / L. paracasei.*

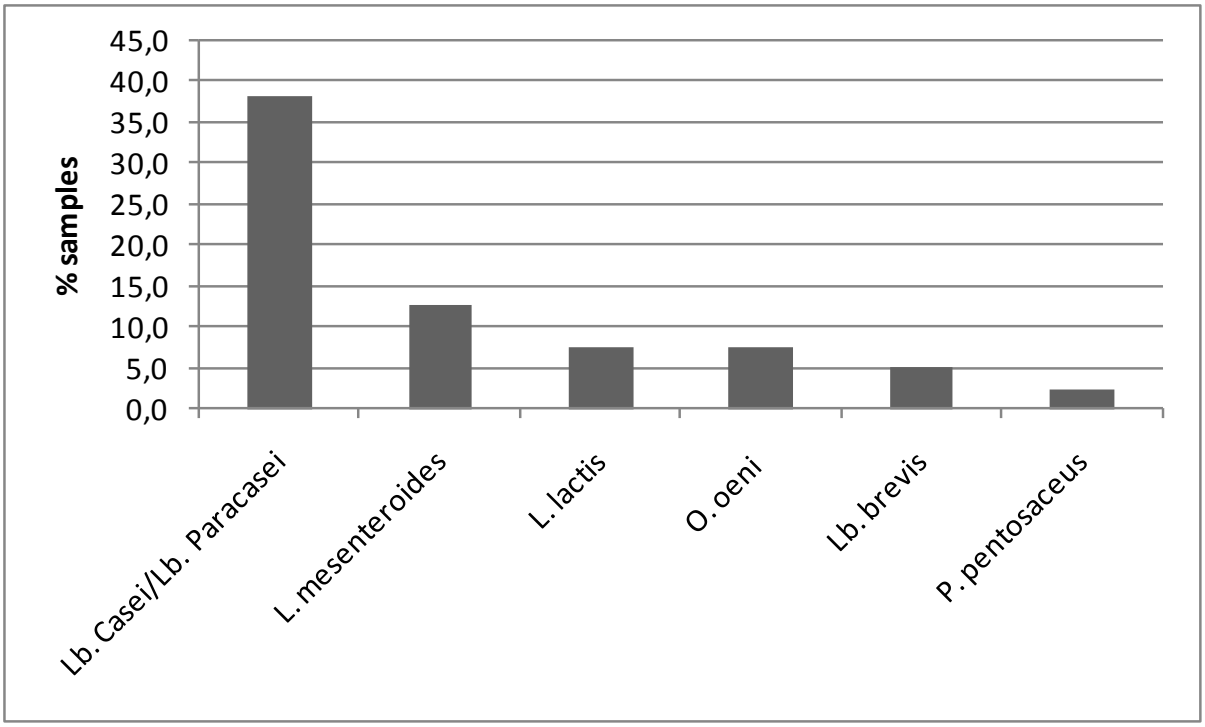


Figure 11: DGGE results expressed in percentage of the total number of isolated samples analyzed

Lactococcus lactis wasn't identified neither in the must nor in the wine, in spite of being in 2 samples of the vineyard and in a contact plate (CP6) of the winery. On the other hand, *Leuconostoc mesenteroides* was found in the must and in the wine. It came solely from the bark and leaves of the vineyard.

Regarding *Oenococcus oeni,* only 2 samples of the vineyard that came from the buds and one contact plate (CP4) that was put on the wall above the barriche cellar door, were identified as *O. oeni*.

Finally, both *Pediococcus pentosaceus* and *Lactobacillus brevis* were identified solely in the winery, without seeing its presence neither in the must nor the wine.

The direct DNA picolit wine confirms what we have seen earlier. Only the *Lactobacillus casei/ Lactobacillus paracasei* and the *Lactobacillus mesenteroides* band were identified in the DGGE profile.

Species that didn't correspond to a reference strain used were sent for sequencing to Eurofins Genomics (Ebersberg, Germany). Results obtained by DNA sequencing are reported in Table 3.

