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DEPARTMENT OF FOOD SCIENCE, TECHNOLOGY AND MICROBIOLOGY

XIV CYCLE

SPARKLING WINE PRODUCTION BY *CHAMPENOISE* METHOD IN LOMBARDY REGION: YEAST POPULATION BIODIVERSITY AND TECHNOLOGICAL ASPECTS OF INDIGENOUS *SACCHAROMYCES CEREVISIAE* AS POTENTIAL STARTERS

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"Nuestra recompensa se encuentra en el esfuerzo y no en el resultado. Un esfuerzo total es una victoria completa".
Mahatma Gandhi.

A mis padres, hermanos y familiares en Colombia
A ti, mi familia en Italia.
Este esfuerzo es dedicado a ustedes.

Chapter 1

General Introduction

1.1 Lombardy wines

Lombardy (figure 1), in the mountainous north of Italy, is a wine producing region from a variety of local and international grapes such as: *Pinot Bianco*, *Pinot Grigio*, *Pinot Nero*, *Chardonnay*, *Moscato Bianco*, *Malvasia*, *Sauvignon*, *Riesling* and *Müller-Thurgau* for white wines; *Barbera*, *Bonarda*, *Cabernet Sauvignon*, *Croatina*, *Gropello*, *Merlot*, *Nebbiolo*, *Sangiovese* and *Vespolina* for red wines. The principal wine-producing areas in Lombardy region are *Franciacorta*, *Garda*, *Oltrepò Pavese*, *Valtellina* and *Valcalepio*, where twenty titles between *DOC (Denominazione di Origine Controllata)* and *DOGC (Denominazione di Origine Controllata e Garantita)* are present covering an impressive range of wine styles, demonstrating just how versatile the production is. Among these twenty *DOCs* there are a still red wine (*Valtellina Superiore*), two sparkling whites (*Franciacorta* and *Oltrepò Pavese Metodo Classico*), a sweet wine (*Moscato di Scanzo*) a dry *Passito* wine (*Sforzato di Valtellina*) a rosé wine (*Garda Classico Chiaretto*) and a red wine (*Garda Classico Rosso*).

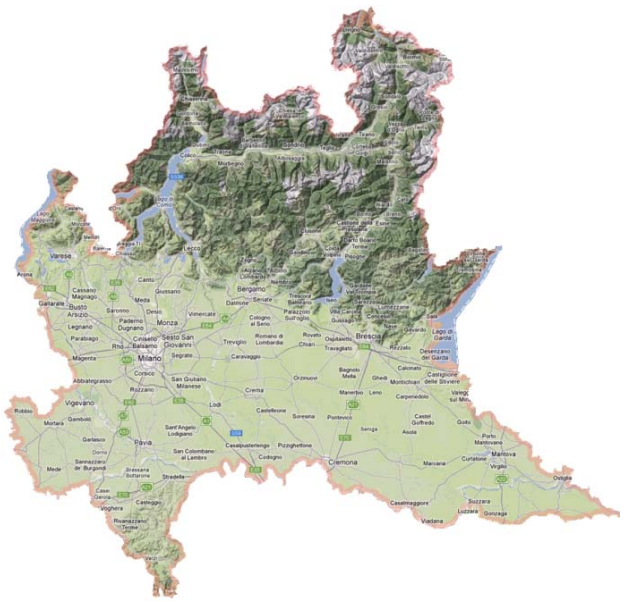

Figure 1.
Lombardy region[1]

The region annually produces over 28 million gallons (1.1 million hectolitres) of wine and is known particularly for its sparkling wines made in *Franciacorta* and *Oltrepò Pavese* areas by *Champenoise* method.

[1] http://maps.google.it (accessed: 01/12/11)

1.2 *Franciacorta* area

Franciacorta (figure 2) is a region of gentle hills in the district of *Brescia*. The area is limited eastward by rocky and drifty hills, westward by the river *Oglio*, northward by the banks of the *Iseo* lake, the last parts of *Alpi Feniche*, and southward by the alluvional plain that ends on the state highway *Brescia-Bergamo*. Watching *Franciacorta* from above, a double drifty amphitheatre can be noticed; it was formed during the last ice age, thanks to the action of a large glacier that, falling from *Val Camonica*, dug the basin of the lake *Iseo* and pushed its drifts to form the present hill bars. Life has been present in these valleys since ancient time. This is proved by the findings of pre-historic era and the many evidences in classical authors: *Plinio*, *Columella* and *Virgilio*. That area represents one of leader region in the Italian winery industry because of its high quality production. It has about 2.500 hectares of vineyards planted with *Chardonnay* (85%), *Pinot nero* (10%), and *Pinot bianco* (5%) of which, approximately, 2.100 are for *Franciacorta* sparkling wine production and 400 for "*Curtefranca*" white and red wine.

Figure 2.
Franciacorta area[2]

The grapes for *Franciacorta* have grown in strictly delimited vineyards in the following villages: *Adro*, *Capriolo*, *Cazzago San Martino*, *Cellatica*, *Coccaglio*, *Cologne*, *Corte Franca*, *Erbusco*, *Gussago*, *Iseo*, *Monticelli Brusati*, *Ome*, *Paderno Franciacorta*, *Paratico*, *Passirano*, *Provaglio d'Iseo*, *Rodengo Saiano*, *Rovato*, and *Brescia*. Within the same communes, but in separately defined vineyards, grapes have grown for *Terre di Franciacorta DOC* (a still red blended from *Cabernet Sauvignon*, *Cabernet Franc*, *Merlot*, *Barbera*, and *Nebbiolo*, and a still dry white made from one or more of *Chardonnay*, *Pinot Bianco*, and *Pinot Nero*)

[2] http://maps.google.it (accessed: 01/12/11)

1.3 *Oltrepò Pavese* area

The *Oltrepò Pavese* (figure 3) is an area of the province of *Pavia* with a total surface of approximately 1097 km² and a population of about 146.579 habitants, which owes its name to the fact of being located south of the River *Pò*, wedged between *Emilia-Romagna (*province of *Piacenza)* and Piedmont *(*province of *Alessandria*). *Oltrepò pavese* area has always been suitable for the production of grapes and wine: its wine-growing history is at least as old as 2000 years. The morphological areas that characterize the *Oltrepò* are mainly two: the lands on the low hills, made up of marine sedimentary rocks with a high level of clay, and the higher lands containing gypsum. Both these terrains perfectly guarantee the typological features of different white wines, sparkling white wines and red wines produced here. About 13.000 cultivated hectares of the *Oltrepò Pavese* corresponds to 55% of the 24.000 cultivated areas of Lombardy. If the surface registered at the *Denominazione di Origine* (*DO)* list is taken, the cultivated area of the *Oltrepò Pavese* (About 11.000 hectares) corresponds to more than 70% of the Lombard cultivated areas (15.000). With 600.000 hectoliters of *DOC* and *DOCG* wines, and the 300.000 hectoliters of *Indicazione Geografica Tipica* (*IGT)* wines, which are produced on average every year, the *Oltrepò* (with the 42 villages located on the hills here) becomes the third most important one in Italy for *DOC* wines, after *Chianti* and *Asti* (Scienza et al., 2008).

Figure 3.
Oltrepò Pavese area[3]

[3] http://maps.google.it (accessed: 01/12/11)

The *Oltrepò Pavese DOC* list includes a significant number of wine typologies: from the traditional *Bonarda*, obtained with *Croatina* grapes, to other traditional red wines like the *Buttafuoco*, the *Sangue di Giuda*, the vintage *Rosso* obtained with *Croatina* and *Barbera* grapes, other local grapes like *Uva Rara* and *Vespolina*, and the traditional white wines like the *Riesling* or the aromatic *Moscato*. For sure, though, the most important one is the *Pinot Nero*: a versatile vineyard in the *Oltrepò* area, which is particularly suitable for red wines and noble sparkling wines as the *Oltrepò Metodo Classico DOCG*. The cultivation of *Pinot Nero* has increased from about 600 hectares in the mid 20th century to about 2,200 today, a number that tends to increase steadily. This vineyard can be found almost in the whole territory of the *Oltrepò* although it is particularly grown in *Valle Versa*, *Valle Scuropasso* and on the high level of *Valle Coppa*. From the numerous experiments carried out in the *Oltrepò* area by the Department of Vegetable Productions of the Faculty of Agricultural Science in Milan, it has been established that this variety adapts well to thick cultivation on high levels (due to its scarce vigor) and low pruning with lots of low buds. In these conditions, and only in areas that are suitable for its cultivation, it is possible to reach high quality levels with different variety.

1.4 Sparkling wine

For a general definition of the product, sparkling wine could be described as the result of two consecutive fermentation steps obtained through *Champenoise* method (in Italy nominated *Metodo classico*). In the first fermentation, a base wine is made by standard winemaking procedures. In the second step, commonly denominated "*prise de mousse"* or "*tirage*", a mixture of yeasts, sucrose and, in some cases, clarifying agents, is added to the base wine. This mixture is then bottled to allow the refinement. At the end of this period, yeast lees are removed through a procedure called "*remuage*" (Lallement, 1998). At this point, some parameters of the wine are adjusted by adding wine, liquors, and sugar in various proportions, and then, the bottles are closed for marketing. The final product has an ethanol concentration of about 9.5 to 11.5% vol/vol, and the pressure in the bottle is approximately from 5 to 6 atmospheres (atm). An interesting characteristic of sparkling wine production through this method is that the yeast autolysis takes place in the bottle. Yeast autolysis involves the release of different products, resulting in a degradation of yeast macromolecules, into the wine. Different compounds such as proteins, peptides, amino acids, polysaccharides, nucleic acid derivatives, and lipids released during autolysis, have a positive effect on the quality of the aroma, flavor, and foam of the final product (Cebollero et al., 2005).

1.4.1 Legislation in Europe

Over thirty years ago the European Community established a series of regulations that certified the quality of products on sale for the consumer and that safeguarded the interest of those producers who, with commitment and diligence, created genuine and valuable products. The wine sector has always been in the forefront with regard to the enhancement of the typical aspects of the different geographical areas and to the tradition of production systems.
According to the Council regulation (EC) No 479/2008 of 29 April 2008 of the European Union, sparkling wine shall be the product:

- which is obtained by first or second alcoholic fermentation:
  — from fresh grapes,
  — from grape must,
  — from wine;
- which, when the container is opened, releases carbon dioxide derived exclusively from fermentation;
- which has an excess pressure, due to carbon dioxide in solution, of not less than 3 bar when kept at a temperature of 20°C in closed containers;
- for which the total alcoholic strength of the *cuvées* intended for their preparation shall not be less than 8,5 % vol.

The European Union has regulated the following classification for the sparkling wines:

Quality sparkling wine:

- is obtained by first or second alcoholic fermentation:
  — from fresh grapes,
  — from grape must,
  — from wine;
- releases carbon dioxide derived exclusively from fermentation when the container is opened;
- has an excess pressure, due to carbon dioxide in solution, of not less than 3,5 bar when kept at a temperature of 20°C in closed containers;

- the total alcoholic strength of the *cuvées* intended for their preparation shall not be less than 9 % vol.

Quality aromatic sparkling wine:

- is obtained only by use, when constituting the *cuvée*, of grape must or grape must in fermentation which are derived from specific wine grape varieties on a list to be drawn up in accordance with the procedure referred to in article 113[4]. Quality aromatic sparkling wines traditionally produced using wines when constituting the *cuvée*, shall be determined in accordance with the procedure referred to the article 113;
- has an excess pressure, due to carbon dioxide in solution, of not less than 3 bar when kept at a temperature of 20°C in closed containers;
- the actual alcoholic strength may not be less than 6 % vol.;
- the total alcoholic strength may not be less than 10 % vol. Specific rules concerning other supplementary characteristics or conditions of production and circulation shall be adopted in accordance with the procedure referred to the article 113.

Aerated sparkling wine:

- is obtained from wine without a protected designation of origin or a geographical indication;
- releases carbon dioxide derived wholly or partially from an addition of that gas when the container is opened;
- has an excess pressure, due to carbon dioxide in solution, of not less than 3 bar when kept at a temperature of 20°C in closed containers.

Semi-sparkling wine:

- is obtained from wine provided that such wine has a total alcoholic strength of not less than 9 % vol.;
- has an actual alcoholic strength of not less than 7 % vol.;
- has an excess pressure, due to endogenous carbon dioxide in solution of not less than 1 bar and not more than 2,5 bar when kept at a temperature of 20°C in closed containers;
- is put up in containers of 60 liters or less.

Aerated semi-sparkling wine:

- is obtained from wine;
- has an actual alcoholic strength of not less than 7 % vol. and a total alcoholic strength of not less than 9 % vol.;
- has an excess pressure of not less than 1 bar and not more than 2,5 bar when kept at a temperature of 20° C in closed containers due to carbon dioxide in solution which has been wholly or partially added;
- is put up in containers of 60 liters or less.

Member States may limit or exclude the use of certain oenological practices and provide for more stringent restrictions for wines authorized under community law produced in their territory with a view to reinforce the preservation of the essential characteristics of wines by means of a protected designation of origin or protected geographical indication of sparkling wines and liqueur wines. Member States shall communicate those limitations, exclusions and restrictions to the commission, which shall bring them to the attention of the other Member States.

[4] Council regulation (EC) No 479/2008 of 29 April 2008

In the case of Italy, there are special regulations for the production of *DOC* and *DOCG* wines. These are local instruments of control based on the European regulations, establishing standards that wine producers have to respect in order to indicate on their label the *DO*: the principal norms relate to the precise geographical area of production, the choice of the varieties of grape to cultivate in order to obtain a given wine, winemaking and bottling methods, a chemical and organoleptic analysis carried out to control the wine before launching it on the market. All of this has done to safeguard both the denomination as a collective good and the final consumer.
The geographical origin and the strict control of every phase of production through the regulations are characteristics that allow *DO* wines to be distinguished from "table wines", which can claim neither a precise geographical origin of the grapes nor a regulation that certifies the origin of the product. According to the community directives regulating this subject, on the label of still wines must appear the abbreviation *VQPRD*., which stands for *Vino di Qualità Prodotto in Regione Determinata* (Quality Wine Produced in a Specific Region), an "international" hallmark valid throughout the European Union. In this sense, wine quality can be imagined as a pyramid at whose base are table wines, followed higher up by wines with *IGT*, where a geographical origin is indicated and that, unlike *DOC* wines, do not undergo a physicochemical and organoleptic control, then by *DOC* wines and finally, at the top, by *DOCG* (Figure 4) wines for which production criteria are even more restricting. In Italy there are organizations called *Consorzi di Tutela* which functions are regulating the use of *DO* seal, safeguarding against fraudulent use, guaranteeing the compliance of the requirements for the quality production, promoting the production area, and instilling in consumers knowledge and image of the *DO* wines. These functions are performed according to the 164/92 Law provided by the *MiPAF (Ministero delle Politiche Agricole e Forestali)* (Ferrazi, 2009).

Figure 4.
DOGC (Denominazione di Origine Controllata e Garantita) labeled[5]

1.4.2 Sparkling wine in Italy

Sparkling wine obtained through the *Champenoise* method, represent a relevant cultural and outstanding economical fact in Italy. Most of Italy's sparkling wines are produced in the cooler regions of Northern Italy, particularly Piedmont, Veneto, and Lombardy. Economically and culturally, this typology of wine plays an important role and is mainly consumed during holidays and special occasions.
The balance of 2010 showed 376 million bottles sold in Italy and around the world, by both *Champenoise* (classic) and "Italian" (*Charmat*) methods. According to the data revealed in the

[5] http://www.prodottiregionali.net/marchi-tutela/(accessed: 13/03/12)

annual survey conducted by *OVSE* (Observatory of Effervescent Sparkling Wines)[6], 24 million of bottles produced by the *Champenoise* method were sold during the year, of which 10,200,000 *Franciacorta* and 8,100,000 *Trento DOC*. Nearly 22.750.000 bottles were sold on the domestic market and only 1,180,000 bottles on foreign markets. Lombardy is confirmed as the leading Italian region of the *Champenoise* method with 15,300,000 bottles (64% of the total), out of a total of 18 million. 352 million bottles of the so-called " Italian" (*Charmat*) method were sold: 127 million on the domestic market and 225 million on 78 foreign markets. The main markets were Germany, U.S.A., U.K., Russia and Switzerland.
The market for Italian sparkling wines has a value of 2.9 billion euro, of which the wines produced by the *Champenoise* method represent 225 million euro. It is important to notice that Italy exports into 78 countries, while Spain does it in 117 and France in 180.
In the first quarter of 2011 the growth trend of Italian sparkling wines is confirmed once again. Also, sparkling wines have taken a major step forward: highlighted in 2010, they now seem to be the key for entering certain new markets, where consumers are wine novices. It is easier to break down entrance barriers with simple wines, red and white, sparkling, ideal for every palate, suitable to any food combination - sushi, roast meats or vegetables with sauces - and affordable, even for those who could not afford a bottle of wine in the recent past.
Italian production is on a high and low swing: there is strong growth in prices of base wines for sparkling *Prosecco DOC* wines and especially *Cartizze Superiore* of *Valdobbiadene DOCG*, which hit a record of around 7 euro per gallon in bulk, while the production capitals of the *Champenoise* method, from *Franciacorta* to *Trento* and from *Altalanga* to *Oltrepò Pavese*, decreased. In the first quarter of 2010 there was an 18% growth in shipments recorded by *OVSE*, especially for *Asti* and *Prosecco*. The main destinations are Russia (champagne and sparkling increased 25%), Germany (12%) and Brazil (9%). Origin prices have not increased; the retail price through traditional channels is increasing slightly and consumer prices on the international large-scale distribution are in decline. There is growth in "generic" sparkling wines, or those without a clear origin of the grapes that sell fancy named brands made from a blend of Muscat or *Prosecco-Glera* grapes. Sparkling wines made from *Malvasia* grapes and Italian *Riesling* are both doing well on European markets. In Italy, sales of sparkling wines are increasing on domestic markets, in supermarkets and Cooperatives. Champagne wine has also registered a recovery but only of the well-known brands and vintage products. There is a decline in new brands and small producers showed a weakening as well. *DOC* sparkling wines have made an enormous jump (+7%), while in Italy sparkling wines without a clear source of origin, felt down during the first quarter of 2011. The first positive notes have been recently heard in restaurants and wine shops after the decline in 2009 and 2010: there is a slow but promising recovery for sparkling wines, "Vintage" wines and top Italian brand names are also performing well with affordable prices: *Ferrari* and *Berlucchi* lead the category of the classic method with the most famous brands and *Mionetto*, *Bortolomiol*, *Ruggeri* and *Val d'Oca*, for the world of *Conegliano Valdobbiadene Prosecco Superiore DOCG*. A relevant case is the all-time record for *Cartizze Spumante* Extra Dry *DOCG*, with an average price of 12.50 to 14.50 euro a bottle on the shelf in supermarkets.

1.4.2.1 Some commercial aspects

In France the name "*Champagne*" conveys exactly the style of wine it talks about, and makes a direct connection between the wine type and its place of origin. The same is true for other French sparkling wines such as *Crémant de Bourgogne*, *d'Alsace*, *de Limoux* and so on, as well as for the highly effective name "*Cava*" which refers specifically to wines from the *Penedès* region of Catalonia in Spain. This, unfortunately, is not the case of the Italian word *"spumante*." In addition to the numerous and diverse products made by the *Charmat* system, there are also a multitude of

[6] http://www.ovse.org (accessed: 15/01/12)

wines produced by the *Champenoise* method, originated mostly in the northern regions of *Piemonte*, *Lombardia*, *Trentino-Alto Adige* and *Friuli*, but also in smaller quantities from *Valle d'Aosta*, *Veneto*, *Emilia Romagna*, *Toscana*, *Umbria* and even *Sicilia*. Producers of these wines have been aware of the need to find an alternative to the generic term "*spumante*" in order to distinguish their products from the rest of the category and so presenting a clear image to consumers in Italy and, above all, abroad. On the other hand, there are other problems which are holding back the quality sparkling-wine sector. One is the seasonal pattern of sales. Consumers in Italy understand sparkling wines as a part of the ritual celebration of the major annual festivities, rather than an anytime drink and, as a consequence, two thirds of the entire national production is sold in the last three months of the year. The other problem is the size of the production. With an output of between 16 and 17 million bottles, Italy just does not have the critical mass of a production like, for instance, France does with the 300 million bottles of *Champagne* or Spanish *Cava*'s 200 million bottles.
More than half of all the *Champenoise* method wine made in Italy comes from the cellars of two producers, *Ferrari* in *Trentino* and *Guido Berlucchi* in *Franciacorta*. The regional breakdown sees 6,300,000 bottles (of which 4.3 million labeled *Ferrari*) from *Trento DOC*, just over 4 million from *Franciacorta DOCG*, and around one million from *Oltrepò Pavese*, with the rest of the production shared between *Piedmont*, *Veneto*, *Friuli*, *Alto Adige* and other minor zones. The combination of the seasonal sales, fragmented production, and relatively limited total output has put a brake on advertising and promotion, which has meant that the sector has not been able to benefit, in terms of growth, from the significant improvements in quality of the last 10-15 years. In *Trentino* and *Franciacorta* in particular, there are numerous boutique wineries and grower/producers with productions ranging from 50,000 to 100,000 bottles of impeccable quality.

1.4.2.2 *Franciacorta* sparkling wine

"Franciacorta" is one of the two sparkling *DOCG* wines produced in Lombardy region. It has rapidly acquired a reputation as Italy's finest sparkling wine, with due respect still paid to the sweeter, light-hearted wines such as *Moscato d'Asti*. *Franciacorta* was promoted to the highest level of Italian wine classification in 1995, after much hard work and lobbying by the *Consorzio per la tutela del Franciacorta*. The *Consorzio per la Tutela del Franciacorta* (*CVF*) with its unmistakable logo (figure 5) – a battlemented F, symbol of the ancient crenellated towers of the Middle Ages – was constituted on March 5, 1990 as an aggregation of a group of vine-growers united by passion and common stimuli, interested above all in the protection, exploitation and promotion of the wine-producers of *Franciacorta* and its territory as a whole.

Figure 5.
Franciacorta DOCG labeled[7]

This gave rise to a new and independent inter-professional organization, able to provide services, good image, and aggregation that was strong and equal for all those concerned. Over time the Consortium has grown, has cultivated its own precise vocation between study and research and between control and vigilance over denomination, also, and above all, in collaboration with local

[7] http://www.franciacorta.net (accessed: 26/09/11)

institutions and university bodies. The *CVF* has been based in *Erbusco* (BS) since 1993 and has been joined by three professional categories: vine-growers, wine makers and bottlers who were interested in the productivity of the Franciacorta *DOCG*, *DO*, *Curtefranca DOC* e *Sebino IGT*. There are various firms of various dimensions ranging from family-run businesses to large-scale companies renowned throughout the world, steeped in tradition and determined to obtain the best possible quality. The Associated wineries of the Consortium of *Franciacorta* are the most active in the area and are always ready to approach the market with the best product of the vine-growing area, the *Franciacorta*. The Consortium is responsible for a variety of activities: on the one hand, it checks production and on the other one it protects and promotes the denomination and the wines. Checks take place in the vineyard throughout the year, on the grapes and in the cellars, during the winemaking process (Ferrazi, 2009). Furthermore, the "*Strada del Franciacorta*" offers itself as a support organization for individual tourists, groups, and tour operators, ensuring them efficient technical and organizational assistance. It supplies information and services, plans itineraries, and helps them to personally discover the broad array of possibilities that Franciacorta offers, with its renowned, centuries-old viticulture and nonpareil location between the Lake Iseo and the art-city of Brescia.