Table 3

Lactobacillus hilgardii
Lactobacillus mali
Pediococcus parvulus

2. *Saccharomyces* differentiation

Potentially *Saccharomyces* sensu stricto yeasts that were planted in a WL Nutrient Agar showing classical color and colony morphology related to *Saccharomyces* were extracted and DNA was amplified with ShafGC and Shar specific primers. Also DNA extracted directly from must, wine and the starter used for inoculation were amplified with ShafGC and Shar primers.

DNA extracted directly from wine and from the starter, and also DNA samples coming from the winery contact plates isolates gave the expected 210bp band for *Saccharomyces sensu strico* (Figure 12).

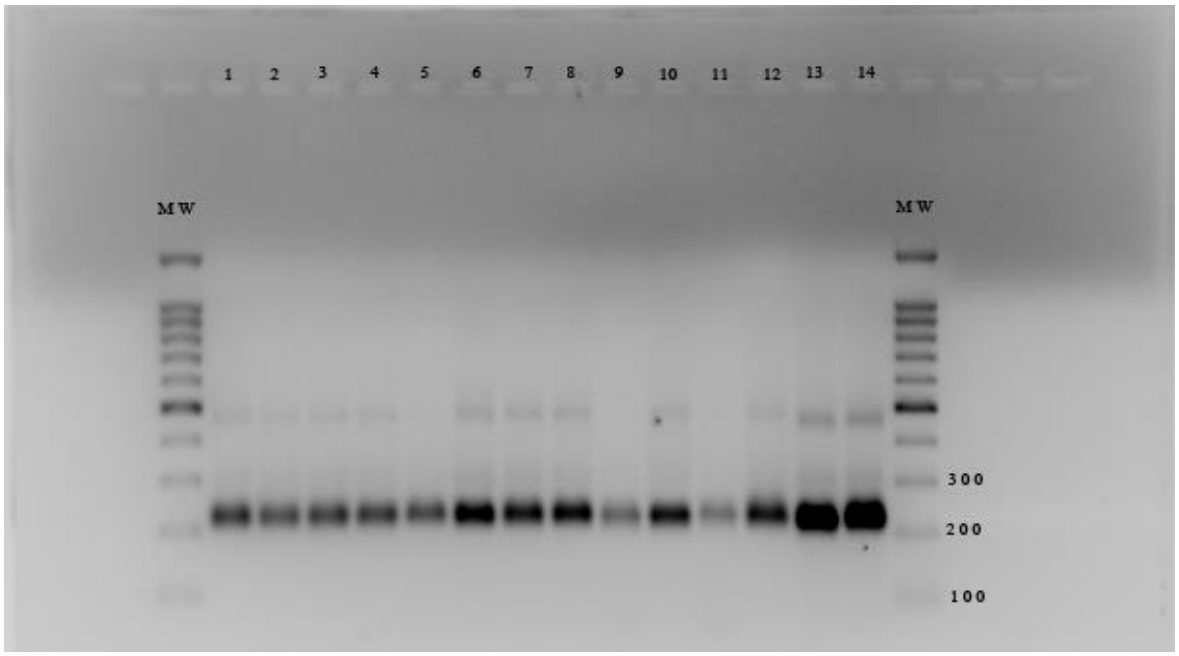


Figure 12: DNA amplification of *Saccharomyces sensu stricto* samples using the forward primer ShafGC and reverse primer Shar. Lane 1 to lane 11, contact plate samples; lane 12, *S. cerevisiae* ATCC51; lane 13, *S. bayanus* DBVPG 6171; lane 14, Starter.