The types *Franciacorta* sparkling wine are classified as:

- *Franciacorta*
- *Franciacorta Satèn*
- *Franciacorta Rosé*
- *Franciacorta millesimato*
- *Franciacorta riserva*

Type	Minimum period of aging (in months)
Franciacorta	18
Franciacorta Rosé	24
Franciacorta Satèn	24
Franciacorta millesimato, Franciacorta Rosé millesimato, Franciacorta Saten millesimato	30
Franciacorta riserva, Franciacorta Rosé riserva, Franciacorta Satèn riserva	60

Table 1.
Franciacorta sparkling wine classification and minimum period of aging[8]

All *Franciacorta* is aged on its lees (18 months for the non-vintage wines and 30 months for those bearing a vintage) and is not released for further seven months after this period is complete. The result of lees ageing is a more complex, rounder wine style, although few *Franciacorta Cuvéees*

[8] http://www.franciacorta.net (accessed: 26/09/11)

match the weight and body of good *Champagne*. *Franciacorta* has its own version of *Champagne*'s *Blanc de Blancs – Franciacorta Saten –* made only from white *Chardonnay* and *Pinot bianco* grapes. This name was originally restricted for use only among members of the consortium mentioned earlier, but has now been opened up to all *Franciacorta* producers. A pink *rosato* (*rosé*) style is also produced, using at least 15% *Pinot nero* for adding color.

Franciacorta DOCG was the first Italian wine – and up to now the only one – produced exclusively with slow fermentation in bottles to be awarded, in 1995, by the *DOCG* qualification, the highest recognition of quality for a wine. *Franciacorta DOCG* becomes the name that represents all the exceptional characteristics of this special wine but it is strictly prohibited to indicate the method of processing and the term "*vino spumante*" (Stevenson, 2008). Since 1st August 2003, the word *Franciacorta* has been the only means to identify the wine, the area and the method of production. *Franciacorta* is made from the grapes of *Chardonnay* and/or *Pinot bianco* and/or *Pinot nero*, harvested only by hand in small boxes, and after a selected and soft pressing. The sensory profile presents straws yellow with greenish or golden highlights, and a delicate, long-lingering bead of bubbles. The bouquet boasts classic notes of refermentation in the bottle "the impressions of fresh-baked bread and yeastiness", enlivened with subtle hints of citrus, dried white fig, and mixed roasted nuts, including almond and hazelnut. On the palate it is full-flavoured, refined, and remarkably well-balanced. The following styles of taste are admitted: No Dosage, Extra Brut, Brut, Extra Dry, Sec, Demi-sec (Table 2).

Style	Characteristic
No Dosage	Sugar up to 3 grams/liter. It is the driest of the *Franciacorta* range. It has an exceptional character, pungent bubbles obtained by not adding syrup which would make it sweeter and softer. This applies only to *Franciacorta* wines. The dry wine with the typical aroma of the refermentation in the bottle
Extra Brut	Sugar up to 6 grams/liter. Very dry and apart from being an excellent aperitif it also goes extremely well with delicate food; it is an ideal companion to fish dishes, shellfish and raw shellfish. It can attempt to savour it with cooked cured meats like *"cotechino"*.
Brut	Sugar up to 15 grams/liter. Dry but slightly more "*soft"* than Extra Brut, it is certainly the most versatile: as well as being an aperitif it also goes very well with delicate dishes and throughout the meal.
Extra Dry	Sugar 12-20grams/liter. *Franciacorta "soft"* with a slightly larger dosage than the classical Brut. It is ideal with savoury flans and ovenbaked vegetables. Excellent as an aperitif for those who dislike a very dry taste. In fact, the slight sweetness tones limit the strong flavours and enhances the taste of the food.
Sec or Dry	Sugar from 17- 35 grams/litre Less dry or slightly "*abboccato*", it is particularly indicated to be served with soft cheeses, piquant and full fat cheeses, similar to *Taleggio* or *Gorgonzola*. It is also recommended with liver *patè*. Excellent with not very sweet desserts and usually served at the end of a meal with dry *pasteries*, fruit tarts or sweet "*focacce"*.
Demi-sec	Sugar from 33-50grams/litre. Thanks to its "abboccato" taste which is due to the high sugar content, it goes well with typical tarts and cakes like the "*Panettone*" and the "*Pandoro*". Ideal with snacks, piquant cheeses and it blends well with a great number of foods.

Table 2
Types of taste of *Franciacorta* sparkling wine on the basis of residual sugar[9]

[9] http://www.franciacorta.net (accessed: 26/09/11)

Franciacorta DOCG Satén

Satén is one of Franciacorta's greatest expressions of harmony, pleasure and taste. It is made only from *Chardonnay* grapes (prevalent) and *Pinot bianco*, with a pressure less than or equal to 4.5 atm and a sugar content no higher than 15 g/liter, as in the production of brut, only. Creamy, smooth and harmonious, it can also be "*millesimato*". The *Satèn* mark, registered by the *CVF* in 1995 to identify this particular type of *Franciacorta*, is reserved only for producers associated with the Consortium and that can adhere to the strict production regulations. The sensory profile presents straw yellow in appearance, sometimes deep in hue, and with greenish highlights at times. It releases a creamy, long-lasting bead of notably delicate bubbles. A soft-contoured bouquet offers emphatic notes of well-ripened fruit, enriched by delicate nuances of spring flowers and of mixed nuts, including roasted almond and hazelnut. In the mouth, lively flavours and a refreshing crispness are in admirable balance with a texture that gives the impression of luxurious silk. This velvety quality is due to the fact that the internal pressure is less than 5 atmospheres. *Satèn* is produced only in the Brut style

Franciacorta DOCG Rosé

White and red grapes are made into wine separately according to the *Franciacorta* disciplinary and the preparation of the *cuvée* with *Chardonnay* wine, *Pinot bianco* wine and at least 15% of *Pinot nero* wine takes place at the end of fermentation. The *Pinot nero* grapes are made to ferment in contact with the skins for the necessary time for the attainment of a pink or red wine base capable of conferring to the final product a pale pink hue. During the formation of the foam, *Franciacorta Rosé* has a very slightly pinky colour. *Franciacorta Rosé* can be "*millesimato*" and produced in all the taste versions. The presence of *Pinot nero* confers to this *Franciacorta* a significant body and vigour. *Franciacorta Rosè* is produced in the style of No Dosage, Extra brut, Brut, Extra Dry, Sec or Dry, Demi-sec.

Franciacorta DOCG Millesimato

Millesimato, or vintage-dated *Franciacorta*, is composed of base wines that are at least 85% from one single growing year. It may be released only after a minimum of 37 months from harvest. The Sensory profile describes bouquet and palate of *Franciacorta Millesimato* reflects in a striking fashion the weather conditions of its growing year and the sensory expressiveness of the grapes from that particular vintage. It is produced in the styles of No Dosage, Extra Brut, Brut, Extra Dry. In the case of *Satèn*, only Brut has been produced. Both *Satèn* and *Rosé* can be *Millesimato*, which increases their complexity, body, cellar ability, and elegance.

Franciacorta DOCG Riserva

Riserva is a *Franciacorta Millesimato*, which can include *Satèn* and *Rosé*, that has matured a minimum of 60 months. A *Riserva* is released, therefore, a full 67 months (5 and a half years) after harvest. Since many *Franciacorta Millesimato* rest lie far longer than the required minimum of 30 months, this designation was created to highlight this unique type of wine. *Riserva* is produced in the styles of No Dosage, Extra Brut, Brut. In the case of Satèn, only Brut style has been produced.

1.4.2.3 *Oltrepo Pavese* sparkling wine

The first and certain date connected to the production of sparkling wine in the *Oltrepò Pavese* area dates back to 1870 when *Domenico Mazza di Codevilla*, an engineer, started the production of the "*Oltrepò Champagne"*. It was during the early years of the 20th century that the *Pinot nero* widely spread in the area of the *Oltrepò Pavese*, thanks to companies that produced sparkling wine from *Pinot nero*. This area then proved to be extremely suitable for its cultivation. Until then, other

producers have undertaken the slow fermentation in bottles. In 1930 *La Versa* wine-producers' co-operative, started a correct and professional sparkling process. Furthermore, it is worth remembering that the *"Società Vinicola Italiana di Casteggio"* (Italian Winery of the town of *Casteggio*, *SVIC*) produced and exported classic sparkling wines during the early years of last century from what used to be the Italian Reign even to the United States. After them, there was the historical winery of the *Ballabio* family. This tradition has lasted to the present day, and the *Oltrepò* has been acknowledged - at both national and international level - as an excellent area for the production of sparkling wine *champenoise* method from *Pinot nero* grapes. *Oltrepò Pavese Metodo Champenoise* was awarded with its *DOCG* status in 2007. The preservation and enhancement of wine denomination is carried out by the *Consorzio Tutela Vini Oltrepò Pavese (*figure 6*)* (*Oltrepò Pavese* consortium for the Preservation of wines), whose headquarters are in the town of *Broni* (in the province of *Pavia*). Nowadays, the Consortium - which was founded as a volunteer association in 1961 and instituted in 1977 - includes 237 companies among which there are six wine-producers' co-operatives with their 1480 associated wineries. For 10 years (Ministerial Order 256/1997) the consortium has accepted the proposal of the *Mipaaf (Ministero delle Politiche Agricole Alimentari e Forestali[10])* to have an authority for the preservation, the inspection as well as the enhancement of the whole production. In December 2003, the *Mipaaf* instructed the Consortium to carry out inspections for the *Oltrepò Pavese DOC* among all the producers. The preservation policy is carried out by the Consortium through inspections on vineyards, wineries as well as bottles. As of May 1st, 2005 the application of an alphanumeric label on each *DOC* bottle has become mandatory: it was authorized by the *Mipaaf* in order to be able to verify the origin of a given product from vineyard to the moment when it's bottled. The enhancement of the wines of the *Oltrepò* area is carried out by the Consortium through the organization and participation to events as well as exhibitions on a local, regional, national and international level.

Figure 6.
Consorzio Tutela Vini Oltrepò labeled[11]

The area of production for the grapes used in the DOCG *Oltrepò Pavese Metodo Classico*, contains a large part of the hill side area of the *Oltrepò Pavese* and the territories of the following 42 municipalities in the Province of Pavia: *Borgo Priolo, Borgoratto Mormorolo, Bosnasco, Calvignano, Canevino, Canneto Pavese, Castana, Cecima, Godiasco, Golferenzo, Lirio, Montalto Pavese, Montecalvo Versiggia, Montescano, Montù Beccaria, Mornico Losana, Oliva Gessi, Pietra de Giorgi, Rocca de Giorgi, Rocca Susella, Rovescala, Ruino, San Damiano al Colle, Santa Maria*

[10] http://www.politicheagricole.it (accessed: 16/12/11)
[11] http://www.vinoltrepo.it/ (accessed: 10/01/11)

della Versa, Torrazza Coste, Volpara, Zenevredo and part of the territories of these other municipalities*: Broni, Casteggio, Cigognola, Codevilla, Corvino San Quirico, Fortunago, Montebello della Battaglia, Montesegale, Ponte Nizza, Redavalle, Retorbido, Rivanazzano, Santa Giuletta, Stradella, Torricella Verzate.*

There are two categories under this *DOCG*: *Oltrepò Pavese Metodo Classico* and *Oltrepò Pavese Metodo Classico Pinot nero*, which can both be transformed into *Rosato Spumante* using the traditional method (*champenoise*). The former must be made by 70% Pinot nero using the white-wine vinification method, with the addition of up to 30% *Pinot grigio*, *Pinot bianco* and *Chadonnay*. The latter requires at least 85% *Pinot nero* by up to 15% *Pinot grigio*, *Pinot bianco* and *Chardonnay* as the remainder (Table 3).

Type	Minimum percentage of *Pinot Nero*	Other percentages of Grapes
Oltrepò Pavese Metodo Classico *Oltrepò Pavese Metodo Classico Rosè*	70%	*Chardonnay*, *Pinot grigio* and *Pinot bianco* jointly or separately up to a maximum of 30%
Oltrepò Pavese Metodo Classico Pinot Nero *Oltrepò Pavese Metodo Classico Pinot Nero Rosè*	85%	*Chardonnay*, *Pinot grigio* and *Pinot bianco* jointly or separately up to a maximum of 15%

Table 3.
Oltrepò Pavese sparkling wine classification[12]

The characteristic of the *Oltrepò Pavese* sparkling wine *DOCG* are shown in Table 4.

[12] http://www.vinoltrepo.it/ (accessed: 24/01/12)

Type	Characteristic
Oltrepò Pavese Metodo Classico	It is characterized by a fine and persistent froth; the color is more or less intense straw yellow; the scent is a mix of subtle, soft, and broad bouquets; the flavor is savoury, fresh and harmonious; alcohol content is a minimum total volume of 11.50%; the minimum total acidity is 5.0 g/l and the minimum non-reducing extract is 15.0 g/l.
Oltrepò Pavese Metodo Classico Rosè	It has a fine and persistent froth, a more or less intense pink colour, the bouquet's scent is subtle, soft, the flavour savoury, harmonious, and moderately full-bodied, the minimum total alcohol volume: 11.50%, total minimum acidity of 5.0 g/l and minimum non-reducing extract 15.0 g/l
Oltrepò Pavese Metodo Classico Pinot Nero	It has a fine and persistent froth, a straw yellow colour with more or less orangeish reflections, the bouquet's scent is that of the fermentation in the bottle, soft, broad and persistent, with a savoury taste, good structure, fresh and harmonious, with a total minimum alcohol volume of 12.00%, total minimum acidity of 5.5 g/l and minimum non-reducing extract 15.0 g/l.
Oltrepò Pavese Metodo Classico Pinot Nero Rosè	It has a fine and persistent froth, a more or less intense pink colour, soft, broad and persistent, with a savoury taste, good structure, fresh and harmonious, with a total minimum alcohol volume of 12.00%, total minimum acidity of 5.5 g/l and minimum non-reducing extract 15.0 g/l.

Table 4.
Characteristic of *Oltrepò pavese* sparkling wine *Metodo Classico DOGC.*

One of the best known typologies of sparkling wine within *Oltrepò pavese* wines, is the so called *Cruasé*. *Cruasé* is a unique denomination that identifies a dry style of Sparkling Rosè (classic method) obtained by a natural selection of *Pinot Nero Rosè*, defining the producer's desire to give a form to the highest quality for the uniqueness of *Pinot Nero* in the *Oltrepò Pavese*. The *Cruasé* is nowadays the only Sparkling Rosé exclusively obtained from this noble red berried grape (*Pinot Nero*) with a 100% natural procedure and not as a *Cuvée*. Their organoleptic characteristics are: a bright tender pink colour with purple glints. An intense aroma, delicate but penetrating and flavour: intense and persistent, mellow and harmonious.

1.4.3 Technological characteristics of production

The *Champenoise* method (as previously said, also known in Italy as classic method) involves many specialized steps in both viticulture and enology; it has taken centuries to evolve, through the contributions of inventors, innovators, and workers, both famous and nameless. Modernization and refinement of the "traditional" sparkling wine process continues to this day, although its beginnings are in antiquity.

The first stage for obtaining a sparkling wine is to produce a base wine and, just as with all wine making, the quality and general conditions of the fruit have remarkable impact on the final quality of the product. Following the first alcoholic fermentation, various base wines are often blended together and reserve wines are sometimes added, until the desired style has been created. This process generally takes place in stainless steel tanks. The second stage, in which bubbles are formed throughout a secondary fermentation, occurs in the bottle. The *Champenoise* method is also the most time consuming and expensive one. A mixture of sugar and yeast, known as *liqueur de tirage*, is added to the blended wine, which is then divided into individual bottles. These bottles are then sealed with an interim crown seal and a second alcoholic fermentation (also called "*prise de mousse*") takes place in the bottle (Tabera et al., 2006). The fermentation process generates carbon dioxide, which dissolves in the wine under pressure and creates the important bubbles. Following this secondary fermentation, the wine is matured in bottle on the yeast lees, adding character to the sparkling wine. The length of the storage on yeast lees, or ageing, varies depending on the desired wine style and quality. Eventually, the lees are removed from the bottle using a process known as riddling or *remuage*. This originally used to take several weeks to shake the sticky lees to the neck of the bottle but mechanical machinery has shortened the process down to around ten days. The removal of this sediment is known as *degorgement*. The cap of the inverted bottle is snap-frozen, the crown seal removed and the lees ejected. A small dose of wine with a final adjustment of sweetness is added and a cork, cage and foil are put in place. Before the bottles are closed for marketing, the final character of the wine is adjusted by adding wine, liquors, and sugar in various proportions. The amount of sugar added determines the sweetness of the wine. The wine is now ready for shipping in the same bottle that was used for the secondary fermentation (Figure 7). This method produces a finer, softer bead in the finished wine.

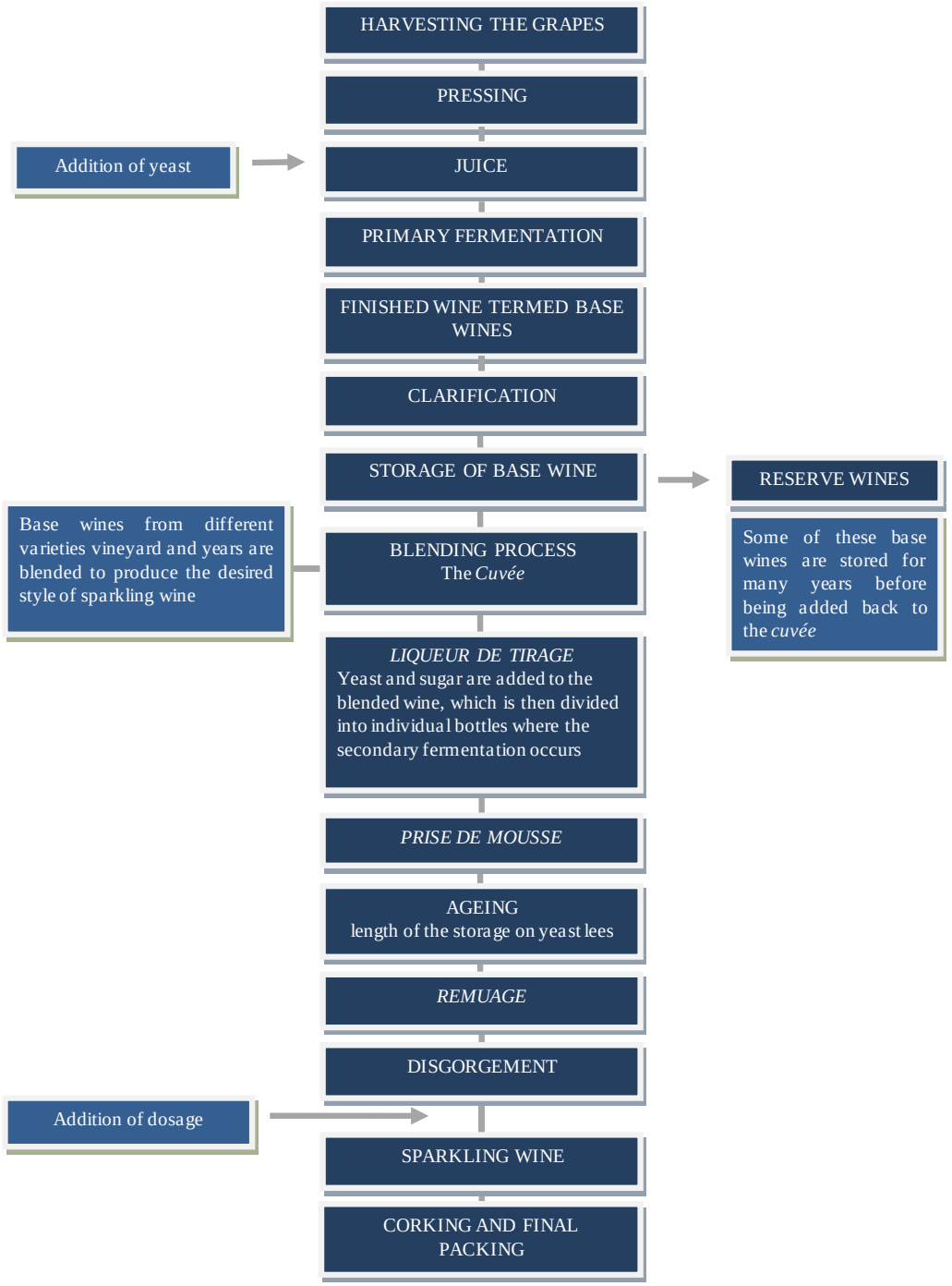

Figure 7.
Schematic diagram of sparkling wine production by *Champenoise* method[13]

[13] Adapted from http://www.wineaustralia.com (accessed: 10/01/12)

The contribution of yeasts to the properties of sparkling wines during "*prise de mousse*" also takes place in two steps. First, a secondary fermentation of the added sucrose leads to the production of ethanol, carbon dioxide, and minor secondary products. At the end of this step, the sparkling wine has an ethanol concentration of about 9.5 to 11.5% vol/vol, and the pressure in the bottle can reach 5 to 6 atm (Lallement, 1998). After fermentation has been completed, there is an aging period. During aging, yeast cells die and undergo autolysis, which releases intracellular compounds into the external medium. Improvement in the sensorial quality of the wines has been correlated with the products of the hydrolytic degradation of yeast cells, including free amino acids, peptides, mannoproteins, nucleic acid derivatives, and lipids (Leroy et al., 1990; Moreno-Arribas et al., 2000, 1996; Pueyo et al., 1995). However, autolysis in enological conditions is a very slow process that leads to long aging periods involving expensive storages of the wines.

1.5 Oenological yeast

Yeast is defined as unicellular ascomycetous or basidiomycetous fungi whose vegetative growth results predominantly from budding or fission, and which do not form their sexual states within or upon a fruiting body (Kurtzman and Fell, 1998). There are about 1,500 recognized species and the size can vary greatly depending on the species, typically measuring 3–4 µm in diameter, although some yeasts can reach over 40 µm.

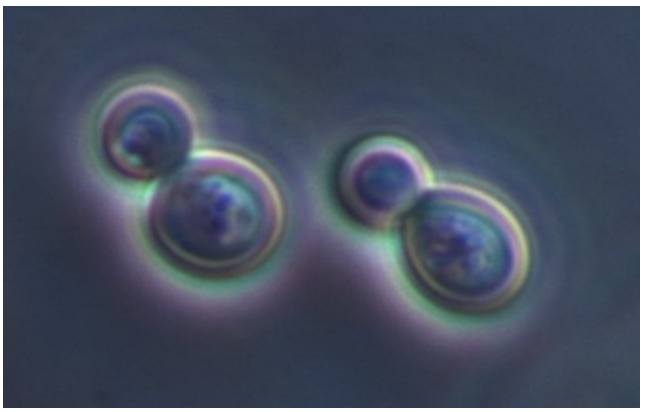

Figure 8
Microscopic image of *Saccharomyces cerevisiae* budding cells[14]

During the fermentation processes, some genuses of yeast are involved in winemaking. Classically, non-*Saccharomyces* yeasts, such as *Hanseniaspora ⁄ Kloeckera*, *Candida*, *Metschnikowia*, *Torulaspora*, *Kluyveromyces*, and *Zygosaccharomyces*, are the starting microflora in wine must, and when alcoholic content exceeds 5%–7% v ⁄ v in fermentation, the *Saccharomyces* species are the predominant yeasts (Fleet and Heard, 1993; Romancino et al., 2008; Sabate et al., 1998; Demuyter et al., 2004).

1.5.1 *Saccharomyces sensu stricto* group

The genus *Saccharomyces* can be divided into two major groups: *sensu stricto* and *sensu lato* according to complex criteria (Barnett, 1992.). The *sensu stricto* group comprises some of yeast species closely related to *S. cerevisiae* (*S. cerevisiae, S. paradoxus, S. bayanus, S. kudriavzevii* and *S. mikatae*) and the *sensu lato* group involves heterogeneous yeast species that are more diverging from *S. cerevisiae* (Naumov, 1996; Naumov et al., 2000; Kurtzman and Robnett, 2003). Among the yeasts in *sensu stricto* group, the species *S. cerevisiae*, *S. bayanus* and *S. pastorianus* are associated with anthropic environments because of their high fermenting capabilities (Naumov et al., 2000). For the food industry, namely, *S. cerevisiae* is the agent of wine, bread, ale beer, and sake fermentations; *Saccharomyces bayanus* is involved in wine and cider fermentations; *Saccharomyces pastorianus* is responsible for lager beer fermentation, instead (Ranieri et al., 2006).

[14] http://genome.ucsc.edu (accessed: 09/09/10)

1.5.1.1 Genomic characteristics

The *sensu stricto* yeasts contain at least 16 distinctive nuclear chromosomes of small, medium, and large sizes, and each species appears to contain a unique karyotype (Vaughan-Martini et al., 1993) Their mitochondrial DNA (mtDNA) molecules range in size from 64 to 85 kb and contain a number of G+C clusters, among them three to nine *ori-rep* sequences (Piskur et al., 1998). Molecular polymorphism is widespread among the *sensu stricto* yeasts, especially among yeast strains associated with the wine industry (Dubourdieu et al., 1987; Vezinhet et al., 1990), and almost every isolate has a characteristic karyotype and restriction pattern of digested mtDNA (Piskur et al., 1998). However, among isolates belonging to the same species, similar karyotypes and restriction patterns are observed. In the laboratory, members of the *sensu stricto* group can be mated at low frequency and can generate viable offspring (Naumov, 1996). The lager brewing strain *S. pastorianus* (syn. *S. carlsbergensis*) is a partial amphitetraploid, which was generated upon an interspecific fusion-cross between two different yeasts (Kielland-Brandt et al., 1995). One of the parental strains in this fusion-cross was *S. cerevisiae* and the second was a member of the *S. bayanus* species complex, possibly *S. monacensis* (Hansen and Kielland-Brandt, 1994**;** Pedersen, 1986; Piskur et al., 1998). In the characterized strains of *S. pastorianus* (syn. *S. carlsbergensis*), both sets of parental chromosomes are present (Kielland-Brandt et al., 1995) but the mtDNA molecule was inherited only from the non-*S. cerevisiae* parent. Initially, the hybrid zygote was possibly heteroplasmic regarding the mitochondrial genome, but apparently only one parental type was transmitted to the progeny.

Chromosomal DNA of S. cerevisiae

S. cerevisiae has a relatively small genome, a large number of chromosomes, little repetitive DNA and few introns (Petering et al., 1991). Haploid strains contain approximately 12±13 megabases (mb) of nuclear DNA, distributed along 16 linear chromosomes. Each chromosome is a single deoxyribonucleic acid (DNA) molecule approximately 200±2200 kilobases (kb) long. The genome of a laboratory strain of *S. cerevisiae* has been completely sequenced and found to contain roughly 6000 protein-encoding genes (Goffeau et al.,1996). The *S. cerevisiae* genome, which is relatively rich in guanine and cytosine content (%G+C of 39±41) is much more compact when compared with the genomes of other eukaryotic cells (Pretorius, 2000).

Extrachromosomal elements in S. cerevisiae

Several non-Mendelian genetic elements are known to exist in the nucleus, mitochondria and cytoplasm. The genome of *S. cerevisiae* contains approximately 35±55 copies of retrotransposons (Ty elements). These transposable elements move from one genomic location to another via an RNA intermediate using reverse transcriptase. The 2 mm plasmid DNA is the only naturally occurring, stably maintained, circular nuclear plasmid in *S. cerevisiae*. This 6.3 kb extrachromosomal element is also inherited in a non-Mendelian way and, although most strains of *S. cerevisiae* contain 50- 100 copies of 2 mm DNA per cell, its biological function has not yet been discovered (Friedl et al., 2010)

1.5.2 Yeast metabolism

Yeast carries out a biochemical assimilation (in anabolic pathways) and dissimilation (in catabolic pathways) of nutrients through enzymatic reactions, and regulation of the underlying pathways. Anabolic pathways include reductive processes leading to the production of new cellular material, while catabolic pathways are oxidative processes which remove electrons from substrates or intermediates that are used to generate energy. Preferably, these processes use Nicotinammide Adenina Dinucleotide Fosfato (NADP) or Nicotinammide Adenina Dinucleotide (NAD), respectively, as co-factors.

Most of the yeasts uses sugars as their main carbon and hence energy source, but there are particular yeasts which can utilize non-conventional carbon sources. With regard to nitrogen metabolism, most of the yeasts is capable of assimilating simple nitrogenous sources to biosynthesize amino acids and proteins (Table 5). Aspects of phosphorus and sulphur metabolism as well as aspects of metabolism of other inorganic compounds have been studied in some details, predominantly in the species *S. cerevisiae.*

Substrate	Intermediates	Enzymes	Products
Saccharose		Invertase	Glucose + Fructose
Maltose		Maltase	Glucose
Melibiose		Melibiase	Glucose + Galactose
Glucose			Products of Glycolysis
Ethanol	Acetaldehayde > Acetyl-CoA> Oxaloacetate>	Alcohol-Dehydrogenase	
Lactate	Pyruvate>	Lactate-Dehydrogenase	
Glycerol	Glycerol-3-phosphate> Dihydroxyacetonphosphate		
Amino acids			
Glutamate			
Ammonium			

Table 5
Nutrients for growth of yeast (*S. cerevisiae*) cells[15]

Energetic metabolism

In *S. cerevisiae* the main source for energy production is glucose and glycolysis is the general pathway for conversion of glucose to pyruvate; hence production of energy in form of Adenosine Triphosphate (ATP) is coupled to the generation of intermediates and reduces the power in form of Nicotinamide adenine dinucleotide-reduce form (NADH) for biosynthetic pathway.
The glycolytic pathway and its individual enzymes are conserved during evolution, although mechanisms controlling carbon and energy metabolism have adapted to the needs of each species or cell type. Aerobic organisms respire pyruvate completely to CO_2 with oxygen (O_2) as the terminal electron acceptor (figure 8), thereby making maximal use of energy transformations for ATP production (Pretorious, 2000). However, facultative anaerobic organisms may add fermentation for fast energy production. For instance, glucose is fermented to lactate by human muscle cells. Similarly, the yeast *S. cerevisiae* switches to a mixed respiro-fermentative metabolism, resulting in ethanol production (figure 9), as soon as the external glucose concentration exceeds 0.8 mM (Verduyn et al., 1984) Hence, *S. cerevisiae* chooses the fermentation or the respiration process primarily according to the sugar level.

[15] Adapted from http://biochemie.web.med.uni-muenchen.de (accessed: 29/01/12)

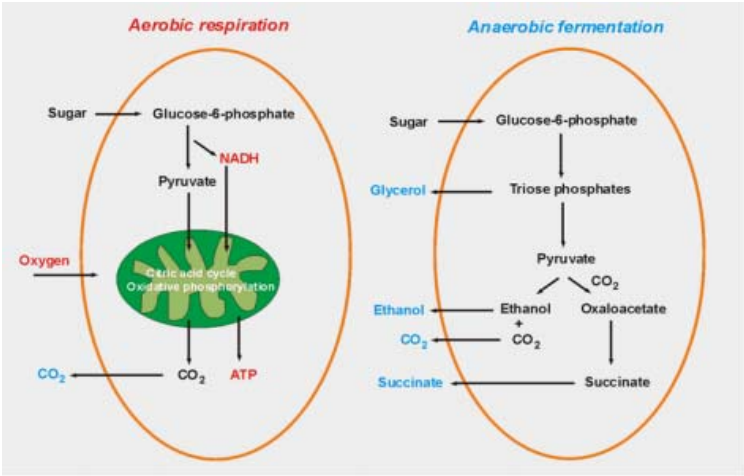

Figure 9.
Metabolism in yeast under aerobic and anaerobic conditions[16]

During alcoholic fermentation of sugars, yeasts re-oxidize NADH to NAD in a two-step reaction from pyruvate, which is first decarboxylated by pyruvate decarboxylase followed by the reduction of acetaldehyde, catalyzed by alcohol dehydrogenase (ADH). Concomitantly, glycerol is generated from dihydroyacetone phosphate to ensure production of this important compound. In fact, glycerol is a solute (by-product) compatible in osmoregulation in osmotolerant yeasts that are capable of growing in high sugar or salt environments. Many yeasts can grow on glycerol as a sole carbon source under aerobic conditions, but glycerol is a non-fermentable carbon source for many yeast species, including *S. cerevisiae*. Glycerol is synthesized by reducing dihydroxyacetone phosphate to glycerol 3-phosphate which is catalyzed by a NAD dependent cytosolic G3P dehydrogenase (ctGPD), followed by dephosphorylation of glycerol 3-phosphate by a specific phosphatase (GPP) (Gancedo et al., 1968). This pathway seems to be the only route for producing glycerol in *S. cerevisiae*.

Secondary compounds

Secondary compounds are those arising from the vinification process, usually a results of yeast metabolism. Although ethanol, glycerol and carbon dioxide are quantitatively the most abundant of these compounds and play a fundamental role in wine aroma, their contribution to the secondary aromas is relatively limited (Esteve-Zarzoso et al., 2000). Volatile fatty acids, higher alcohols, esters, and, to lesser extent, aldehydes, have a greater contribution to secondary aroma (Rapp and versini, 1991).

Volatyle fatty acids

The principal is the acetic acid which represents about 90% of them (Radler, 1993). The remaining fatty acids, such as propanoic and butanoic acid, are present in small quantities as products of yeast metabolism. Long-chain fatty acids (C16 and C18) are essential precursors for the synthesis of many lipid compounds in yeast. In general, these acids do not appear in wines, but they are found in products distilled in the presence of yeast lees. In contrast, intermediate-chain fatty acid (C8, C10 and C12) do appear alongside their ethyl esters as components of wine. Delfini and Cervetti (1991) classified *Saccharomyces* yeast strains into three groups according to their production of

[16] http://biochemie.web.med.uni-muenchen.de (accessed: 29/01/12)

acetic acid: low (0-0.3 g/L), intermediate (0.31-0.60 g/L), and hight (>0.61 g/L). In each case, these types of compounds represent an undesirable aspect for the sensorial quality of wine.

Higher alcohols

Higher alcohols are composed of aliphatic and aromatic alcohols such as propanol, isobutyl alcohol and amyl alcohol; the aromatic alcohol of which Phenethyl alcohol is the most important (Nikanen and Nikanen, 1977). Isoamyl alcohol is the main aliphatic fusel alcohol synthesized by yeast during fermentation. Depending on the nature of the beverage, it comprises 40-70% of the total fusel alcohol fraction (Lambrechts and Pretorius, 2000). Other important higher alcohols are propanol and isobutyl alcohol. Higher alcohols are also important precursors for ester formation (Soles et al., 1982) and the esters of higher alcohols are associated with pleasant aromas.

Esters

Esters are a group of volatile compounds found in wine and that impart a mostly pleasant smell. Most esters found in alcoholic beverages are produced by yeast during fermentation as secondary products of sugar metabolism and constitute one of the largest and most important groups of compounds affecting flavor (Engan, 1974; Peddie, 1990). However, a particular aroma property can only rarely be associated with a specific ester (Van Rooyen et al., 1982). In general, the concentration of esters in wine is above the perception threshold (Salo, 1970). The fresh, fruit aroma of young wines derives in large part from the presence of the mixture of esters produced during the fermentation, which is the reason why it is usually called “fermentation aroma/bouquet”(Marais and Pool, 1980).

1.5.3. Ecology

The wine fermentation is a complex ecological and biochemical process of microbial species that are affected for the environmental conditions. Yeast flora of grapes, fermenting musts and wines have been widely investigated in wine-producing countries to analyze the distribution and the evolution of yeast composition during the winemaking process, and also to differentiate useful yeast flora or yeast strains for wine fermentation (Fleet and Heard, 1993; Kunkee and Bisson, 1993; Parish and Carroll, 1987). The distribution of *Saccharomyces* and non-*Saccharomyces* yeast in must and wines varies according to vineyard, grape variety, harvesting practice and winemaking technology. The origin of wine yeast can be different. “Generalist” yeasts are endowed with a broad niche and occupy many habitats, whereas “specialist” yeasts occur in unique habitats (Walker, 1998). There are three main sources where wine yeast can be found: soil, grape and cellar. The microflora of grapes vary according to the grape variety; temperature, rainfall and other climatic influences; soil, fertilization, irrigation and viticultural practices development stage at which grapes are examined; physical damage caused by mould, insects and birds; and fungicides applied to vineyards (Pretorius et al., 1999). It is also important to note that harvesting equipment, including mechanical harvesters, picking baskets and other infrequently cleaned delivery containers can also represent sites for yeast accumulation and microbiological activity before grapes reach the winery (Fugelsang, 1997). This becomes more important as travelling time to the winery increases.

1.5.4. Controlled and spontaneous fermentations

Enological practices consist of a controlled fermentation through the inoculation of dry selected starters (Esteve-Zarzoso et al., 2001) that ensure a reproducible product and reduce the risk of wine spoilage. Inoculation is recommended, but there is still some concerns about missing some desirable characters due to the spontaneous fermentations. In any case, wine fermentation is a complex ecological and biochemical process that involves a sequential contribution of different yeast species (Fleet and Heard, 1993). *Hanseniaspora*, *Candida* and *Pichia* genera grow during the early stages of the fermentation but die with high concentration of alcohol, and are replaced by

stronger fermenting and more alcohol-tolerant yeasts. In latter stages, fermentation is always controlled by yeasts of the *Saccharomyces* species (Blanco et al., 2006). In particular, *S. cerevisiae* is the dominant yeast that is able to complete the alcoholic fermentation (Sabate et al., 1998). When fermentations are seeded with commercial yeasts, the inoculated strains have to compete with the natural population of must. Although inoculated strains were actually responsible for the fermentation, showing a gradual dominance, they did not suppress the development of indigenous strains during the first stages (Querol et al., 1992). Today, the origin of the *S. cerevisiae* yeasts involved in spontaneous fermentation is still controversial. On one hand, ecological studies show that *Saccharomyces* is rarely found on intact grapes or soils in the vineyard (Rosini et al., 1982; Martini, 1993; Sabate et al., 2002), but is found to a larger extent on the winery equipment of the cellar (Fleet and Heard 1993; Vaughan-Martini and Martini, 1995; Sangorrin et al., 2002), driving to suggest a winery origin. On the other hand, *S. cerevisiae* is more abundant on damaged berries (Mortimer and Polsinelli, 1999), and spontaneous fermentations are observed in new built wineries (Beltran et al., 2002) or at laboratory in sterilized vessels (Polsinelli et al.,1996; Povhe Jemec et al., 2001) were winery strains could not be involved. The identification of yeasts involved in alcoholic fermentation is fundamental since is proved the relationship between yeast and the chemical composition and sensory qualities of the resulting wine (Lurton et al., 1995).
Even in *Franciacorta* and *Oltrepò pavese,* some cellars still prefer to use only indigenous yeasts to produce wines with a more complexity aromatic profile that those carried out with controlled fermentations using commercial yeasts. Others prefer to start the spontaneous fermentation and, at a later date, to provide inoculation with a yeast starter. In large-scale production cellars, where a rapid and constant fermentation of the product yet with high quality is essential, commercial yeasts are usually preferred. In fact, few wineries take the potential risk of spontaneous fermentation to produce a product where the final outcome is difficult to be predicted.

1.5.5 Oenological yeasts selected

As already described, yeasts play a central role in the fermentation process during sparkling wine production. *S. cerevisiae,* ‘‘the wine yeast’’ is the most important species involved in the process. The use of active dry yeasts, also called “starters”, is a common practice during “*prise de mousse*” (Torija et al., 2000). The starters are the selected yeasts, characterized by physiological, biochemical and oenological optimized properties for the technological requirements. They must be able to conduct the fermentation with predictable and programmable results (Pretorius and Westhuizen, 1991).

Criteria strategies for oenological yeasts selection

A good starter strain for sparkling wine production should:

- Conduct a vigorous fermentation
- Conduct fermentation to dryness (from low level to no-residual fermentable sugar)
- Possess reproducible fermentation characteristics and behave predictably.
- Possess good ethanol tolerance
- Produce no off-flavors
- Be SO2 tolerant
- Flocculate to be easily removable

The identification of a good wine strain having the above mentioned characteristics supposes that a conscious selection of a certain wine yeast strains as a starter culture has been made. If so, the strain may be commercially available in a dried form; or it may be from a culture collection, and a liquid inoculum needs to be prepared. Whichever type of starter is used, the amount should be large enough to assure the winemaker that the fermentation is made and completed by inoculated strain.

Until recently, this assurance was not easily demonstrated. However, with the available new biotechnologies, the identity of the dominant yeast strain at the end of fermentation can be determined. Recent researches employing genetic markings and karyogamic typing confirm that under normal conditions, the inoculated strain commands the fermentation and constitutes most of the total yeast population at the end (Delteil and Aizac, 1989; Vezinhet et al., 1994; Querol and Ramon, 1996; Boulton et al., 1996). On the other hand, the use of selected yeasts potentially may represent some disadvantages because, among all *ADY*, those actually used by winemakers around the world are relatively few. This could lead to a standardization process with the microbial agent resulting in a reduction of biodiversity of yeast strains associated with the cellar environment. The prospect, however, is that the vineyard will suffer the most important loss of biodiversity; in fact, after the harvest, about 73% of commercial yeast starter can be found in the winemaker environment, of which about 94% is scattered by agricultural machine for harvesting grapes in a radius ranging from 10 to 200 m from the cellar (Valero et al., 2005). In addition, commercially available starters, even though they have characters of undoubted oenological importance, are not always able to fully develop the flavors and aromas of a wine, just because they come from foreign wineries realities (Pretorius, 2000). To overcome these problems the microbiologists, the winemakers, and the wine sales managers believe that it is appropriate to introduce the use of selected ecotype starters following the typical characteristics of the local product. This requires reliable techniques able to identify and differentiate the selected strains from the remaining microflora during the fermentation process.

1.5.5.1 Starter cultures used in the sparkling wine production

In 1965, the first two oenological starters under active dry yeast form (*ADY*) were offered and sold in California. These two strains, "*Montrachet*" and "*Pasteur Champagne*" soon spread around the world. Thanks to a high viability (> 50%), the long shelf life due to the low moisture content (4-8%) and vacuum packaging system, allowed wide dissemination of the *ADY*. In Italy, the rapid widespread use of selected yeasts began in 1978, after the entry of the law *DM* of *10^{ht} October 1977* that authorized their use.

The utilization of selected yeast strains as starter cultures in the second fermentation is nowadays a common and widely used practice. Grape must microflora comprises many *S. cerevisiae* strains and it is well established that each of those strains contributes differently to the final flavour profile of the base wine (Howell et al., 2006; Romano et al., 2003; Golueke and Diaz, 1989). In order to preserve the typical characteristics of certain wines produced in a certain region, many enologists recommend the selection of strains within the local microflora of grape musts (Pretorius, 2000). Furthermore, some wineries select indigenous *S. cerevisiae* strains and use them in consortia to inoculate their grape musts. The use of starter cultures containing strains in consortium, displaying desirable and complementary characteristics, requires reliable techniques able to identify and differentiate the selected strains from the remaining microflora during the fermentation process (Xufre et al., 2011).

In contemporary market, it is possible to choose selected strains for sparkling wine production. The main strains used worldwide are:

- *IOC 18-2007 Institut oenologique de Champagne*: this yeast strain produces high quality wines, preserving both the grape variety and *terroir*. Renowned for the sparkling wine production by the traditional method, this yeast is also valued for the production of still wine worldwide. It copes well with difficult fermentation conditions (low pH and temperature) facilitating a complete utilization of sugar without undesiderable secondary compounds. The principal oenological characteristics are high alcohol conversion, low production of volatile acid, high resistance to alcohol, contains active killer factor, good production of glycerol, resistance to sulphur dioxide, very low production of sulphur

dioxide, very low foam formation, controlled and regular fermentation across the range 10 to 30°C.

- *Lalvin DV10***:** This is "the original *Champagne* isolate," known in other contexts as Epernay. Its kinetics of fermentation are strong over a wide temperature range (10-35 °C) with relatively low oxygen and nitrogen demands. It is one of the most widely used strains in *Champagne* and is known for clean fermentations that respect varietal character while avoiding bitter sensory contributions associated with many other strains. It is highly recommended for both premium white and red varietals, mead and cider production, and many fruit, berry, vegetable, and herb wines. It is a fast fermenter with an 18% alcohol tolerance, is famous for its ability to ferment under stressful conditions of low pH, high total SO2, and is low foaming with low volatile acid production.

- *Lalvin EC-1118 (Prise de Mousse)*: This is the original strain, steady, low foamer, excellent for barrel fermentation or for working on heavy suspended pulps. It is one of the most popular wine yeasts in the world. It ferments well at low temperatures, flocculates well, and produces very compact lees. It is good for *Champagne* bases, secondary (bottle) fermentations, restarting stuck fermentations, and for late harvest grapes. It is also the yeast of choice for apple, crabapple, cranberry, hawthorn, and cherry wines. It has excellent organoleptic properties. Alcohol toxicity is 18% and it ferments relatively fast. It tolerates temperatures from 3,9°C-35°C. It is not, however, tolerant of concurrent malolactic fermentation.

- *Epernay* : Epernay is a *Champagne* yeast, meaning it was isolated in *Champagne*, France and it is used in *Champagne* production. Epernay is used in bottle fermenting because it ferments slowly and it is tolerant to cold temperatures with moderate foaming. It is also used for primary fermentations of still white wines.

- *Pasteur Champagne* : *Champagne* yeast is the second most common yeast strain. It was isolated in Champagne, France and technically it is a mixed-population culture. It is common in sparkling wine production because of its ability to induce fermentation quickly and because of its effectiveness in low temperatures as well as its tolerance of medium-high alcohol conditions. These conditions are common in sparkling wine production. Temperature range is 15-30°C, low to medium flocculation, and alcohol is 13-15%.

- *Premier Cuvée* : Also known as *Prise de Mousse*, this is a *Champagne* yeast that is strong acting, low foaming and therefore qualified for barrel fermentations. It imparts a strong yeasty aroma and is useful for secondary fermentation in both still and sparkling wine production. Good for reds and whites alike and for restarting stuck or sluggish fermentations. Temperature range is 7,2-35°C (equal to Lalvin EC-1118), flocculation is low, and alcohol is reliably 18%.

1.5.6. Genetic identification

Until the 1990s, traditional analysis based on morphology, biochemical and sexual reproduction characteristics were used for wine yeast identification. Such methods only allowed discrimination between species, and they were difficult because required long lead times and were imprecise (Barnett et al., 1990; Deak, 1993). To date, numerous molecular methods have been proposed for the identification of wine yeasts. They have allowed to optimize the analysis and to know the distribution of the yeast during winemaking. (Guillarmon et al., 1994; De Barros Lopes et al., 1996; Giudici and Pulvirenti, 2002; Xufre et al., 2011). “Polymerase Chain Reaction” (PCR) has

revolutionized the methods used in microbiologic laboratories. This technique has been developed for Kary Mullis in 1983 and is used to amplify a specific region target from a DNA template through a DNA polymerase named *Taq* polymerase. From this technique has been possible to develop molecular methods for the identification of different microorganism. Some of these are utilized for identification at specie level: Restriction Fragment Length Polymorphism (RFLP) of Internal Transcribed Spacer (ITS), Sequencing of *D1/D2* domain of 26S rDNA gene.