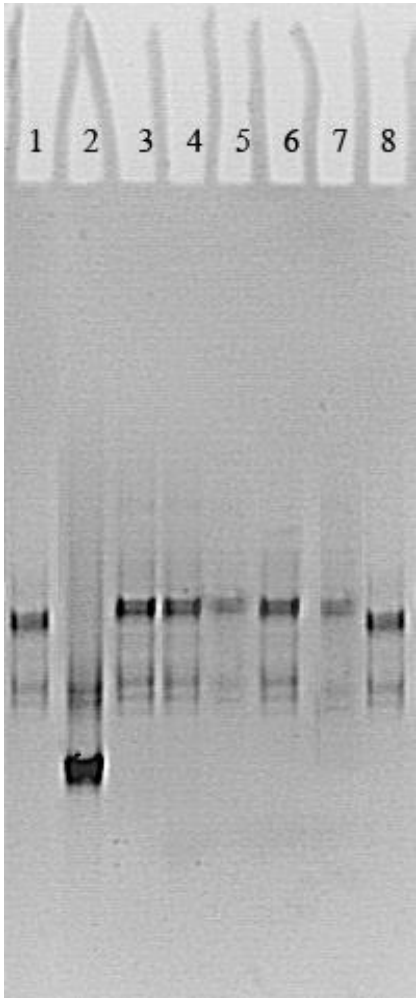


Figure 13: Line 1 and line 8 *S. cerevisiae* ATCC51; Line 2 *S. bayanus* DBVPG 6171; Line 3 to line 6 contact plate samples; Line 7 Starter

The PCR products were run in DGGE and migration profiles of samples were compared with the reference strains as shown in Figure 13.

The isolated samples coming from the winery were the same as the one that came from the starter, indicating the influence of the starter in the winery resident flora. All samples corresponded to the *Saccharomyces cerevisiae* species, although the bands did not have the exact same height as the reference strain.

These results support the conclusion that there was only one strain of *Saccharomyces cerevisiae* present in the winery, which is identical to the commercial starter.

3. Non *Saccharomyces* differentiation

All yeast showing morphology and color colony characteristics not related to *Saccharomyces* and yeast that did not annealed with ShafCG and Shar primers although showing *Saccharomyces* morphologies, were amplified using the Nested PCR protocol with primers NL1 and NL4 for the first step, and NL1GC and LS2 for the second step.

Samples coming from the vineyard, the winery, as well as from the direct DNA samples from must and wine that gave the expected 250bp amplicon are reported in Figure 14.

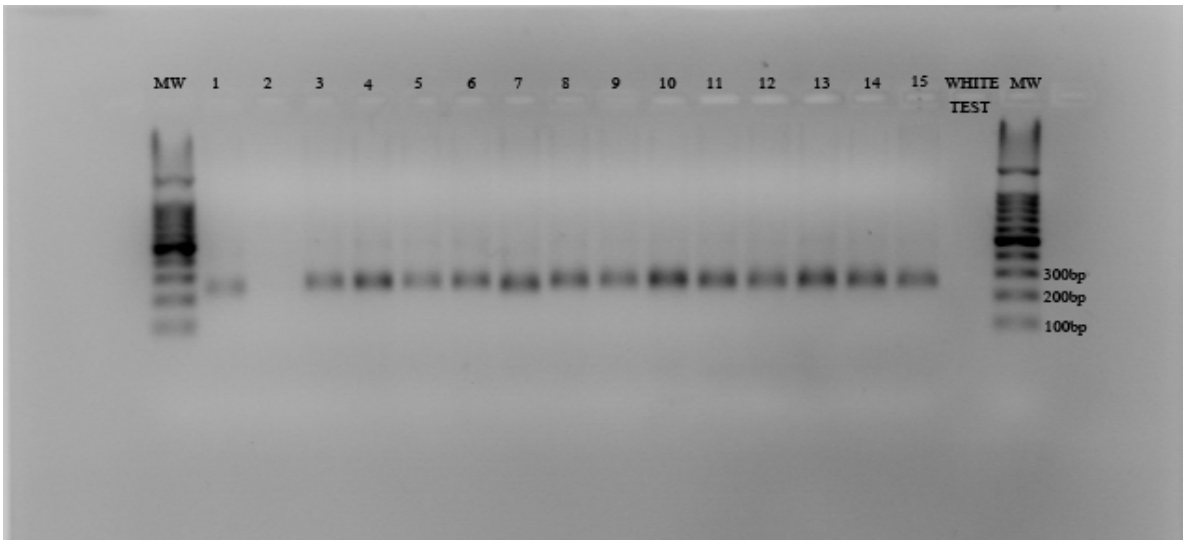


Figure 14: Second step of the DNA amplification of non-*Saccharomyces* samples extracted from vineyard and winery isolates. Line 1, 3 and 4 Contact plate isolates. Line 5 to 15, vineyard isolates. Line 2 showed no amplification indicating that it wasn't yeast, but bacteria.