1.5.6.1 RFLP-ITS analysis

The analysis of ITS restriction fragment length polymorphism (RFLP) is a very good tool for the identification of wine yeasts at the species level, although as ribosomal regions show a low degree of polymorphism within the same species (Marinangeli et al., 2004). Previous results have demonstrated that the complex ITS regions (non-coding and variable) and the 5.8S rDNA gene (coding and conserved) are useful in measuring close fungus genealogical relationships since they exhibit far greater interspecific differences than the 18S and 25S rDNA genes (Cai et al., 1996; James et al., 1996; Kurtzman, 1992, 1993). Because ribosomal regions evolve in a concerted way, they show a low intraspecific polymorphism, and a high interspecific variability (Li, 1997), has been proved to be very useful for the classification of *Saccharomyces* species (Huffman et al., 1992; Molina et al., 1992; Valente et al., 1996; Wyder and Puhan, 1997), *Kluyveromyces* species (Belloch et al.,1998) and, for the identification of a small collection of wine yeast species (Guillamon et al.,1998).

1.5.6.2 Sequencing of *D1/D2* domain of 26s gene

The analysis of divergence of nucleotide of D1/D2 domain, allows identifying about 500 species of ascomycetes yeast (Giudici, et al 1998). The data indicate that most yeast species can be identified from sequence divergences on this domain, and show that 55 currently accepted species are either synonyms or sister species of earlier described species(Kurtzman and Robnett, 1998). In addition, a phylogenetic analysis of the dataset provides an overview of close species relationships. These genetic analyses have been used to generate phylogenetic databases for ascomycetous and basidiomycetous yeasts. In figure 10 the phylogenetic tree of some oenological yeast species is shown.

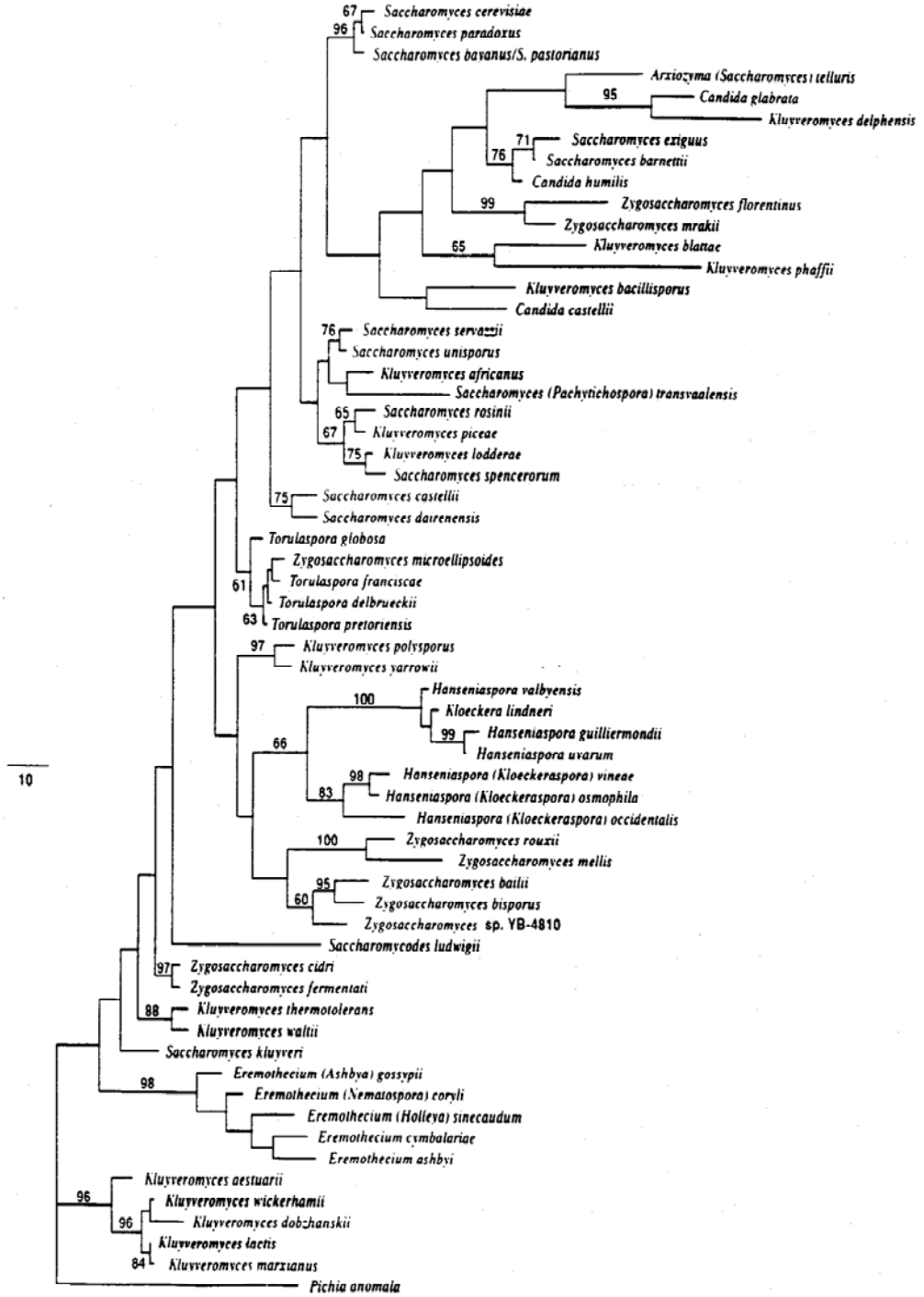

Figure 10.
Phylogenetic tree of the *Saccharomyces* clade from nucleotide sequences of 26S rDNA D1/D2 (Kurtzman and Robnett, 1998).

1.5.7. Genetic typing

In order to discriminate the strains belonging to the same species, some molecular techniques have been developed. This capability is useful to study the evolution of yeasts population during a fermentative process or to identify a particular strain with special characters or to evaluate the biodiversity within a geographic area or technology.

1.5.7.1 Analysis of delta sequences

Ness et al. (1993) proposed for the first time this molecular technique for typing of *S. cerevisiae* strains via the analysis of interdelta polymorphism. Genome of *S. cerevisiae* contains repetitive DNA sequences, such as delta sequences, that are frequently associated with the *Ty1* transposon (Cameron et al., 1979). Delta elements form the Long Terminal Repeats (LTRs) flanking retrotransposons *TY1* and *TY2* in yeast, but can also be found separate from these retrotransposons and are called only "delta elements" (Legras and Karst, 2003). Depending of the strain examined, *S. cerevisiae* can contain between two and 30 copies of at least five retrotransposons (Ty1–Ty5). Recombination events expel the central sequence at one LTR, leaving a single LTR behind, explaining the dispersed presence of many copies of LTRs throughout the genome (figure 11). As demonstrated by different authors (Legras and Karst, 2003; Schuller et al., 2004; Cameron et al., 1979), the number and location of these elements have a certain intraspecific variability that can be used as a genetic fingerprint to identify *S. cerevisiae* strains (Xufre et al., 2011).

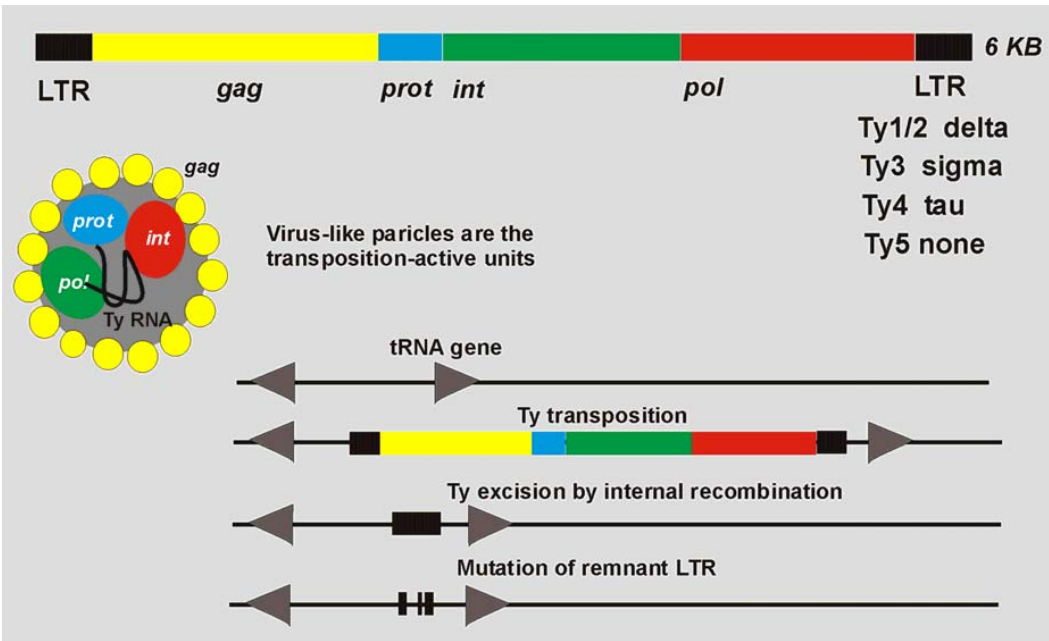

Figure 11[17]**.**
Ty elements in yeast. Five distinct families of retrotransposons are existent in *S. cerevisiae*, currently grouped into four Ty1-copia type elements (Ty1, Ty2, Ty4 and Ty5) and one "gypsy" type element (Ty3). Among the five families of *S. cerevisiae* Ty elements, only three are known to be transpositionally active, namely Ty1, Ty2 and Ty3.

[17] http://biochemie.web.med.uni-muenchen.de/Yeast_Biol/11%20Yeast%20Retrotransposons.pdf (accessed:29/01/12)

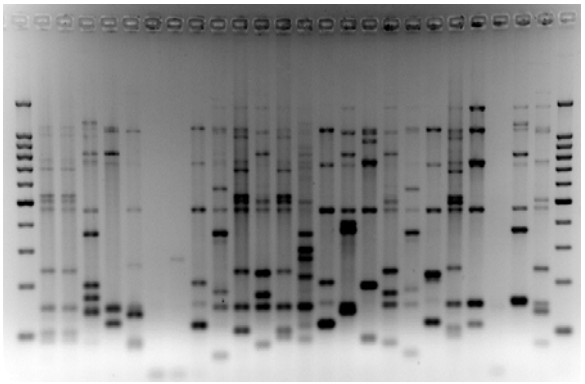

Figure 12
PCR-Interdelta profiles from *S. cerevisiae*

In a survey of molecular methods for typing wine yeast strains, Schuller et al. (2004) showed that both microsatellite typing and interdelta analysis have a discriminatory power similar to that of mtDNA restriction analysis and karyotyping. Those authors concluded that PCR amplification of delta sequences (figure 12) is a very convenient method to use in standard control of industrial wine fermentations since it is rapid, reproducible and very sensitive.

1.5.7.2 Mitochondrial DNA (mtDNA) restriction analysis

Mitochondrial DNA (mtDNA) polymorphisms have been used extensively to characterize brewing yeasts (Aigle et al., 1984; Lee and Knudsen, 1985) and wine strains of the species *Saccharomyces* (Querol et al., 1992, 1994; Martinez et al., 1995; Guillamon et al., 1996; Ibeas et al., 1997). Several methods have been developed to isolate yeast mitochondrial DNA (Aigle et al., 1984; Gargouri, 1989; Querol and Barrio, 1990). However, Querol et al. (1992) have developed a mtDNA restriction analysis method for *Saccharomyces* which does not require previous isolation of mitochondria or purification of mtDNA. One of the enzymes commonly used is *Hin*f *I* since it has revealed a high level of restriction fragment length polymorphism for strains belonging to species of the genus *Saccharomyces sensu stricto* (Guillamon et al., 1994).

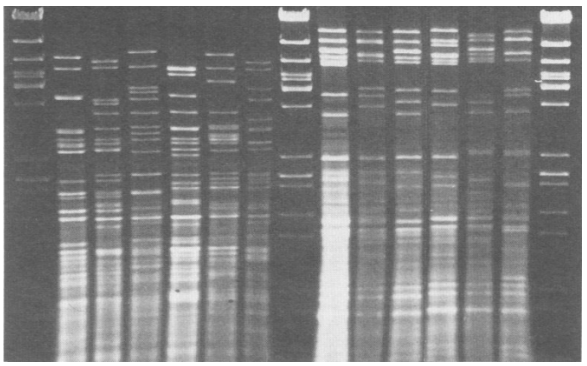

Figure 13
mtDNA patterns from *S. cerevisiae* (Querol et al., 1992)

1.6 Outline of the thesis

Selected yeast starters are nowadays widely used since they possess very good fermentative and oenological capabilities, contributing to both standardization of fermentation process and wine quality, in order to ensure a reproducible product and to reduce the risk of wine spoilage. However, the study of biodiversity within microbial populations involved in winemaking have recently become an object of growing interest due to the possibility of obtaining new strains with useful capabilities for the wine industry. Starters currently used in Italy for sparkling wine production have been isolated from French territories on the basis of the quality characteristics of *Champagne* wine and a biodiversity analysis of indigenous yeast populations in Lombardy region has not still been carried out.
This PhD thesis was focused on the study of biodiversity and technological aspects of yeasts involved in sparkling wine production by *champenoise* method in Lombardy region. It is part of the wider program of the *Regione Lombardia* in collaboration with the *Consorzio per la tutela del Franciacorta* and the *Consorzio Tutela Vini Oltrepò Pavese*, aimed at to enhancing the *DOCG Franciacorta* and *DOCG Oltrepò Pavese Metodo Classico* through the use of autochthonous starters to improve the sparkling wine production and as "*marcatori di tipicità*" (typicality markers).
Accordingly, the present PhD thesis describes:

- In chapter 2, the genetic identification and typing of indigenous yeasts isolated from Lombardy's oenological areas. The study was performed through three years during 2009, 2010 and 2011 vintages.

- In chapter 3 a monitoring of evolution of yeast population during controlled and spontaneous fermentation to determine their genetic diversity and the dominant specie in the process.

- In chapter 4 the results from *tirage* proves, at pilot scale, of indigenous *S. cerevisiae* as potential starters. The tests were performed in different *Franciacorta* and *Oltrepò Pavese* wineries.

- In chapter 5, the development of a protocol for recovery of yesast DNA from sparkling wines, aimed to improve the traceability for the *DOCG* products of Lombardy.

- In chapter 6 the main results presented in this thesis are concluded and put into future perspectives.

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Chapter 2
Biodiversity of yeast population involved in winemaking process in *Franciacorta* and *Oltrepò Pavese*

2.1 Background

The quality of wine is strongly correlated to the yeasts involved in the fermentation process. Accordingly, there is a growing interest to increase knowledge about biodiversity of wine yeast strains (Agnolucci et al., 2007; Capello et al., 2004; Capece et al., 2010; Guerra et al., 1999; Mercado et al., 2007; Vilanova et al., 2003). From one side, assessment of yeast biodiversity is relevant for understanding the evolution in winemaking and consequently increasing the control capacity on such processes. On the other hand, autochthonous strains with typical oenological characters could be representative of a particular vine growing region and hence, their presence enhances the "local value" (typicality), thus promoting the diversification of wine products. Research conducted so far has been focused mainly on monitoring of alcoholic fermentation. However, many others have been addressed in the study of yeasts ecology on grapes, must and also in the air of the cellar.

Winemaking is a process in which a series of complex microbiological transformations take place involving interactions between yeasts and bacteria (Fleet and Heard, 1993; Garijo et al., 2008). The first step of this process is the conversion of must into wine through the alcoholic fermentation. This is effectuated by alcohol-tolerant species of *Saccharomyces*, mainly by *Saccharomyces cerevisiae*. Geographical distribution of *S. cerevisiae* strains within specific wine-producing regions has been analyzed (Caruso et al., 2002; Redzepovic´ et al., 2002; Versavaud et al., 1995) and changes in this microflora composition in different vineyards have been observed (Versavaud et al., 1995). Different studies demonstrated the presence of non-*Saccharomyces* yeast during the early stages of fermentation (Fleet, 2008). For example, Jolly et al.(2003) found four different yeast species, i.e. *Kloeckera apiculata, Candida stellata, Candida pulcherrima* and *Candida colliculosa* that predominated in grape must at the start of fermentation. Moreover, it has been found that freshly crushed grape juice harbors a diversity of yeast species, principally within the genera *Hanseniaspora* (anamorph *Kloeckera*), *Pichia*, *Candida*, *Metschnikowia*, and *Kluyveromyces*. Occasionally, species of other genera such as *Cryptococcus*, *Rhodotorula, Debaryomyces*, *Issatchenkia*, *Zygosaccharomyces*, *Saccharomycodes*, *Torulaspora*, *Dekkera*, *Schizosaccharomyces* and *Saccharomyces* have also been isolated from wine grapes of several wine-producing areas (Jolly et al., 2006; Fleet et al., 2002; Fleet, 2003; Fleet, 2008; Prakitchaiwattana et al., 2004; Ribéreau-Gayon et al., 2000). Instead, Garijo et al. (2008) studied the presence of microorganisms of enological interest (yeasts, bacteria and molds) and their evolution in the air of a wine cellar. In that study the yeasts isolated from the air belonged to both the *Saccharomyces* genus and the non-*Saccharomyces* group.

Biodiversity and composition of yeast population associated with an oenological environment may vary considerably due to different factors such as, climatic conditions, grape variety and geographical location (Guillamon et al., 1996; Martinez et al., 2007). The yeast biodiversity of a wine-producing regions is relevant for several reasons, such as preserving the indigenous flora and determine the non-*Saccaromyces* yeasts present in the process, since it has been suggested that

metabolites formed by these species may contribute to wine quality (Fleet et al., 1984; Gil et al., 1996; Lema et al., 1996; Soden et al., 2000).

As mentioned in chapter 1, (section 1.5.6) advances in molecular biology techniques and their higher resolving power have contributed significantly to the studies of biodiversity and genomic properties of the wine yeasts. Restriction patterns generated from the region between the Internal Transcribed Spacers (ITS) and the 5.8s rRNA gene are used as a quick and easy method to identify yeasts (Belloch et al., 1998; Esteve-Zarzoso et al., 1999; Fernandez-Espinar et al., 2000; Giudici and Pulvirenti, 2002). Furthermore, Kurtzman and Robnett. (1998) have identified 500 species of ascomycetous yeasts through the analysis of D1/D2 domain of large subunit (26S) ribosomal DNA. Their study demonstrated that nearly all currently recognized ascomycetous yeasts can be identified from their unique D1/D2 sequences.

With regard to the typing of wine yeasts, several methodologies, based on DNA polymorphisms, have been developed for discriminating among closely related yeast strains (Querol et al., 1992, Ness et al., 1993; Schuller et al., 2004). The studies are focused mostly within the species *S. cerevisiae* as it is the most important winemaking yeast. Various studies have shown significant molecular polymorphisms of the indigenous *S. cerevisiae* strains from different vine-growing regions and a strong correlation between their genomic and phenotypic properties (Esteve-Zarzoso et al., 2000; Nadal et al., 1996). The amplification of δ sequences is known to be highly specific for the identification of *S. cerevisiae* strains respect to other yeasts (Ness et al., 1993). For example, Pramateftaki et al. (2000) analyzed the δ sequences from 500 isolates of vine-growing areas of Greece. In that study, genetic profiles were obtained only from *S. cerevisiae* isolates and never from the non-*S. cerevisiae* isolates. Since the amplification of the δ sequences seems to be highly efficient for typing, many studies have been focused on optimizing of this technique. Legras and Karst. (2003) have designed new primers to show a clear improvement of interdelta analysis. Tristezza et al. (2009) investigated the application of capillary electrophoresis (CE) of the interdelta markers (IDM) for the genotyping of *S. cerevisiae* strains. The adaptation of the IDM amplification protocol for the CE analysis represented an important step towards increasing sensitivity and precision of the assay and decreasing scoring time and errors.

As above mentioned in the case of *S. cerevisiae*, a comprehensive study of biodiversity gave interesting results, due to the possibility of isolating new indigenous strains that could replace commercial starters that are currently used for the wine production, considering that native starter cultures are potentially better adapted to the growth in a specific grape must, and reflect the biodiversity of a given region. Until now, many countries such as Argentina (Combina et al., 2005; Mercado et al., 2007), Spain (Blanco et al., 2006; Garijo et al., 2008; Gonzales et al., 2007; Torija et al., 2001;Sabate et al.,2002), France (Valero et al., 2007), Austria (Lopandic et al., 2007), Croatia (Redzepovic et al., 2002), Slovenia (Raspor et al., 2006), Hungary (Csoma et al., 2010), Greece (Nikolaou et al., 2007; Nisiotou and Nychas, 2007), South Africa (Jolly et al., 2003a, 2003b; Pretorius et al., 1999), China (Shi-Li et al., 2010), India (Chavan et al., 2009) and Japan (Shinohara et al., 2003) have carried out studies of wine yeasts biodiversity in several regions. In Italy, some studies of this type have been developed in the region of *Marche* (Guerra et al., 1999), *Basilicata* (Caruso et al., 2002), *Puglia* (Cappello et al., 2004) and *Sicilia* (Romancino et al., 2000). Despite the Lombardy is a very important region for sparkling wines production, a biodiversity analysis of yeast involved in the winemaking process in this region, has not still been carried out.

Therefore, this study regarded the evaluation of the yeast biodiversity involved in winemaking process in *Franciacorta* and *Oltrepò pavese* areas . Molecular identification at species level was carried out for yeasts collected during vintages of 2009, 2010 and 2011, from the air of vineyard, must before the addition of SO_2 and base wine. Furthermore, molecular identification at strain level was carried out for the isolates identified as *Saccharomyces cerevisiae*, isolated during the vintages 2009 and 2010.

2.2 Materials and Methods

The study was conducted during the vintages of 2009, 2010 and 2011 in *Franciacorta* (province of *Brescia*) and *Oltrepò Pavese* (province of *Pavia*) in Lombardy, Italy. For each year, the months of sampling were July, August and October in correspondence with the beginning of the veraison of the berries, the time of grape harvest and the base wine production.

2.2.1 Microbiological Methods

Air sampling

Air samples were taken using the "*MAS 100 Eco*" (figure 2.1) from VWR® International. For each year, the same vineyards (eleven in *Oltrepò pavese* and thirteen in *Franciacorta*) were investigated and, in each vineyard, two aliquots of 100 and 500 liters of air each were collected for analyses.

Figure 1.
Air Sampler "*MAS 100 Eco*" of VWR International for air sampling.

Yeasts isolation was carried out using the YEPD medium [1% (w/v) yeast extract, 2%(w/v) peptone and 2% (w/v) glucose,] added with 2% agar and modified in the following characteristics: 3.6 pH, addition of 200 mg/l $K_2S_2O_5$ and 100 mg/l of chloramphenicol. The plates obtained from the air sampling were transferred in laboratory and incubated at 25 °C in anaerobic conditions (GasPak system) for 5 days. This modification was done to avoid the invasive growth of moulds on the plates.

Must sampling

Sampling was possibly done before of SO_2 addition, transferring 50 ml of must (two samples in each winery) in sterilized flasks transported at 4°C to the laboratory. Decimal dilutions were performed in peptone water (.Merck, Germany.). 100 µl of the appropriated dilutions were spread on WL agar (Merck, Germany). Plates were incubated during 3/5 days at 25°C.

Base wine sampling

Each year, the base wine was collected in the month of October. 100 ml of wine (two samples in each winery) was transferred in sterilized flasks and transported to the laboratory. Decimal dilutions were performed in peptone water (Merck, Germany). 100 µl of the appropriated dilutions were spread on WL agar (Merck, Germany)Plates were incubated during 3 days at 25°C.

2.2.2.1 Yeasts isolation and collection

After incubation, the number of colony-forming units (CFU) was registered. The morphological characteristics of each colony type were recorded, counted and observed at optical microscope. A number corresponding to square-root of the number of each type of colonies was selected. Colonies were re-streaked and purified on WL agar (figure 2.2). Purified isolates were maintained at -80° C in YEPD broth added with 20% (v/v) glycerol.

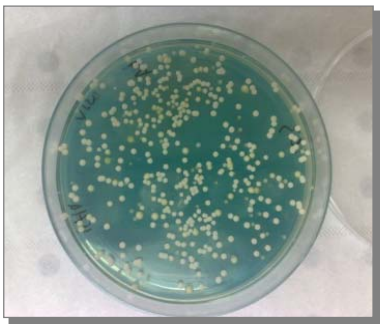

Figure 2.
Typical colonies of *S. cerevisiae* on WL Agar.

2.2.1.2 Commercial yeast strains collection

Commercial starter strains (active dry yeasts) were included in this study (Table 1). Most of them are used for sparkling wine commercial production by the winery involved in the experimentation. The same microbiological protocols described above were used for isolation and maintaining of the yeasts.

Commercial strain[1]	Species[2]
SC1	*S. cerevisiae killer*. Var. *bayanus*
SC2	*S. bayanus*
SC3	*Saccharomyces cerevisiae* var. *bayanus*
SC4	*Saccharomyces cerevisiae* var. *bayanus*
SC5	*S. cerevisiae*
SC6	*Saccharomyces bayanus*
SC7	*S. cerevisiae*

Table 1.
Comercial starter strains used as reference in this study.

[1] Designation given to this study
[2] Information reported in the package

2.2.2 Molecular Biology Methods

2.2.2.1 DNA isolation

A protocol for DNA extraction from yeast was proposed for Querol et al.(1992). Cells were grown in an overnight culture of 5 ml of YEPD [1% (w/v) yeast extract, 2%(w/v) peptone and 2% (w/v) glucose, 5.5 pH and added of 0.1 g/l of chloramphenicol]. Culture was centrifuged (Hettich zentrifugen, rotina 380r, Germany) at 3500 *g* for 15 min. The pellet was suspended in 500µl of a solution containing 0.9 M sorbitol-0.1 M EDTA, pH 7.5 to which 500 µg/ml of zymolyase 100T (USBiological,USA) and 1µl/ml of 14mM β-mercaptoethanol were added. Micro tubes were incubated at 37°C for 60 min in order to lyse the cells. Then they were centrifuged (Hettich zentrifugen, mikro 200, Germany) at 3500 *g* for 15 min and the pellet was suspended in 0.5 ml of 0.05M Tris-HCl-0,02M EDTA. After suspension, 0.05 ml of 10% sodium dodecyl sulfate was added and the mixture was incubated at 65°C for 30 min. Immediately thereafter, 0.2 ml of 5 M potassium acetate was added and tubes were placed on ice for 30 min. Tubes were centrifuged at 14000*g* in a microcentrifuge for 5 min. Supernatants were transferred into new tubes and the DNA was precipitated by adding 1 volume of isopropanol. After gently agitation, tubes were centrifuged at 14000 *g* for 10 min. DNA was washed with 70% ethanol, and then again centrifuged at 14000 *g* for 5 min. The pellet was dried at 55°C for 15 min,dissolved in 50 µl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) and maintained at 4°C for 12 h. 0.5 mg/ml RNase (Fermentas, Lithuania) was added and tubes were incubated at 37°C for 30 min. Finally, DNA was conserved at -20°C. DNA concentrations were determined by measuring the A_{260nm}.

2.2.2.2 Determination of the *ITS rDNA* gene sequences

For the preliminary identification of isolates, the internal transcribed spacers between the 18S and 26S rDNA genes (ITS1-5.8S-ITS2) were amplified directly from genomic DNA. Amplification was performed in a 25 µl reaction mixture containing 1X Buffer (5 Prime, Hamburg), 2.5 mM of $MgCl_2$ (5 Prime, Hamburg), 200 µM of dNTPs (Fermentas, Lithuania), 0.1 µM of each primer (Table 2), 2 U of Taq-DNA Polymerase (5 Prime, Hamburg) and 80 ng of DNA. A T Gradient Biometra Thermocycler[3] (Biometra, Germany). The temperature profile was: denaturation step at 95°C for 1 min, annealing step at 50 °C for 1 min and extension step at 72°C for 1 min and this was repeated for 35 cycles. Electrophoresis was run in 1.5% (w/v) agarose gel in 1X TAE buffer (40 Mm Tris–acetate, pH 8.2; 1 mM EDTA) stained with 0.4 µg/ml ethidium bromide. PCR products were photographed under GelDoc UV transilluminator[4] (Bio-rad, Hercules, CA, USA). ITS amplifications were subject to restriction (RFLP) as described by Fernandez-Espinar et al. (2000) using *Hin6*I restriction endonuclease (Fermentas, Lithuania) according to the supplier's instructions. Restriction fragments were separated and visualized on 1.5% (w/v) agarose gel in 1X TAE buffer. Yeast isolates showing the same RFLP profile were grouped together and one sample per each cluster was subjected to the partial amplification and sequencing of the 26S rDNA D1/D2 domain.

2.2.2.3 D1/D2 of *26S rDNA* sequence analysis

Amplification was performed from genomic DNA and the analyses were done in a 25 µl reaction mixture containing 1X Buffer (5 Prime, Hamburg), 2.5 mM of $MgCl_2$ (5 Prime, Hamburg), 200 µM of dNTPs (Fermentas, Lithuania), 0.1µM of each primer (table 2), 2 U of Taq-DNA Polymerase (5 Prime, Hamburg) and 80 ng of DNA. The temperature profile was: denaturation step at 94°C for 1 min, annealing step at 52 °C for 1 min and extension step at 72°C for 2 min and this was repeated

[3] Instrument used in all PCR sequence analysis

[4] Instrument used to visualize all amplification products

for 35 cycles. Amplification products were resolved by electrophoresis in 1.0% (w/v) agarose gels in 1xTAE buffer. PCR products were subject to sequencing by an external service (Primm s.r.l. Milan, Italy) and sequences were compared through BLAST software with the ones listed in databases[5] for the different species. The ascription to a species was done considering two percentages resulting from the comparison; the coverage and identity assignment was performed only when these two rates were equal or greater than 97%.

2.2.2.4 Interdelta sequences typing

Typing was carried out for all isolates identified as *S. cerevisiae* using Interdelta analysis (δ-PCR) according to Legras and Karst. (2003). Amplification was performed from genomic DNA and analyses were performed in a 25 µl reaction mixture containing 1X Buffer (5 Prime, Hamburg), 2 mM of $MgCl_2$ (5 Prime, Hamburg), 200 µM of dNTPs (Fermentas, Lithuania), 1 mM of each primer (table 2) , 1 U of Taq-DNA Polymerase (5 Prime, Hamburg) and 80 ng of DNA. The temperature profiles was: 5 cycles of denaturation step at 95°C for 30 s, annealing step at 42°C for 30 s and extension step at 72°C for 2 min, followed for 30 cycles of denaturation step at 95°C for 30 s, annealing step at 42°C for 30 s, extension step at 72°C for 2 min and finish step at 72°C for 30 min. Amplification products were separated by electrophoresis in 2.0% (w/v) agarose gels in 1X TAE buffer

Primer	Nucleotide sequence (5'-3')	References
ITSL1	GTTTCCGTAGGTGAACCTGC	Montrocher et al.(1998)
ITSL2	ATATGCTTAAGTTCAGCGGGT	
NL1	GCATATCAATAAGCGGAGGAAAAG	Kurtzman et al.(1998)
NL4	GGTCCGTGTTTCAAGACGG	
DELTA 12	TCAACAATGGAATCCCAAC	Legras and Karst.(2003)
DELTA 21	CATCTTAACACCGTATATGA*	

Table 2.
DNA amplification primers. *According to Tristezza et al.(2009) the delta21 primer was 5′-dye-labelled with 6-Carboxyfluorescein (6-FAM, Primm, Milan, Italy) in order to run the products amplification in Capillary electrophoresis

2.2.2.5 Capillary Electrophoresis

Capillary electrophoresis of the amplified fragments from δ-PCR was carried out on an ABI Prism 310 Genetic Analyzer (Applied Biosystems – Life Technologies, Unites States) using POP-4 polymer, 310 Genetic Analyzer Buffer with EDTA, and a 47 cm x 50 µm capillary (Applied Biosystems – Life Technologies, Unites States). Samples were prepared in a solution of 0.9 µl of δ amplified, 20 µl of formamide (Applied Biosystems – Life Technologies, Unites States) and 0,75 µl size standard GeneScan-1200 LIZ (Applied Biosystems, Life Technologies). The solution was incubated at 95°C for 3 min in order to denature DNA fragments and then snap cooled on ice prior to loading them into the autosampler tray. Following, samples were injected for 20 s at 1.5 kV and separated at 8 kV for 80 min with a run temperature of 60°C. Data elaboration was performed using

[5] www.ncbi.nlm.nih.gov

ABI PRISM GeneMapper 3.7 (Applied Biosystems – Life Technologies, Unites States) software. For elaboration of peaks, fragments between 50 and 1200bp were scored. Peaks with fluorescent intensity value less than 100 were not taken account.

Repeatability of method

The repeatability was validated by multiple testing as follows. The genomic DNA, extracted from four distinct *S. cerevisiae* commercial starters, was used as template in four independent PCR amplifications (see section 2.3.4) using delta primers (table 2). PCR products were separated by electrophoresis in 2.0% (w/v) agarose gels in 1X TAE buffer and run in capillary electrophoresis.
The genetic similarity among replicates belonging to the same clonal type (each of four distinct *S. cerevisiae*), was calculated according to Dice´s coefficient (Dice, 1945). The lowest percentage on which these were grouped, was considered the discrimination threshold among *S. cerevisiae* isolates. Isolates that were placed above this percentage were considered clones of the same strain.

Data analysis

Electrophoretic patterns from agarose gel analysis and GeneMapper 3.7 software were transformed in binary matrices (1 presence, 0 absence of fragment) and used to create a similarity matrix. NTSYS-pc 2.1(Rohlf, 1998) software was used to imported binary matrices and calculate genetic similarity of isolates according to Dice´s coefficient (Dice, 1945) using the SIMQUAL routine. The SAHN-clustering program was used to clustered the matrices which were visualized by a UPGMA (unweighted pair group method with arithmetic means) dendrogram and then displayed by TREE PLOT program.

2.3 Results

The present study was focused on biodiversity evaluation of yeast involved in winemaking process in *Franciacorta* (province of *Brescia*) and *Oltrepò Pavese* (province of *Pavia*) in Lombardy, Italy, in 2009, 2010 and 2011 vintages. During the months of July August and October, for each year of the research, some samplings were performed in correspondence with the beginning of the veraison of the berries, the time of grape harvest and the base wine production, respectively. Molecular identification at species level (ITS rDNA gene sequences and D1/D2 of 26S rDNA sequence analysis) was carried out for yeasts collected from air of vineyard, must and base wine. Furthermore, molecular identification at strain level (interdelta sequences typing and Capillary Electrophoresis) was carried out for the isolates identified as *Saccharomyces cerevisiae*, isolated during the vintages 2009 and 2010 from the same types of samples.

2.3.1 Yeasts isolation and collection

During this study was possible to create a collection of 492 yeast isolates from air, must and base wine (table 3)

Vintage, origin and samples	Yeast isolates (n)	Percentage
2009	**178**	**36%**
FCR	**95**	**19%**
AIR	8	2%
BASE WINE	48	10%
MUST	39	8%
OLT	**83**	**17%**
AIR	9	2%
BASE WINE	52	11%
MUST	22	4%
2010	**186**	**38%**
FCR	**112**	**23%**
BASE WINE	21	4%
MUST	91	18%
OLT	**74**	**15%**
AIR	3	1%
BASE WINE	15	3%
MUST	56	11%
2011	**128**	**26%**
FCR	**58**	**12%**
BASE WINE	23	5%
MUST	35	7%
OLT	**70**	**14%**
AIR	6	1%
BASE WINE	22	4%
MUST	42	9%
Total	**492**	**100%**

Table 3.
yeast isolates collected during 3 years vintages of *Franciacorta* (FCR) and *Oltrepò Pavese* (OLT).

The highest isolates percentage were obtained during the vintage 2010 (38%), but only 2% of difference was observed in vintage 2009 (36%). Regarding the sample type, the greater percentage of isolates during 2009 vintage came from wine base (10% and 11% for *Franciacorta* and *Oltrepò Pavese* samples respectively); during 2010 and 2011 vintages, the greater percentage of isolates came from must (18%-7% *Franciacorta* 2010-2011 vintages respectively and 11%-9% *Oltrepò Pavese* 2010-2011 vintages respectively), instead. As expected, during all vintages the fewest isolates came from the air samples.
On the other hand, in *Franciacorta* area 8 wineries denominated *BVT, FGT, UBT, CBC, MTR, MJL, VLA* and *VZL* took part into this study. In particular, out of the 265 isolates collected during the three vintages, the highest percentage came from the winery *BVT* with 74 yeast isolates (corresponding to 28%) of which 65% were collected from must, 34% from base wine and only 1% were collected from air. *FGT* turned out to be the winery where the second rate (22%) of isolates was observed, corresponding to 57 yeasts isolated during the three years (figure 3).

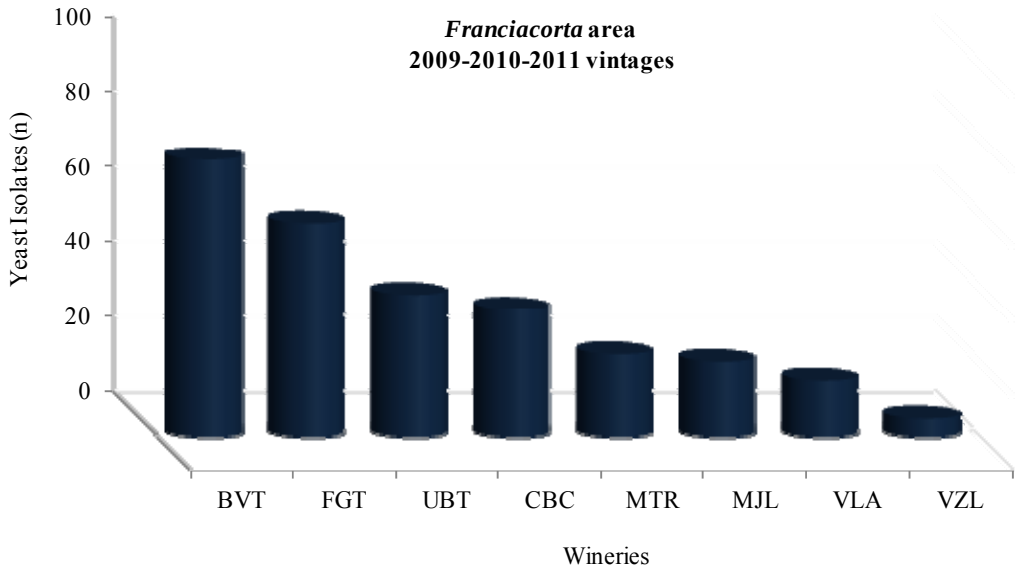

Figure 3.
Wineries and yeast isolates (n) during 2009-2010-2011 vintages in *Franciacorta* area.

Regarding to *Oltrepò Pavese* area, 11 wineries denominated *ATO, CSO, MSL, QRN, VTN, MLN, IBC, SGG, IBD, TVN* and *VRD* were included in the sampling. During the three years, 227 isolates were collected, of which the highest percentage came from the winery *ATO* with 28 yeast isolates (corresponding to 12%) of which 50% were collected from base wine, 46% from must and 4% were collected from air. From *CSO* winery the same percentage of *ATO* winery was isolated. In fact, as shown in figure 4 in the *Oltrepò Pavese* wineries, the percentage of isolation was homogeneous during the three years.

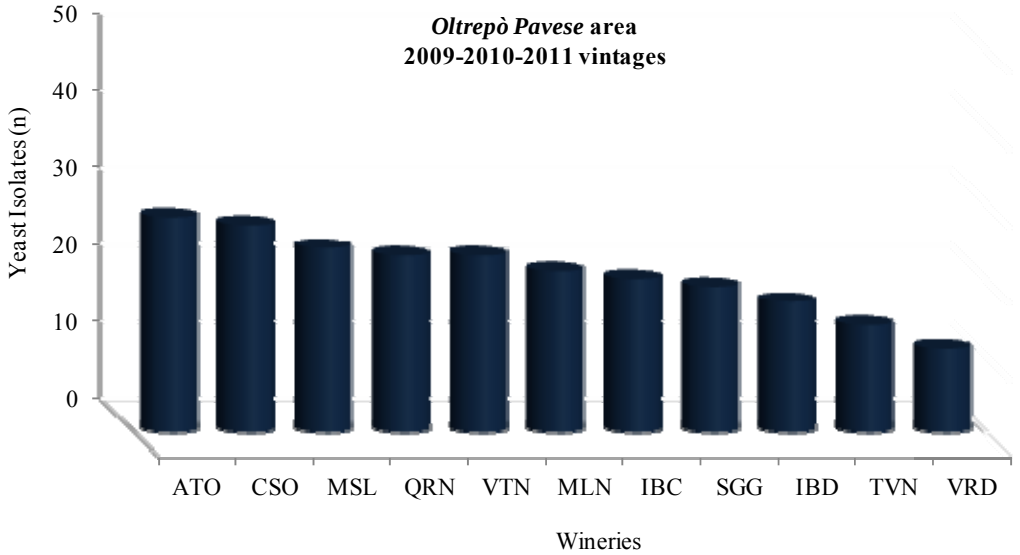

Figure 4.
Wineries and yeast isolates (n) during 2009-2010-2011 vintages in *Oltrepò Pavese* area.

The isolates collected during the three years of vintages, were maintained at -80° C in YEPD medium added with 20% (v/v) glycerol. The complete yeast isolates collection is shown in detail in appendix A.
Furthermore, a small collection of commercial starter strains used mainly for sparkling wine production, were collected and maintained under the same conditions. These were used as reference in this study.

2.3.2 Genetic identification

Genetic identification was performed from extracted genomic DNA according to Querol et al. (1992). Out of 492 isolates collected during the three vintages in both *Franciacorta* and *Oltrepò Pavese* areas, 86% of them were identified through ITS rDNA gene sequences and D1/D2 of 26S rDNA sequence analysis**.** At the time of writing, the genetic identification of the remaining 14% of yeast isolates collection (belonging to 2011 *Oltrepò Pavese* vintage) is being carried out.

2.3.2.1 Yeast isolates from air

Air samples were taken using the "*MAS 100 Eco*" (figure 1) from VWR® International. During 3 vintages, thirteen vineyards called *Paiù, Tralici, Lechi, Zenighe, Le selve sotto, I piani, Sottomonte CH, Piana cantina, Cologne, Capannina Erbusco, Capannina Adro, Capanina M Rot* and *Derbusco* (belonging to 8 wineries, see figure 3) were sampled in *Franciacorta* area. Eleven vineyards called *Chiozzo, Sottocasa, Giardino, Molinello, Crocioni, Bellaria, Il Portico, Montagnera basso, Regina, Vigna del pozzo* and *Castello* (belonging to 11 wineries, see figure 4) were sampled in *Oltrepò pavese* instead**.** In all the vineyards, two aliquots of 100 and 500 liters of air were collected for analyses. From samples, 26 isolates (5% of the yeast isolates collected) were collected during the three vintages in *Franciacorta* and *Oltrepò pavese* .

Vintage and origin	Yeast isolates (n)	Percentage
2009	**17**	**65%**
FCR	**8**	**31%**
Aureobasidium pullulans	4	15%
Cryptococcus laurentii	4	15%
OLT	**9**	**35%**
S. cerevisiae	7	27%
Aureobasidium pullulans	1	4%
Issatchenkia spp	1	4%
2010	**3**	**12%**
OLT	**3**	**12%**
S. cerevisiae	1	4%
Issatchenkia terricola	1	4%
Pichia fermentans	1	4%
2011	**6**	**23%**
OLT	**6**	**23%**
Yeast isolates unidentified	6	23%
Total	**26**	**100%**

Table 4.
Genetic identification of yeast isolates from air in *Franciacorta* (FCR) and *Oltrepò Pavese* areas during 2009, 2010 and 2011 vintages.

The most interesting data of air sampling was the isolation of *S. cerevisiae* specie (figure 5). The highest percentage of yeast isolates during three years was observed in 2009 vintage (65%), where 7 isolates (*1OA-MZZ* ;*1OA-MZZ-I* ; *1OA-MZZ-II* ; *1OA-MZZ-IIA* ; *1OA-SG-IIA* ; *1OA-SG-VS* ; *1OA-TRV-IA*), corresponding to 27% of this specie, was isolated from *Oltrepò Pavese* area (wineries *MLN, SGG* and *TVN* 57%, 29% and 14% respectively). During 2010 vintage, 4% was obtained again in *Oltrepò pavese* from winery *VTN* (2OA-VIS-1). In addition to *S. cerevisiae* specie, other species such as *Aureobasidium pullulans, Cryptococcus laurentii, Issatchenkia spp* (both 2009 and 2010 *Oltrepò Pavese* vintages), *Issatchenkia terricola*, and *Pichia fermentans* were successfully isolated from air samples. Particularly in *Franciacorta* area, yeast isolation was possible in 2009 vintage, only.

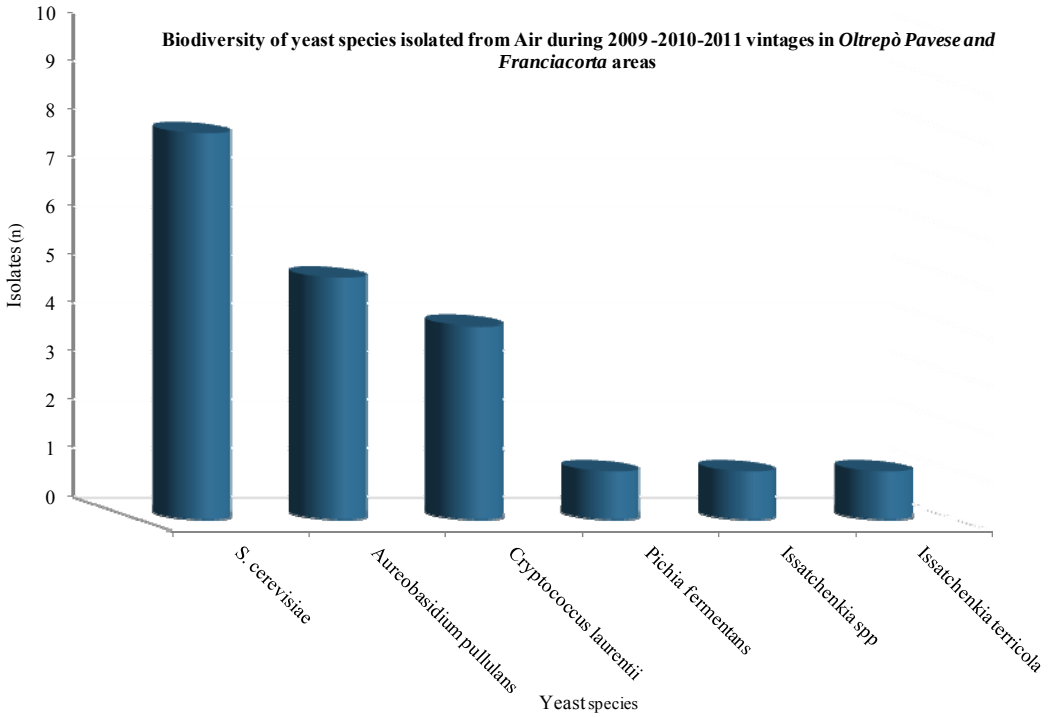

Figure 5.
Biodiversity of yeast species isolated from air in *Franciacorta* and *Oltrepò Pavese* area during three vintages.