All PCR products were analyzed with DGGE. In each DGGE run, amplicons with same band profile were regrouped and one amplicon of each group was used in a final DGGE next to the reference strains. As shown in Figure 15 two reference strains of *Saccharomyces cerevisiae* ATCC 51, amplified with the same protocol used for non-*Saccharomyces*, were put at the

beginning and the end of the gel to allow the evaluation of the sample positions after migration.

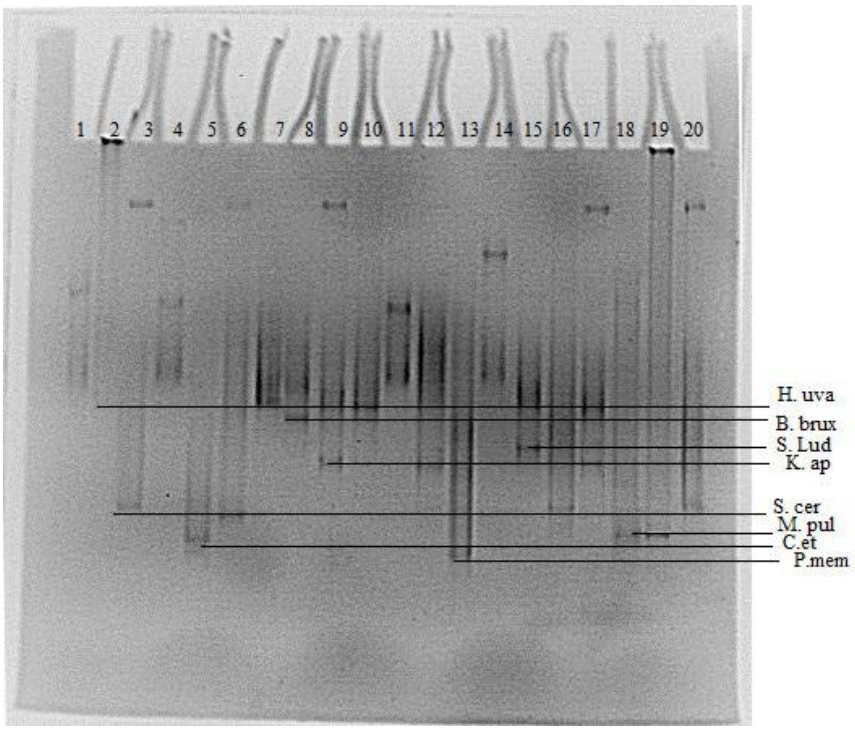


Figure 15: Line 3 and 20, *Saccharomyces cerevisiae* ATCC 51; Line 5, *Candida etanolica* UCD 7; Line 7, *Hansenula uvarum* UCD 6717; Line 8, *Brettanomyces bruxellensis* DSMZ 70726; Line 9, *Kloeckera apiculata* UCD 646; Line 13, *Pichia membranifaciens* UCD 22, Line 15, *Schizosaccharomyces ludwigii* DSMZ 70550; Line 18, *Metchnikowia pulcherrima* DSMZ 70336. Line 1, 4, 11 and 14 did not show any similarity with the reference strains. They were sent to sequencing. The rest of the lines correspond to vineyard and winery isolates.

The DGGE analysis showed that 21% of the samples corresponded to *Kloeckera apiculata* coming from the vineyard as well as from the winery. *Pichia membranifaciens* and *Hansenula uvarum* were found only in the vineyard, whereas *Metchnikowia pulcherrima* was also found in the winery. There was no identification of *Brettanomyces bruxellensis* nor of *Candida etanolica* (Figure 16) indicating the absence of these yeasts in the samples analyzed.

DNA extracted directly from the Picolit end of fermentation and wine before aging showed the same band profile as *Saccharomyces cerevisiae,* indicating the predominance of *S. cerevisiae* above the other yeasts. Also one sample from the winery Contact Plate (CP8) was identified as *S. cerevisiae* (Figure 16).

DNA extracted directly from the Picolit must showed the presence of *S. ludwigii.* This yeast was also found in the vineyard in one isolated of the Picolit bud.