2.3.2.2 Yeast isolates from must

In each winery, two samples of must were taken transferring 50 ml in sterilized flasks before SO_2 addition, when possible. From samples, 285 isolates (58% of the yeast isolates collected) were collected during the three vintages in *Franciacorta* and *Oltrepò Pavese*. The grand total from genetic identification results of must isolates is present in table 5.

Vintage and origin	Yeast isolates (n)	Percentage
2009	**61**	**21,4%**
FCR	**39**	**13,7%**
S. cerevisiae	22	7,7%
Zygosaccharomyces bailii	11	3,9%
Issatchenkia occidentalis	6	2,1%
OLT	**22**	**7,7%**
S. cerevisiae	14	4,9%
Zygosaccharomyces bailii	4	1,4%
Issatchenkia spp	1	0,4%
Candida zemplinina	1	0,4%
Candida diversa	1	0,4%
Hanseniaspora uvarum	1	0,4%
2010	**147**	**51,6%**
FCR	**91**	**31,9%**
S. cerevisiae	27	9,5%
Metschnikowia fructicola	15	5,3%
Hanseniaspora uvarum	13	4,6%
Pichia kluyveri	10	3,5%
Issatchenkia occidentalis	5	1,8%
Issatchenkia terricola	5	1,8%
Torulaspora delbrueckii	4	1,4%
Pichia anomala	3	1,1%
Pichia kudriavzevii	2	0,7%
Rhodotorula spp	2	0,7%
Candida parapsilosis	1	0,4%
Candida zemplinina	1	0,4%
Zygoascus spp	1	0,4%
Pichia guilliermondii	1	0,4%
Cryptococcus flavescens	1	0,4%
OLT	**56**	**19,6%**
S. cerevisiae	17	6,0%
Candida railenensis	14	4,9%
Torulaspora delbrueckii	6	2,1%
Pichia fermentans	5	1,8%
Hanseniaspora uvarum	4	1,4%
Issatchenkia terricola	4	1,4%
Metschnikowia pulcherrima	3	1,1%
Issatchenkia terricola	2	0,7%
Pichia membranifaciens	1	0,4%
2011	**77**	**27,0%**
FCR	**35**	**12,3%**
S. cerevisiae	16	5,6%
Torulaspora delbrueckii	6	2,1%
Hanseniaspora vineae	5	1,8%
Kluyveromyces thermotolerans	3	1,1%
Metschnikowia pulcherrima	2	0,7%
Candida oleophila	1	0,4%
Hanseniaspora uvarum	1	0,4%
Candida zemplinina	1	0,4%
OLT	**42**	**14,7%**
Yeast isolates unidentified	42	14,7%
Total	**285**	**100,0%**

Table 5.
Genetic identification of isolates from must in *Franciacorta* (FCR) and *Oltrepò Pavese* (*OLT*) during three vintages.

Vintages 2010 was that in which the largest number of isolates were obtained from must with a total of 147 corresponding to 52% of the 3 vintages in *Franciacorta* and *Oltrepò Pavese.* In fact, the greatest biodiversity of yeast species was observed (figure 6). A total of 22 different species were isolated among which the highest percentage corresponded to *S. cerevisiae* (44 isolates; 30%). Second and third percentage was for *Metschnikowia fructicola* and *Candida railenensis* with 10% and 9% respectively. In particular *S. cerevisiae, Torulaspora delbrueckii* and *Issatchenkia terricola* were common species for both areas, while *Metschnikowia fructicola, Hanseniaspora uvarum, Issatchenkia occidentalis, Pichia anomala, Rhodotorula spp, Pichia kudriavzevii, Pichia kluyveri, Zygoascus spp, Candida zemplinina, Candida parapsilosis, Cryptococcus flavescens* and *Pichia guilliermondii* were present only in *Franciacorta* area. *Candida railenensis, Pichia fermentans, Hanseniaspora uvarum, Issatchenkia terricola, Metschnikowia pulcherrima* and *Pichia membranifaciens* were present only in *Oltrepò Pavese* instead.

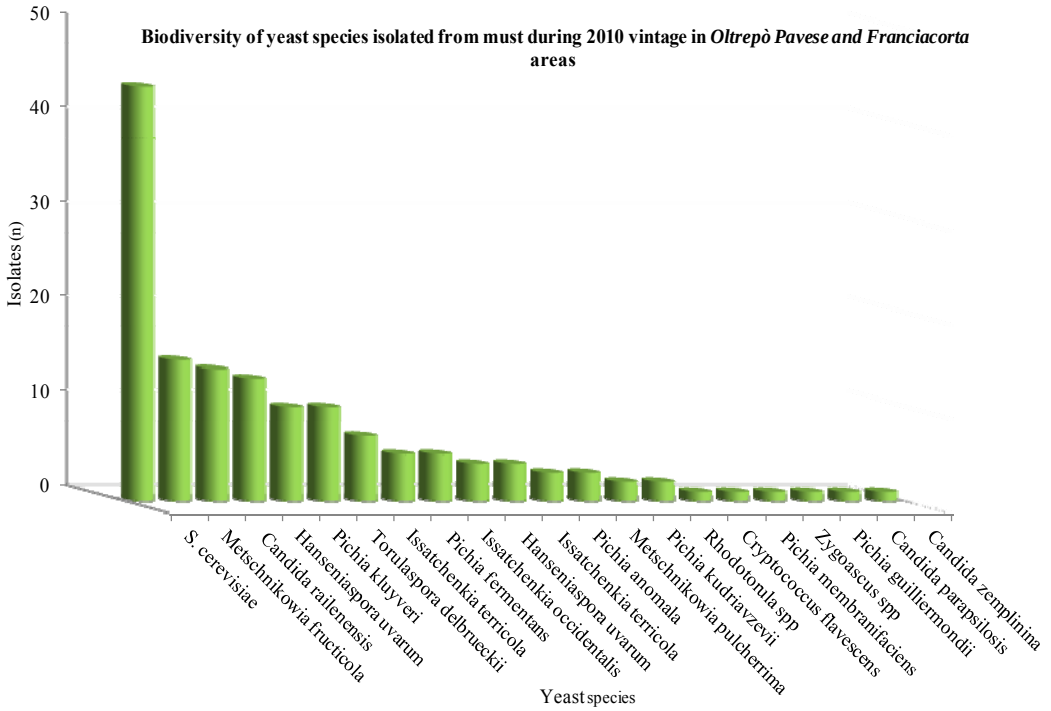

Figure 6.
Biodiversity of yeast species isolated from must samples in *Franciacorta* and *Oltrepò Pavese* area during 2010 vintage

Other yeast species different than those already named, were isolated during 2009 vintage: *Zygosaccharomyces bailii,* commonly present in both areas (25% of 61 isolates) and *Candida diversa* only present in *Oltrepò pavese* (2% of 61 isolates). Regarding the isolates from 2011 *Franciacorta* vintage, 35 were collected from must, of which *Hanseniaspora vineae* (5 isolates; 1.8%), *Kluyveromyces thermotolerans* (3 isolates; 1.1%) and *Candida oleophila* (1 isolate; 0.4%) represented new yeast species never isolated before in past vintages or *Franciacorta* or *Oltrepò pavese*. Tables 6 and 7 shows the percentage of must isolates with respect to the wineries in *Franciacorta* and *Oltrepò Pavese* respectively.

Franciacorta wineries	Yeast isolates (n)	Percentage
BVT	48	29%
FGT	37	22%
UBT	22	13%
CBC	18	11%
MJL	18	11%
MTR	11	7%
VLA	8	5%
VZL	3	2%
Total	**165**	**100%**

Table 6.
Percentage of yeast isolates from must in *Franciacorta* wineries during 3 vintages.

Oltrepò wineries	Yeast isolates (n)	Percentage
MSL	15	13%
CSO	14	12%
IBD	13	11%
ATO	13	11%
MLN	12	10%
QRN	12	10%
VTN	10	8%
IBC	10	8%
SGG	10	8%
VRD	6	5%
TVN	5	4%
Total	**120**	**100%**

Table 7.
Percentage of yeast isolates from must in *Oltrepò Pavese* wineries during 3 vintages.

Regarding to isolates from must in *Franciacorta* wineries, the highest percentages were obtained from *BVT* winery and *FGT* winery where 29% and 22% of 165 isolates were collected respectively. On the other hand, isolates from must in *Oltrepò Pavese* wineries were obtained mainly from *MSL* winery (13%), *CSO* winery (12%); both *IBD* and *ATO* wineries obtained the same percentage (11%).

2.3.2.3 Yeast isolates from base wine

From wineries, samples were collected each year in October. Two samples of base wine were taken transferring 100 ml (duplicate samples) in sterilized flasks and transported to the laboratory. During a period of three years from base wine samples, a total of 181 yeast isolates (37 % of grand total collection) was collectable. The results of genetic identification from wine isolates are shown in table 8.

Vintage and origin	Yeast isolates (n)	Percentage
2009	**100**	**55%**
FCR	**48**	**27%**
S. cerevisiae	32	18%
Pichia membranifaciens	9	5%
Pichia fluxum	3	2%
Zygosaccharomyces bailii	2	1%
Issatchenkia occidentalis	1	1%
Torulaspora delbrueckii	1	1%
OLT	**52**	**29%**
S. cerevisiae	52	29%
2010	**36**	**20%**
FCR	**21**	**12%**
S. cerevisiae	11	6%
Hanseniaspora uvarum	3	2%
Pichia membranifaciens	3	2%
Pichia spp	2	1%
Zygosaccharomyces bailii	1	1%
Issatchenkia occidentalis	1	1%
OLT	**15**	**8%**
S. cerevisiae	6	3%
Candida railenensis	4	2%
Pichia fermentans	3	2%
Hanseniaspora uvarum	2	1%
2011	**45**	**25%**
FCR	**23**	**13%**
S. cerevisiae	20	11%
Pichia membranifaciens	3	2%
OLT	**22**	**12%**
Yeast isolates unidentified	22	12%
Total	**181**	**100%**

Table 8.
Genetic identification of yeast isolates from base wine in *Franciacorta* (FCR) and *Oltrepò Pavese (*OLT*)* areas during 2009, 2010 and 2011 vintages.

Regarding the largest isolates number, the 2009 vintage was the one where the largest number of these was observed. A total of 48 (48%) for *Franciacorta* and 52 (52%) for *Oltrepò Pavese* were collected respectively. As expected, the species of *S. cerevisiae* represented the highest percentage in both *Franciacorta* (67%) and wine Oltrepò Pavese (100%) wine isolates. During this vintage, a total of 6 yeast species were isolated (figure 7).

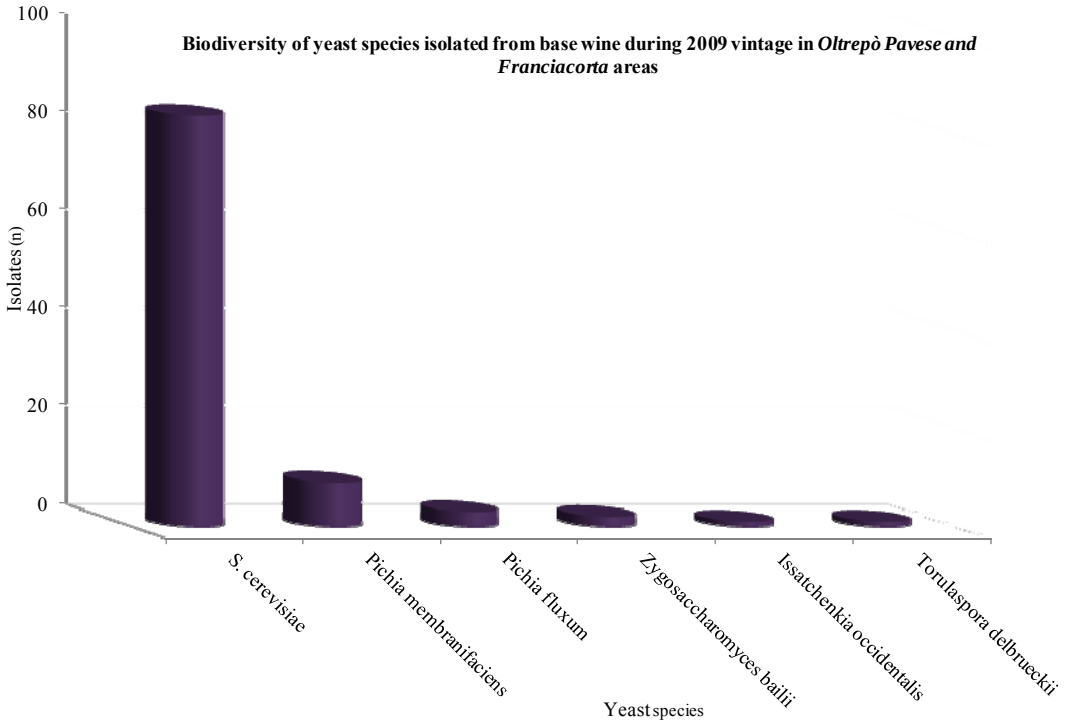

Figure 7.
Biodiversity of yeast species isolated from base wine *Franciacorta* and *Oltrepò Pavese* area during 2009 vintage

Although the largest number of base wine isolates came from the 2009 vintage, actually the greatest biodiversity of yeast species was seen in the 2010 vintage where a total of 8 species were found (figure 8). Between two vintages, the common species isolated were: *S. cerevisiae*, *Pichia membranifaciens*, *Zygosaccharomyces bailii*, and *Issatchenkia occidentalis*. *Pichia fluxum* and *Torulaspora delbrueckii* were isolated only in 2009 while *Hanseniaspora uvarum*, *Candida railenensis* and *Pichia fermentans* only in 2010.
Regarding the sampling of base wine from *Franciacorta* 2011 vintages, 23 isolates were collected, of which 87% of this corresponded to *S. cerevisiae* species and 13% to *Pichia membranifaciens.*

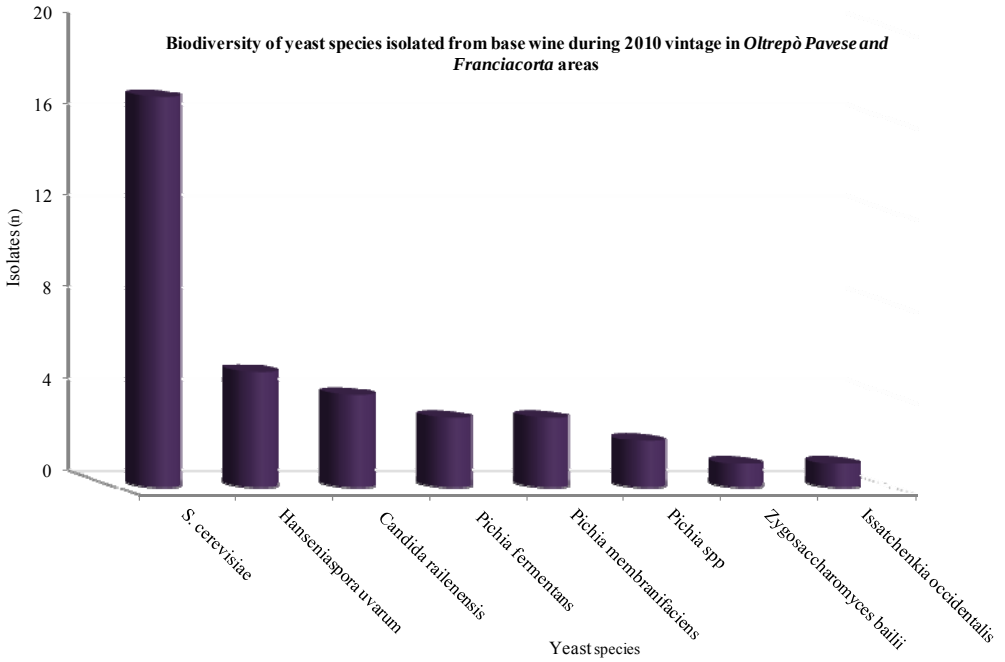

Figure 8.
Biodiversity of yeast species isolated from base wine *Franciacorta* and *Oltrepò Pavese* area during 2010 vintage.

Wineries	Yeast isolates (n)	Percentage
BVT	25	14%
FGT	20	11%
CBC	16	9%
UBT	15	8%
ATO	14	8%
CSO	12	7%
VTN	12	7%
TVN	9	5%
QRN	8	4%
IBC	8	4%
SGG	7	4%
MSL	7	4%
MTR	6	3%
VLA	6	3%
MLN	4	2%
IBD	4	2%
VRD	4	2%
VZL	2	1%
MJL	2	1%
Total	**181**	**100%**

Table 9.
Percentage of yeast isolates from base wine in *Franciacorta* and *Oltrepò Pavese* wineries during 3 vintages.

In table 9, the percentage of base wine isolates with respect to the wineries in both *Franciacorta* and *Oltrepò Pavese* are shown. In particular the same report for *S. cerevisiae* specie is shown in tables 10 (*Franciacorta* wineries) and table 11 (*Oltrepò Pavese* wineries) instead.

Franciacorta wineries	*S. cerevisiae* isolates (n)	Percentage
BVT	19	30%
FGT	14	22%
CBC	11	17%
UBT	10	16%
MTR	5	8%
VLA	2	3%
MJL	2	3%
Total	**63**	**100%**

Table 10.
Percentage of *S. cerevisiae* isolated from base wine in *Franciacorta* wineries during 3 vintages.

Oltrepò pavese wineries	*S. cerevisiae* isolates (n)	Percentage
CSO	10	17%
ATO	8	14%
VTN	7	12%
TVN	7	12%
QRN	7	12%
IBC	6	10%
VRD	4	7%
MSL	4	7%
IBD	3	5%
SGG	1	2%
MLN	1	2%
Total	**58**	**100%**

Table 11.
Percentage of *S. cerevisiae* isolated from base wine in *Oltrepò Pavese* wineries during 3 vintages.

From base wine in *Franciacorta* wineries, as in the must case, the highest percentages of isolates were obtained from *BVT* winery and *FGT* winery where 14% and 11% of 181 isolates were collected respectively. In addiction, the highest percentage of *S. cerevisiae* isolates was found in these same wineries with 30% and 22% respectively. On the other hand, isolates from base wine in *Oltrepò Pavese* wineries were obtained mainly from *ATO* winery (16%) and *CSO* winery (13%); Contrary, the highest percentage of *S. cerevisiae* isolates was found in *CSO* winery (17%) and *ATO* winery in second place with 14%.

2.3.3 Genetic typing

Molecular identification at strain level was carried out for isolates identified as *Saccharomyces cerevisiae* from ITS rDNA and D1/D2 of 26S rDNA sequence analysis isolated during 2009 and 2010 vintages in both *Franciacorta* and *Oltrepò Pavese* territories (table 12). In particular, 127 isolates were analyzed from 2009 vintage and 62 for 2010 vintage

Vintage - origin - sample	*S. cerevisiae* isolates (n)	Percentage
2009	**127**	**67%**
FCR	**54**	**29%**
BASE WINE	32	17%
MUST	22	12%
OLT	**73**	**39%**
BASE WINE	52	28%
MUST	14	7%
AIR	7	4%
2010	**62**	**33%**
FCR	**38**	**20%**
MUST	27	14%
BASE WINE	11	6%
OLT	**24**	**13%**
MUST	17	9%
BASE WINE	6	3%
AIR	1	1%
Total	**189**	**100%**

Table 12.
S. Cerevisiae isolated from *Franciacorta* (FCR) and *Oltrepò Pavese* (OLT) during 2009 and 2010 vintages.

2.3.3.1 Interdelta analysis (δ-PCR)

Typing was carried out using interdelta analysis (δ-PCR) according to Legras and Karst. (2003) modified by use of delta 21 primer which was 5′-dye-labelled with 6-Carboxyfluorescein (6-FAM, Primm, Milan, Italy) according to Tristezza et al.(2009). Fingerprinting analysis involved 189 isolates of *S. cerevisiae* (table 12) and all amplifications were performed from genomic DNA. During *Franciacorta* and *Oltrepò Pavese* 2010 vintages, the highest percentage of *S. cerevisiae* isolates were present in the must with 71% for both areas, while in 2009 vintages the highest percentage of *S. cerevisiae* isolates were present in the base wine with 59% for *Franciacorta* and 71% (as the must case in previous year) for *Oltrepò Pavese*. As examples, in figure 9 and 10 the interdelta profiles from some *S. cerevisiae* isolated in *Franciacorta and Oltrepò Pavese* during 2010 vintage respectively, are shown.

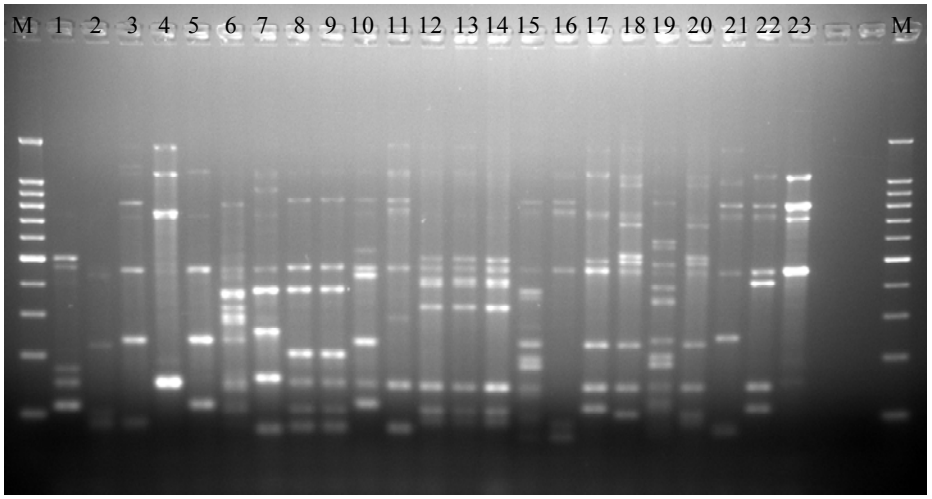

Figure 9.
Agarose gel 2,0% of the PCR amplification from Interdelta analysis. **M:** DNA Molecular Weight (100 bp XL Ladder, 5 PRIME, Italy), **1-23:** *2F-M11-III; 2F-M43-VIII; 2F-M59-I; 2F-M59-III; 2F-MC120-III; 2F-MC122-IV; 2F-MC19-I; 2F-V11-IV; 2F-V11-V; 2F-V34-I; 2F-V34-III; 2F-MC-43-II; 2F-MC43-V; 2F-MC43-VI; 2F-MC59-VI; 2F-MC120-III; 2F-MC122-IV; 2F-V34-IV; 2F-V43-II; 2F-M43-IV; 2F-M43-V; 2F-M122-III; 2F-M122-IV S. cerevisiae* isolated from must (M/MC) and base wine (V) during *Franciacorta* vintage 2010 (2F).