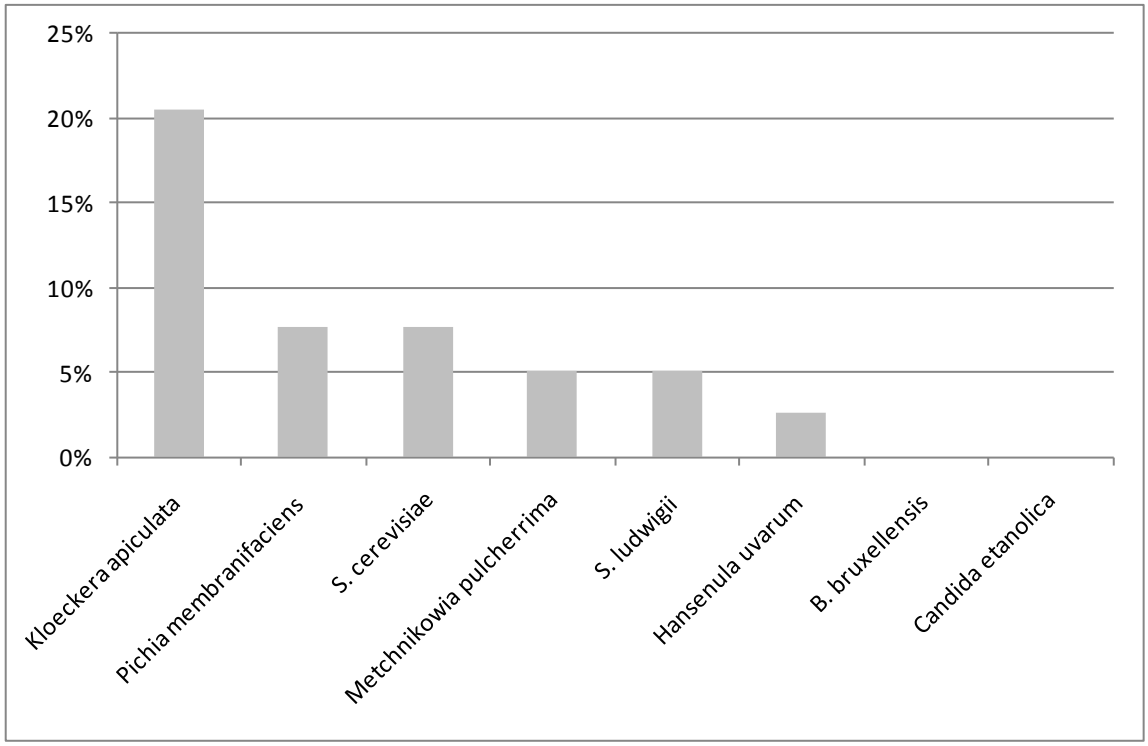


Figure 16: DGGE conclusion expressed in percentage of the total number of isolated samples analyzed.

Yeast samples that didn't correspond to a reference strain used were sent to Eurofins Genomics (Ebersberg, Germany) for sequencing. Results are reported in Table 4.

Table 4

_____

Torulaspora delbrueckii

Debaryomyces hansenii

_____

CONCLUSIONS

The aim of this study was to identify bacteria and yeasts present in the field and surfaces of the cellar to compare these data with the data obtained by a previous analysis on the production of Picolit sweet wine produced in Friuli Venezia Giulia Region. The molecular methods used were useful for the identification of the strains isolated from leaves, barks, bunches, grapes and contact plates. From the data it is possible to know the origin of some microorganisms present in the must or in the wine, indicating the selection due to the yeast fermentation process. In fact only few microorganisms were found at the Picolit wine before aging (after 225 days): *Lactobacillus casei/ Lactobacillus paracasei* and the *Lactobacillus mesenteroides* bacteria.

As expected, the addition of the starter reduced the variability of the yeasts during the fermentation process indicating that the starter was useful in reducing the indigenous flora coming from the bunches, barks, leaves and grapes. In fact, in the wine before aging only *S. cerevisiae* was found. An interesting data is the presence of *O. oeni* on the wall above the barriche cellar door.

ACKNOWLEDGEMENTS

To the Science and Technology Scholarship program of Argentina who funded my two years Masters in Italy.

To my Supervisor and Professor Marisa Manzano, who patiently taught me all about molecular biology and gave me strength and hope whenever something in the analysis went wrong.