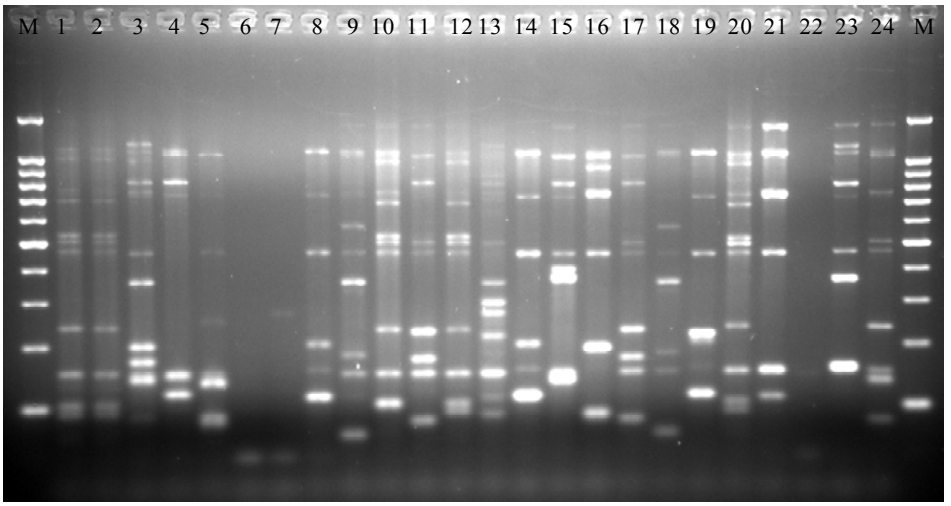

Figure 10.
Agarose gel 2,0% of the PCR amplification from Interdelta analysis. **M:** DNA Molecular Weight (100 bp XL Ladder, 5 PRIME, Italy), **1-24:** *2O-V-VIS-2; 2O-V-CD1; 2O-M+-MON-1; 2O-M+-MON-4; 2O-V-CAS2; 2O-CAS-3; 2O-M-ISM-3; 2O-M-ISM-4; 2OA-VIS-1; 2O-M-CAS-2; 2O-M+-MAZ-1; 2O-V-MON-2; 2O M+-MON-3; 2O-M-ISM-5; 2O-M-MON-1; 2O-M-CAS-1; 2O-M-MAZ-2; 2O-M-MON-2; 2O-M-3IB; 2O-M-MON-3; 2O-V-CAS-1; 2O-M-ISM-2; 2O-M-ISM-6; 2O-V-ANT-1 S. cerevisiae* isolated from air (A), must (M/M+) and base wine (V) during *Oltrepò Pavese* vintage 2010 (2O).

2.3.3.2 Capillary Electrophoresis

The second step for typing *S. cerevisiae* isolates was the separation of Interdelta-PCR amplicons by capillary electrophoresis. As mentioned above, delta21 primer was 5′-dye-labelled with 6-Carboxyfluorescein (6-FAM, Primm, Milan, Italy) according to Tristezza et al.(2009) in order to be detected through a proven multicolor fluorescent labeling (ABI Prism 310 Genetic Analyzer, Applied Biosystems – Life Technologies, Unites States). The run was done using POP-4 polymer (Applied Biosystems – Life Technologies, Unites States)

Repeatability of the technique

In order to validate the method, a multiple test was performed. For this, triple interdelta-PCR profiles from four different *S. cerevisiae* strains (SC1, SC2, SC3, SC4) amplified thorough four independent PCR amplifications, were analyzed in agarose gel and then ran in capillary electrophoresis (figure 11). The detected electrophoresis patterns were transformed in binary matrices (1 presence, 0 absence of fragment) and used to create a similarity matrix, which was visualized by a UPGMA dendrogram (figure 12).

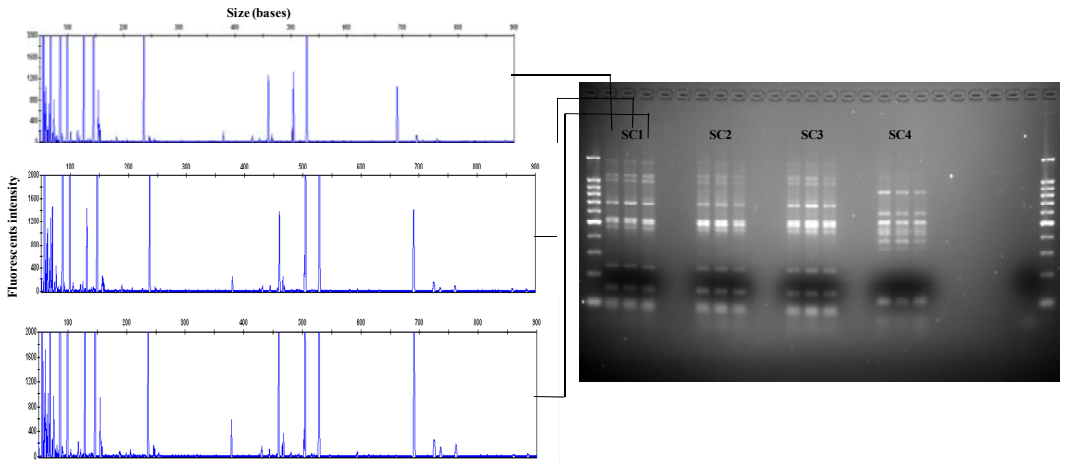

Figure 11.
Repeatability of capillary electrophoresis method. On the right: agarose gel analysis (2% and DNA Molecular Weight 100 bp XL Ladder, 5 PRIME, Italy) of interdelta-PCR profiles from four different *S. cerevisiae* strains (SC1, SC2, SC3 and SC4 in triplicate) amplified in four independent PCRs. On the left: capillary electrophoresis analysis; example of electropherograms from three replicates of SC1 *S. cerevisiae* strain. Molecular size (expressed in base pairs) of the bands is displayed on the X axis, while fluorescents intensity is located in Y axis.

From repeatability assessment, a size range was observed of 2bp of difference between the fragments of each strain replicates, which was considered the same allele form. So, this size value was considered when necessary, as criterion for grouping the fragments resulting from capillary electrophoresis before its transformation in binary matrix in both *S. cerevisiae* repeatability group and *S. cerevisiae* isolates group.

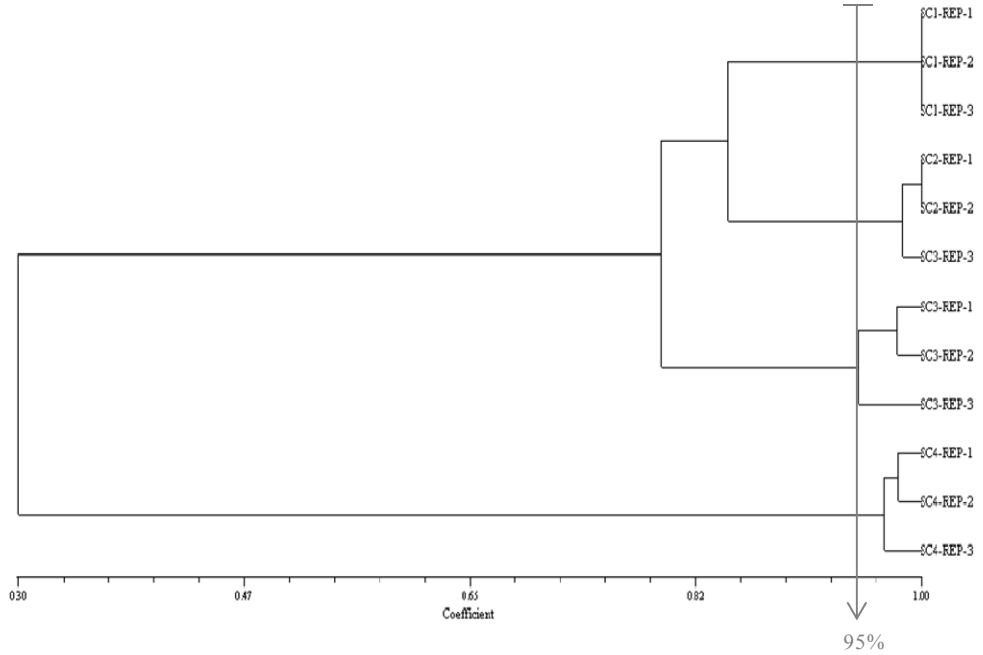

Figure 12.
Dendrogram generated by repeatability assessment of capillary electrophoresis. The coefficient of similarity among interdelta profiles from four different *S. cerevisiae* strains (SC1, SC2, SC3 and SC4 in triplicate) is indicated along the horizontal axis.

The repeatability assessment of capillary electrophoresis evidenced a genetic similarity of 95% for the same clonal type. This result was identical to that obtained by Tristezza et al.(2009) and was the discrimination threshold among *S. cerevisiae* from 2009 and 2010 vintages. Isolates that were placed above this percentage were considered clones of the same strain.

Genetic diversity of S. cerevisiae *isolates*

A grand total of 200 interdelta profiles within isolates of 2009-2010 vintages and commercial starter strains (see table 1 in paragraph 2.2.1.2 of this chapter) were analyzed by capillary electrophoresis. The UPGMA-based dendrogram is shown in figure 13a and 13b.
The dendrogram generated from elaboration of interdelta profiles evidenced a genetic similarity (Dice's coefficient) range from about 0,2% to 95% as the highest percentage. According to the discrimination threshold percentage (which coincides with the maximum percentage of genetic similarity but not correlated each other), only four isolates were considered clones of the same strain: *C117=1OV-QUA-VB2; C118= 1OV-TRV-VB5* isolated from wine (V) during *Oltrepò pavese* 2009 vintage (1O) and *C31= 1FM-40B; C34= 1FM-11C ,* isolated from must (M) during *Franciacorta* 2009 vintage.

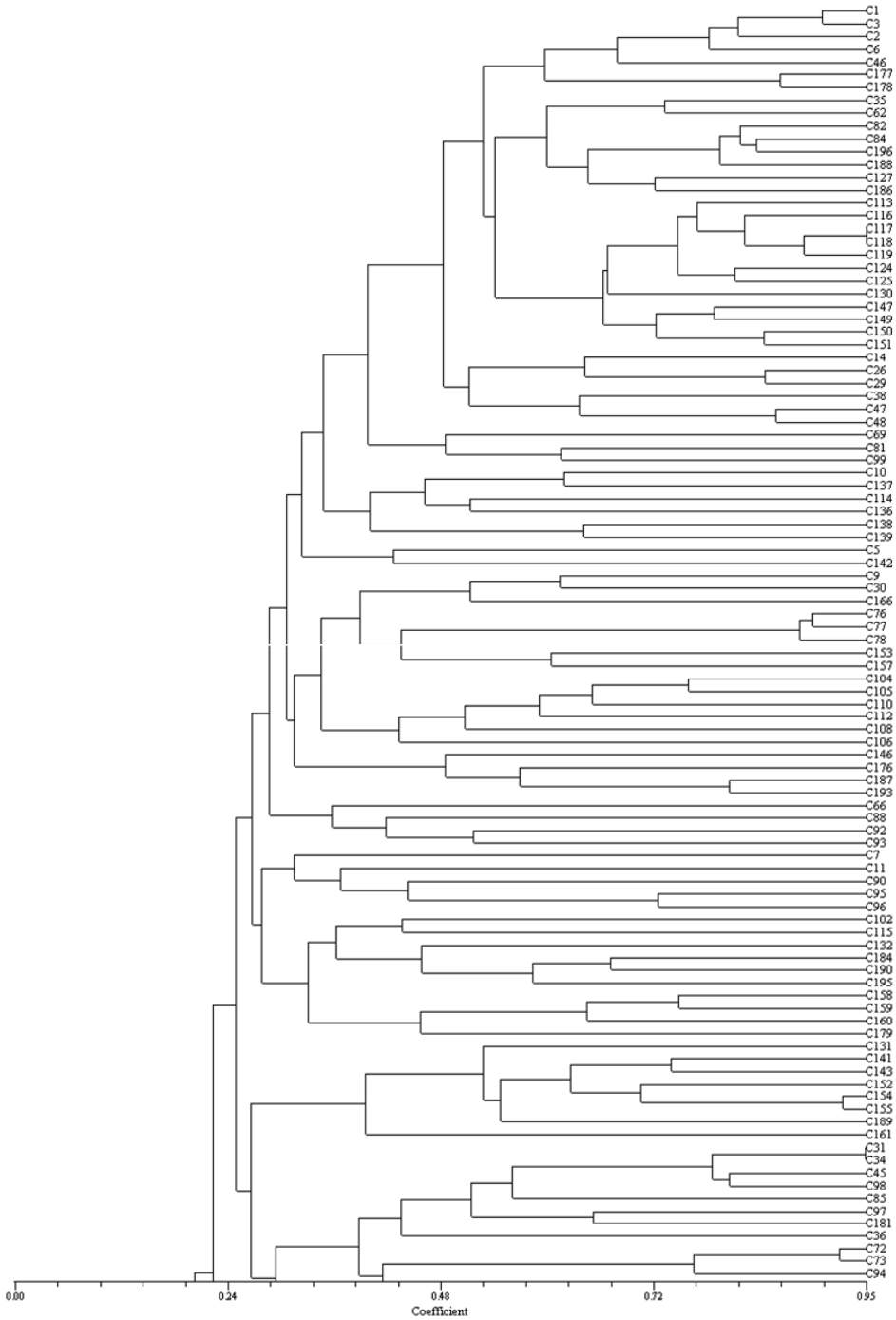

Figure 13a.
Dendrogram from cluster analysis of *S. cerevisiae* patterns. The coefficient (Dice) of similarity among isolates profiles is indicated along the horizontal axis.

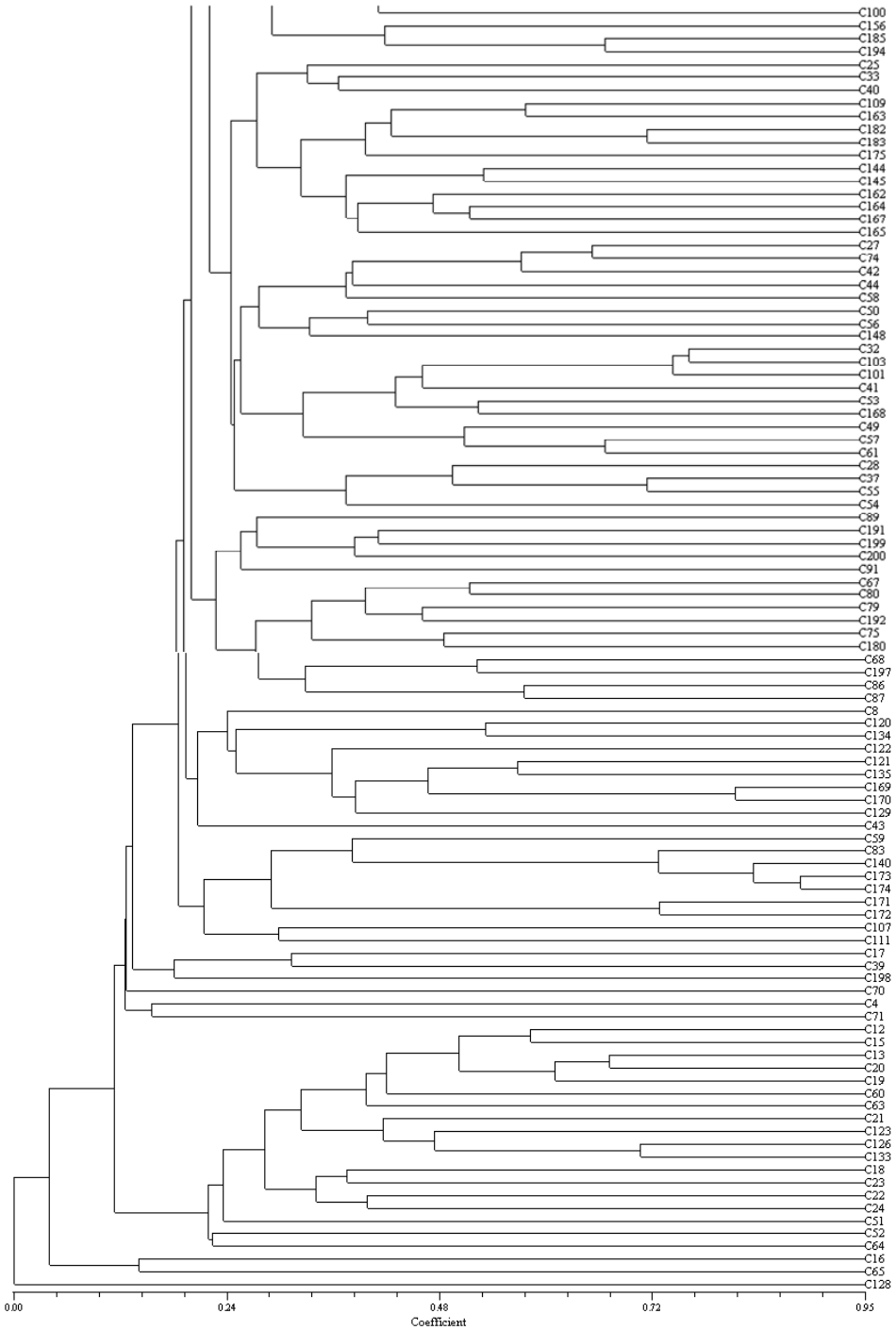

Figure 13b.
Dendrogram from cluster analysis of *S. cerevisiae* patterns. The coefficient (Dice) of similarity among isolates profiles is indicated along the horizontal axis.

The 200 electrophoretic patterns were grouped into 19 groups along the UPGMA- dendrogram, presenting a genetic similarity in a range of 0.2% to 95%. This means that all isolates except 4 (positioned above 95%) are *S. cerevisiae* strains-specific isolated from the vintages of 2009 and 2010 in *Franciacorta* ans *Oltrepò Pavese* areas. In table 13 the strains belonging to first 7 groups are listed as an example of the heterogenic formation of the dendrogram clusters.

vintage–origin-sample	*S. cerevisiae* cluster 1
COMMERCIAL STARTERS	*SC1; SC3; SC2; SC6*
2009-FCR-BASE WINE	*1F-V19-3; 1F-V2-1; 1F-VC2-1*
2009-OLT-BASE WINE	*1OV-CSO-VB6; 1OV-CSO-VB7; 1OV-ISM-VB1; 1OV-ISM-VB2; 1OV-MNS-VB2; 1OV-QUA-VB2; 1OV-QUA-VB5; 1OV-TRV-VB1; 1OV-TRV-VB2; 1OV-TRV-VB3; 1OV-TRV-VB5; 1OV-TRV-VB6; 1OV-VRD-VB1*
2010-FCR-MUST	*2F-M43-IV*
2010-OLT-MUST	*2O-M-CAS-2; 2O M+-MON-3*
2010-FCR-BASE WINE	*2F-V34-IV*
2010-OLT-BASE WINE	*2O-V-CD1; 2O-V-MON-2; 2O-V-VIS-2*
Total	27
vintage–origin-sample	*S. cerevisiae* cluster 2
2009-FCR-MUST	*1FM-11A*
2009-FCR-BASE WINE	*1F-V124-3; 1F-V40-9; 1F-V123-2; 1F-V43-4; 1F-V123-4*
Total	6
vintage–origin-sample	*S. cerevisiae* cluster 3
2010-FCR-MUST	*2F-M59-III; 2F-MC122-IV*
2010-FCR-BASE WINE	*2F-V34-III*
Total	3
vintage–origin-sample	*S. cerevisiae* cluster 4
2009-OLT-BASE WINE	*1OV-CSO-VB2A;1OV-CSO-VB2B; 1OV-CSO-VB5; 1OV-CSO-VB1; 1OV-CSO-VB4; 1OV-CSO-VB8*
Total	6
vintage–origin-sample	*S. cerevisiae* cluster 5
COMMERCIAL STARTERS	*SC5*
2009-OLT-MUST	*1OM-ANT-BB*
Total	2
vintage–origin-sample	*S. cerevisiae* cluster 6
2009-FCR-BASE WINE	*BVT6/5,*
2009-OLT-BASE WINE	*1OV-VST-VB5; 1OV-ANT-VB4; 1OV-ANT-VB5; 1OV-VRD-VB6; 1OV-CSO-VB3; 1OV-VST-VB4; 1OV-ANT-VB6; 1OV-ILB-VB4*
2009-FCR-MUST	*1FM-43C*
2010-FCR-MUST	*2F-MC-43-II; 2F-MC43-V ; 2F-MC43-VI*
2009-OLT-MUST	*1OM-CSO-BA; 1OM-SG-VA; 1OM-SG-BP*
2010-OLT-MUST	*2O-M+-MAZ-1; 2O-M-MAZ-2*
Total	18
vintage–origin-sample	*S. cerevisiae* cluster 7
2010-OLT-MUST	*2F-M11-III; 2F-M122-VI ; 2F-MC59-IV; 2F-MC59-V*
Total	4

Table 13.
S. cerevisiae strains from UPGMA-dendrogram. FCR= Franciacorta; OLT= Oltrepò Pavese.

Regarding to genetic similarity, *S. cerevisiae* strains with the highest percentages of genetic similarity are shown in table 14.

vintage –origin-sample	group	strains	genetic similarity
2009-OLT-BASE WINE	1	*1OV-QUA-VB2* *1OV-TRV-VB5*	95%
2010-FCR-MUST	9	*1FM-40B* *1FM-11C*	95%
2009-OLT-MUST	9	*1OM-MNS-BA* *1OM-MNS-BB*	92%
2010-FCR-MUST-BASE WINE	9	*2F-MC19-I* *2F-V11-IV*	91%
2009-OLT-AIR	13	*1OA-MZZ-I* *1OA-MZZ-IIA*	88%
2010-FCR-MUST	6	*2F-MC-43-II* *2F-MC43-V* *2F-MC43-VI*	87%
2010-OLT-BASE WINE	1	*2O-V-CD1* *2O-V-VIS-2*	86%
2009-FCR-BASE WINE	2	*1F-V43-4* *1F-V123-4*	85%
2009-FCR-BASE WINE	2	*1F-V124-3* *1F-V40-9*	83%
2009-OLT-BASE WINE	1	*1OV-ISM-VB1* *1OV-ISM-VB2*	83%
2009-OLT-BASE WINE	1	*1OV-TRV-VB2* *1OV-VRD-VB2*	80%

Table 14.
S. cerevisiae strains with the highest percentages of genetic similarity.

In terms of genetic biodiversity, the first most relevant result was that among 189 *S. cerevisiae* isolates, none turned out to be genetically identical to other. In fact, the major similarity coefficient was 0.95. The second interesting result was that despite discrimination threshold between isolates proved to be precisely 95% (repeatability of the technique), only 4 isolates resulted to be clones of the same strain. It was found that 98% of the isolates were *S. cerevisiae* strain-specific. Throughout the dendrogram, it was observed that in most cases, the clusters were formed for both wine and must isolates from *Oltrepò Pavese* and Franciacorta area; in one case, a cluster were formed even from the air (group 13). Regarding to isolation origin area, no relationship of genetic similarity was observed among *Franciacorta* strains and *Oltrepò Pavese* strains.