To Priya Vizzini who always answered my questions and helped me implement the molecular analysis.

To my husband who always support me in my decision to study abroad.

REFERENCES

Ciani Maurizio, Comitini Francesca, Mannazzu Ilaria & Domizio Paola (2010) Controlled mixed culture fermentation: a newperspective on the use of non-Saccharomyces yeasts inwinemaking. FEMS Yeast Res 10, 123–133.

Cocolin L., Bisson L.F., Mills D.A. (2000) Direct profiling of the yeast dynamics in wine fermentations. FEMS Microbiology letters 189, 81-87

Deák, T. (2003) Detection, Enumeration and Isolation of Yeasts. In: Yeasts in Food, Beneficial and Detrimental Aspects (Boekhout, T. and Robert, V., Eds.), pp. 39–68. B. Behr's Verlag GmbH & Co., Hamburg, Germany.

Giraffa, G. and Neviani, E. (2001) DNA-based, culture-independent strategies for evaluating microbial communities in food associated ecosystems. Int. J. Food Microbiol. 67, 19–34.

Iacumin L., Cecchini F., Manzano M., Osualdini M., Boscolo D., Orlic S., Comi G.(2008) Description of the microflora of sourdoughs by culture-dependent and culture-independent methods. Food Microbiology 26; 128-135.

Jolly N.P., Augustyn O.P.H. and Pretorius I.S. (2006) The Role and Use of Non-Saccharomyces Yeasts in Wine Production. S. Afr. J. Enol. Vitic., Vol. 27, No. 1, 15-39

Kunkee Ralph E. and. Amerine Maynard A (1968) Sugar and Alcohol Stabilization of Yeast in Sweet Wine. Applied Microbiology, 1067-1075.

Manzano M., Cocolin L., Iacumin L., Cantoni C., Comi G. (2005) A PCR- TGGE (Temperature Gradient Gel Electrophoresis) technique to assess differentiation among enological *Saccharomyces cerevisiae* strains. Int. Journal of Food Microbiology 101, 333-339.

Manzano M., Cocolin L., Longo B. and Comi G. (2004) PCR-DGGE differentiation of strains of *Saccharomyces* sensu stricto. Antonie van Leewenhoek 85: 23-27.

Manzano M., Iacumin L., Vendrame M, Cecchini F., Comi G. and Buiatti Stefano (2011) Craft Beer Microflora Identification before and after a cleaning process. J. Inst. Brew. 117(3), 343-351.

Mills D., Phister T., Neeley E., and Johannsen E. (2008) Wine Fermentation. Molecular Techniques in the Microbial Ecology of Fermented Foods.© Springer 2008, 162-183.

Mortimer Robert, Polsinelli Mario (1999) On the origins of wine yeast. Research in Microbiology.Vol 150, Issue 3, 199-204.

Muyzer G. & Smalla K. (1997) Application of denaturent gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie van Leeuwenhock 73, 127-141

Muyzer, G. (1999) DGGE/TGGE a method for identifying genes from natural ecosystems. Curr. Opin. Microbiol. 2, 317–322.

Muyzer, G. and Smalla, K. (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Van Leeuwenhoek 73, 127–141.

Prakitchaiwattana C., H. Fleet G., M. Heard G. (2004) Application and evaluation of denaturing gradient gel electrophoresis to analyze the yeast ecology of wine grapes. FEMS Yeast Research 4, 865–877.

Rantsiou K., Dolci P., Giacosa S., Torchio F., Tofalo R., Torriani S., Suzzi G., Rolle L. and Cocolin L. (2012) Candida zemplinina Can Reduce Acetic Acid Produced by Saccharomyces cerevisiae in Sweet Wine Fermentations. Appl Environ Microbiol. ; 78(6), 1987-1994.

Ribéreau-Gayon P.; Dubourdieu D.; Doneche B.; Lonvaud A.(2007)Trattato di enologia. Vol 1: Microbiologia del vino Vinificazioni. Il Sole 24 Ore Edagricole; 117, 163-180.

Urso R., Rantsiou K., Dolci P., Rolle L., Comi G. & Cocolin L. (2008) Yeast biodiversity and dynamics during sweet wine production as determined by molecular methods. FEMS Yeast Res 8, 1053–1062.