2.4 Discussion and Conclusion

Biodiversity and composition of yeast population associated with oenological environment of *Franciacorta* and *Oltrepò Pavese area* were investigated. Molecular identification at species level was carried out for the yeasts collected during vintages of 2009, 2010 and 2011. In addition, a molecular identification at strain level was carried out for the isolates identified as *Saccharomyces cerevisiae*, isolated during the vintages 2009 and 2010.Through this study, it was possible to create a collection of 492 yeast isolates. A total of 13 genus and 25 yeast species were isolated and identified during vintages for 3 years. *Aureobasidium pullulans, Candida railenensis, Candida diversa, Candida oleophila, Candida parapsilosis, Candida zemplinina, Cryptococcus flavescens, Cryptococcus laurentii, Hanseniaspora uvarum, Hanseniaspora vineae, Issatchenkia occidentalis, Issatchenkia terricola, Kluyveromyces thermotolerans, Metschnikowia fructicola, Metschnikowia pulcherrima, Pichia anomala, Pichia fermentans, Pichia fluxum, Pichia guilliermondii, Pichia kluyveri, Pichia kudriavzevii, Pichia membranifaciens, S. cerevisiae, Torulaspora delbrueckii, Zygosaccharomyces bailii, Rhodotorula spp and Zygoascus spp* were found in samples from must, base wine and air of 24 vineyards belonging to 19 wineries among *Oltrepò Pavese* and *Franciacorta* areas.

Many ecological studies have been focused on studying the biodiversity of yeast population for both *Saccharomyces* and non-*Saccharomyces* yeasts. Although it is clear that the primary role on winemaking is exercised by *S. cerevisiae* species, it has been widely demonstrated that non-*Saccharomyces* yeasts contribute differently to the wine quality. The diversity and the composition of the yeast flora in winemaking environment, can vary with the grape variety, oenological practices, climatic conditions and geographic area. The primary origin of oenological yeast is the grape which is a natural source of yeast for wine production. In fact, microbiota present in grapes can produce a beneficial or detrimental action on quality of product.

In this study the vineyard air represented one of the sources for oenological yeast isolation. *Aureobasidium pullulans, Cryptococcus laurentii, Pichia fermentano, Issatchenkia terricola* and *S. cerevisiae* were isolated from this sampling typology. The frequency of isolation of these was variable from year to year and usually low. These results indicate that the presence of the yeast found in the air was not directly related to the winemaking processes that were taking place in the wineries. However the main result was the *S. cerevisiae* isolation from vineyard air of four *Oltrepò Pavese* wineries. This yeast specie was present during all the years, instead. One possible explanation could be given for an exchange of microorganisms between the fermentation tanks and the air but this is not supported by the result of fingerprinting because no clones were found among the isolated from the base wine and must. Isolation of *S. cerevisiae* from air was in accordance to the study of Garijo et al. (2008).

Regarding to the results from must sampling, the greatest yeasts biodiversity was verified with a total of 22 species isolated with a variable frequency along the three vintages, among which the highest percentage were obtained for *S. cerevisiae* (40%) and *Hanseniaspora uvarum* (19%) whose frequency was constant during the three vintages. *Torulaspora delbrueckii (*16%), *Zygosaccharomyces bailii* (15%) and *Metschnikowia fructicola* (15%) were isolated, too. The results of must sampling were almost unexpected in the case of *S. cerevisiae* because for both grapes and must, this yeast is not frequently found (Combina et al., 2005). In fact the largest percentage usually occurs for yeast isolates of the apiculate yeasts category. Different studies have demonstrated that non-*Saccharomyces* are the dominant species on the grapes and while crushing, and subsequently these yeasts can be found into the must. For example, Jolly et al.(2003) found four different yeast species, i.e. *Kloeckera apiculata, Candida stellata, Candida pulcherrima* and *Candida colliculosa* that predominated in grape must. Moreover, it has been found that freshly crushed grape juice harbors a diversity of yeast species, principally within the genera

Hanseniaspora (anamorph *Kloeckera*), *Pichia*, *Candida*, *Metschnikowia*, and *Kluyveromyces*. In terms of biodiversity and according to one of the objectives of this research, the high percentage of isolation of *S. cerevisiae* in must represented a very positive result.

From base wine a total of 9 different yeast species was found. As expected, the highest percentage of yeast isolates from base wine belonged to *S. cerevisiae* (76%) following by *Pichia membranifaciens* (9%) whose frequency as *S. cerevisiae*, was constant during the three vintages. In a minor percentage *Hanseniaspora uvarum, Candida railenensis, Zygosaccharomyces bailii, Pichia fermentans, Pichia fluxum, Issatchenkia occidentalis* and *Torulaspora delbrueckii* were also isolated. This result is in agreement with the already known ability of *S. cerevisiae* to dominate the fermentation process. Since wine fermentation is a complex ecological and biochemical process, this involves a sequential contribution of different yeast species (Fleet and Heard, 1993). *Hanseniaspora*, *Candida* and *Pichia* genera, grow during the early stages of fermentation and then die due to the high concentration of alcohol. So, they are replaced by alcohol-tolerant yeasts. Latter stages of fermentation are always controlled by *Saccharomyces* species (Blanco et al., 2006). In fact, *Saccharomyces cerevisiae* is the dominant yeast during the process and it carried out complete alcoholic fermentation (Guillamòn et al., 1998).

During the vintages, the presence of *S. cerevisiae* specie was remarkable for both its constant presence and the percentage of isolates. In this study a total of 189 isolated were identified from air, must and base wine samples of *Franciacorta* and *Oltrepò Pavese* areas. Identification at strain level of *Saccharomyces cerevisiae* is a fundamental step to investigate biodiversity of this yeast and to assess population dynamics during the fermentative process (Lopes et al., 2002; Granchi et al., 2003; Pulvirenti et al., 2001; Cappello et al., 2004; Lopandic et al., 2008). Typing was carried for isolates from 2009 and 2010 vintages and was performed using interdelta analysis (δ-PCR) according to Legras and Karst. (2003). Interdelta amplicons were separated by capillary electrophoresis according to Tristezza et al.(2009) and the percentage of genetic similarity was calculated by the coefficient of similarity according to Dice. (1945). The most important difference observed from capillary electrophoresis technique was its high power of discrimination. While on agarose gel analysis, an average of 10 bands for each interdelta profiles amplified was obtained, in capillary analysis an average of 80 fragments for each electroforetical profile were distinguished. This result supports the high percentage of intraspecific variability reported in dendrogram. In fact, isolates that could appear the same clone from agarose gel analysis actually are genetically different from capillary electrophoresis separation and a great genetic biodiversity was obtained among the patterns analyzed presenting a genetic similarity in a range of 0.2% to 95% by which the 98% of the isolates were *S. cerevisiae* strain-specific. This result was remarkable as well as the incredible biodiversity conservation among autochthonous *S. cerevisiae* strains which was reflected by the fact that there were no clones belonging to the commercial starters strains used in *Oltrepò Pavese* and *Franciacorta* areas to sparkling wine production. In fact, some commercial starters were deliberately included in the interdelta analysis and capillary electrophoresis results elaboration as a reference. Many ecological studies using identification molecular methods have been carried out to select new yeasts better adapted to a particular production area (Pretorius et al., 1999; Khan et al., 2000; van der Westhuizen et al., 2000). These and other studies (Versavaud et al., 1995; Lopes et al., 2002) report a great diversity of genetic patterns among the enological fermentative microbial communities. These investigations revealed the existence of an extensive polymorphism among yeast strains isolated in different areas and also within a specific area from one year to another. Nevertheless in some cases it found that some strains appeared in different areas and they have persisted for several years (Frezier and Dubourdieu 1992; Vezinhet et al. 1990; Sabate et al. 1998). From fingerprinting results, it was not possible to observe the persistence of any indigenous strain during two consecutive years. In addition, no genetic similarity relationship was observed among *Franciacorta* and *Oltrepò Pavese* strains. These results were partially in accordance to

Versavaud et al.(1995) who observed that over several consecutive years, predominant strains have been observed in the same vineyard; despite this, no correlation between strain biodiversity and geographical origin of the *S. cerevisiae* population has been observed.

As a result of this study, the biodiversity of yeast species involved in winemaking process in *Franciacorta* and *Oltrepò pavese* areas was established. This represents a significant result since it has been widely demonstrated that both *Saccharomyces* and Non-*Saccaromyces* species have an important effect on fermentation and quality wine. Since the exploitation of wine yeast biodiversity is a useful tool for the selection of new strains, the great genetic biodiversity found among *S. cerevisiae* isolates during two vintages allows targeting the selection of some representative strains from this areas to be used as starters as well as preservation and study of indigenous wine yeast populations.

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Chapter 3

Yeast population evolution during controlled and spontaneous fermentations

3.1 Background

All wines have been traditionally made by utilizing the natural microflora in spontaneous fermentations. The practice remained prevalent in *old world* wine producing areas until the 1970s. Many *boutique* wineries, depending on vintage variability, still utilize this process today. Spontaneous fermentations are held by the development of indigenous yeasts which inhabit in grapes and winery equipment (Beltran et al., 2002; Sabate et al., 2002; Sangorrin et al., 2002; Vaughan-Martini and Martini, 1995). In spontaneous fermentation there is an early and rapid succession of several yeast species which rarely belong to *Saccharomyces;* but later the increasing level of ethanol limits the growth and activity of almost all non-*Saccharomyces* yeasts (Fleet, 2008). In addition to *Saccharomyces cerevisiae*, other non-*Saccharomyces* species are also responsible for alcoholic fermentation and contribute to the sensory characteristics of the final product. These yeasts are major producers of secondary compounds (Lambrechts and Pretorius, 2000; Plata et al., 2003; Romano et al., 1997; Zohre and Erten, 2002). Many ecological studies show that yeast species with low fermentative activity such as *Hanseniaspora*, *Kloeckera*, *Pichia* and *Candida*, prevailing in the vineyard, begin the process but during the following stages, most ethanol-tolerant yeasts take over the operation and complete the must transformation (Fleet and Heard 1993; Versavaud et al., 1995). More often, the yeast species dominating the late stages of alcoholic fermentation is *Saccharomyces cerevisiae*, but in some cases *Saccharomyces uvarum* is also found at the end of fermentation, associated to *S. cerevisiae* or even alone (Naumov et al., 2000, 2002; Torriani et al., 1999). However, the number of species and their presence during fermentation depends on the production area (Amerine and Kunkee, 1968), the practical winemaking process (Cuinier, 1978) and the type of wine produced (Poulard, 1984). Although the spontaneous fermentation is historically used for the production of wine, the urgent need of large-scale wine production necessitated the use of selected pure yeast inoculated of known ability, to guarantee rapid, reliable, trouble-free fermentations which are essential for consistent wine flavour and predictable quality (Henschke, 1997). Therefore, *S. cerevisiae* strains have been selected for their physiological, biochemical and oenological properties and are used as starters in wine-making process (Le jeune, 2006). In fact, modern enological practices consist of a controlled fermentation through the inoculation of active dry selected yeasts (*ADY)* (Querol et al., 1992). These strains are able to conduct the fermentation with predictable and programmable results and their use reduces the risk of wine spoilage (Pretorius and Westhuizen, 1991). However, their addition will not necessarily prevent the growth of indigenous non-*Saccharomyces* yeasts which will also contribute to the overall fermentation (Fleet and Heard, 1993). Although inoculation is recommended, still there is some concern about missing some desirable characters due to spontaneous fermentations. Analysis obtained from spontaneous and inoculated fermentations has shown significant differences in the chemical composition and sensory properties of wine (Blanco et al., 2006; Egli et al., 1998; Vilanova and Siero, 2006). Earlier studies examined the evolution of native populations of *Saccharomyces* strains evolved during spontaneous wine fermentation (Querol et al., 1992; Querol and Ramon, 1994; Sabaté et al., 1998; Schütz and Gafner, 1993, 1994). Many different strains have been observed, but few of them were predominant in the later stages of the process.

Some had been isolated over several years in the same winery (Frezier and Dubourdieu, 1992; Sabaté et al., 1998) or were widespread in different wineries of the same wine-producing region (Versavaud et al., 1995).

Regarding to fermentation typology used by wineries in *Franciacorta* and *Oltrepò Pavese*, some of them still prefer to use only indigenous yeasts to produce wines with a more complex aromatic profile respect to those carried out with controlled fermentations using commercial yeasts. Other wimakers prefer to start the spontaneous fermentation and to provide inoculation with a colture starter at a later stage.

This study was conducted with two main aims. The first is to investigate the evolution of the yeast population in both, spontaneous and controlled fermentation; the second one is to enlarge the indigenous strain collection that was set up as described in chapter 1. For this, yeast isolates were sampled and molecularly characterized during spontaneous and controlled fermentations from three *Franciacorta* wineries during 2009 vintage.

3.2 Materials and Methods

3.2.1 Microbiological Sampling Methods

Base wine was sampled during spontaneous and controlled fermentation from three *Franciacorta* wineries denominated *VZ*, *V* and *BV* during 2009 vintage. The sampling was done as follows: 50 ml of wine were transferred in sterilized flasks every 48 h from the inoculum day (T=0), until the end of fermentation. The samples were conserved at 2°C until the time of the analysis. In table 1 the wineries and the timing of the monitored fermentation are listed. Appropriate dilutions of the wine samples were done in peptone water (Merck, Germany) and spread on WL agar (Merck, Germany). The plates were incubated during 3 days at 25°C. After incubation, the number of colony-forming units (CFU) was recorded. The morphological characteristics of each colony type were noted, counted and observed through an optical microscope. A corresponding number to square-root of the number of colonies of each type was selected. Colonies were streaked twice on WL agar. Purified isolates were stored at -80° C in YEPD broth [1% (w/v) yeast extract, 2%(w/v) peptone and 2% (w/v) glucose, 5.5 pH and added of 0.1 g/l of chloramphenicol] added with 20% (v/v) glycerol.

Franciacorta winery	Fermentation type	Times monitored
VZ	Controlled fermentation	T0, T1,T2,T3, T4,T5
	Spontaneous fermentation	T0, T1, T2, T3, T4, T5, T6
V	Controlled fermentation	T0, T1, T2, T3
	Spontaneous fermentation	T0, T1, T2, T3, T4, T5, T6, T7
BV	Controlled fermentation*	T0, T1, T2, T3, T4, T5, T6

Table 1.
Wineries, typologies of monitored fermentation and sampled total timing. T0-T1= 2 days; T2 =4 days; T5=10 days; T6= 12 days ; T7= 14 days
*In this winery only controlled fermentation was monitored.

3.2.2 Molecular Biology Methods

3.2.2.1 Genetic identification

Genetic identification of the isolates was carried out as described above in chapter 2. Amplifications were performed from genomic DNA (section 2.3.1) and analyses of *RFLP-ITS* and *D1/D2* of *26S rDNA* sequence (sections 2.3.2 and 2.3.3 respectively) were performed. Furthermore a PCR analysis was carried out using the primers constructed from de *HO* gene as described for De Melo Pereira et al. (2010), in order to distinguish the isolates identified as *S. cerevisiae* from others *S. cerevisiae* sensu strictu species, such as *S. bayanus* and *S. pastorianus* present in alcoholic fermentation,. The protocol was modified as follows: the single colony was used directly in the amplification reaction, after a previous treatment of cellular breaking at 95°C for 30 min; then a PCR reaction was carried out in a 25 µl reaction mixture containing 1X Buffer (5 Prime, Hamburg), 2.5 mM of $MgCl_2$ (5 Prime, Hamburg), 200 µM of dNTPs (Fermentas, Lithuania), 0.5 µM of each primer (5'-3'): *ScHO-F* GTTAGATCCCAGGCGTAGAACAG; *ScHO-R* GCGAGTACTGGACCAAATCTTATG and 1 U of Taq-DNA Polymerase (5 Prime, Hamburg). The temperature profiles were the same described for the authors (Melo Pereira et al.,2010) and a Biometra Thermocycler (Biometra, Germany) was used. Electrophoresis was run in 0.8% (w/v) agarose gel in 1X TAE buffer (40 Mm Tris–acetate, pH 8.2; 1 mM EDTA) stained with 0.4 µg/ml

ethidium bromide. PCR products were photographed under GelDoc UV transilluminator (Bio-rad, Hercules, CA, USA).

3.2.1.2 Genetic typing

For the identification at strain level, Interdelta analysis (δ-PCR) (section 2.3.4) was used. The electrophoretic profiles were compared through software (Gel compare II, Bionumerics Applied Maths, Belgium). Dendrograms were constructed by the un-weighted pair group method using arithmetic averages (UPGMA).

Repeatability of the technique

The repeatability was validated by multiple tests as follows. The genomic DNA from the same strain (S) was used as a template in four independent PCR amplifications (section 2.3.4). The obtained patterns were visualized in different electrophoretic runs and then clustered throughout the elaboration of the data in a unique dendrogram. The percentage at which these profiles were grouped, was the discrimination threshold among the 319 isolates. I.e., the isolates that were placed over this percentage of similaritywere considered clones of the same strain.

3.3 Results

3.3.1 Sampling of yeasts

Samples of base wine (first fermentation) during spontaneous and controlled fermentations were taken in three *Franciacorta* wineries denominated *VZ*, *V* and *BV* during 2009 vintage. The samples were taken every 48 hours, starting from the must inoculation (T=0) until the end of fermentation. Table 2 summarizes the total number of yeast isolates collected for each fermentation type in all the wineries.

Regarding the trend of cell concentration, the size of the populations at the beginning of controlled fermentation was about 10^7 CFU ml^{-1} for all wineries. At the end of fermentation, in the winery *VZ* one logarithmic unit was reduced to 10^6 CFU ml^{-1}; in the winery *V* to 10^2 CFU ml^{-1} (the major reduction observed) and in the winery *BV* to 10^5 CFU ml^{-1}. On the other hand, the size of the populations at the beginning of the spontaneous fermentation was about 10^6 CFU ml^{-1}. For this fermentation type in both *VZ* and *V* wineries, viable cells were decreased to 10^5 CFU ml^{-1} at the later stages of the fermentation (figure 1).

Franciacorta winery	Fermentation type	Code of yeast isolates	Isolates number
VZ	Controlled fermentation	VZI	65
	Spontaneous fermentation	VZN	81
V	Controlled fermentation	VI	32
	Spontaneous fermentation	VN	69
BV	Controlled fermentation*	BV	76
		Total number of collected isolates	323

Table 2.
Yeast isolates collected for each fermentation type in the wineries.

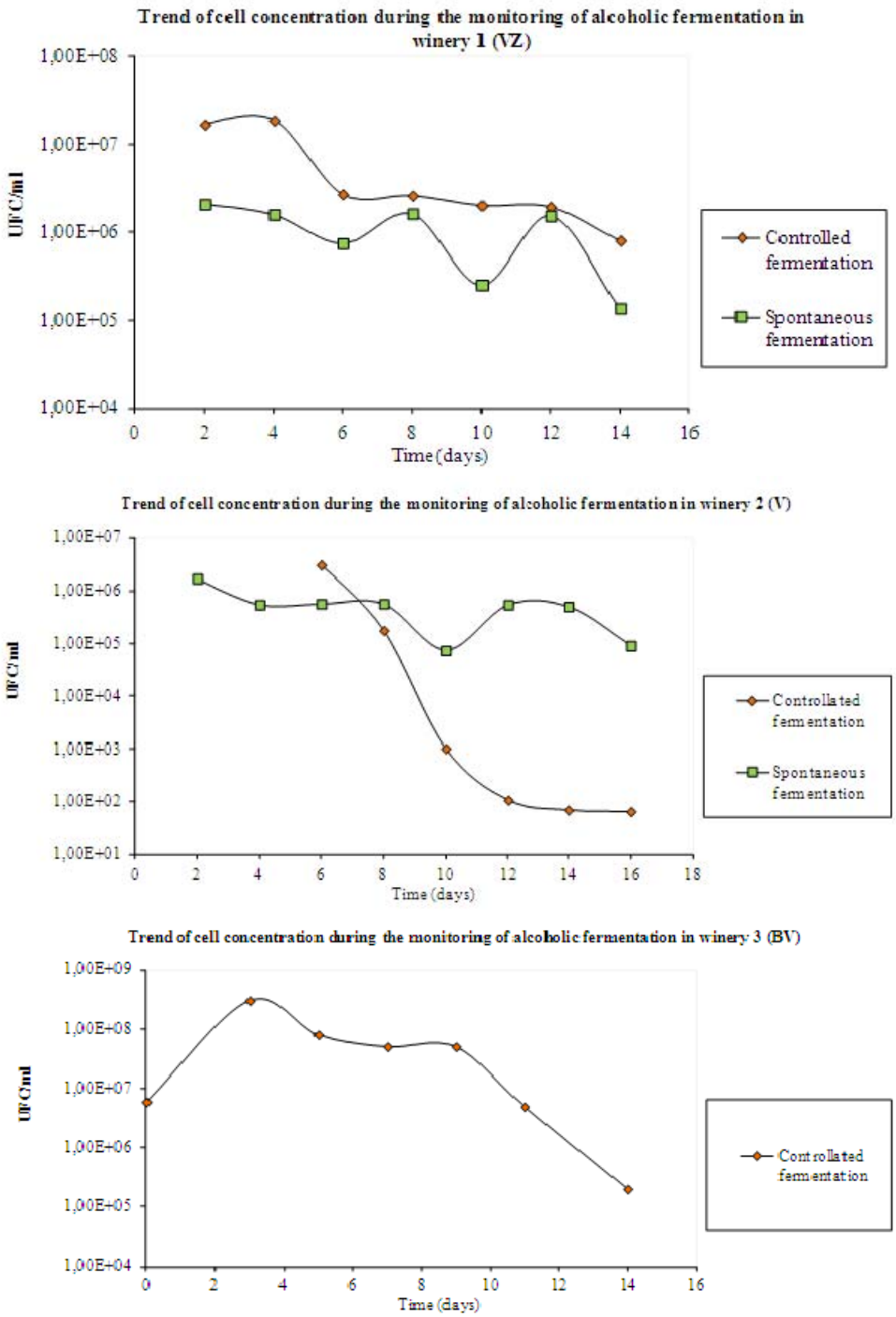

Figure 1.
Trend of yeast population during the monitoring of alcoholic fermentations.

3.3.2 Genetic identification

The identification of yeast populations is reported in Table 3. Through the monitoring 323 isolates were collected, of which ninety-eight percent (98%) were presumptively classified as *S. cerevisiae* according to the results of *RFLP-ITS* of *rDNA* analysis (data not shown). In *VZ* and *BV* wineries, *S. cerevisiae* was predominant (100%) for both types of fermentations. In the case of *V* winery, 3 isolates (*VIT3-2*, *VIT3-6*, *VIT3-8*) from controlled fermentation were belonging to the *Pichia membranifaciens* species (sequence identity 100%, coverage 100%) and 1 isolate (*VZNT0-2*) from spontaneous fermentation in winery *VZ* was ascribed to *Hanseniaspora vinae* species (sequence identity 100%, coverage 99%). Although is common to found *Pichia membranifaciens* in the early stages of winemaking, this result could be due to a late contamination because the sample was collected from a controlled fermentation at sixth day of the fermentation. In the winery *VZ*, the presence of *Hanseniaspora vinae* is a common outcome of the spontaneous fermentation in the early stages of the process, instead. When the fermentation reaches higher concentrations of alcohol, this species are substituted by more alcohol-tolerant strains of genus *Saccharomyces*. Actually *S. cerevisiae* was the predominant yeast with ninety-eight percent (98%) for controlled fermentations and ninety-nine percent (99%) for spontaneous fermentation.

Winery	Type fermentation	Isolates (n)	Identified yeast
VZ	Controlled fermentation	65	*Saccharomyces cerevisiae 100%*
	Spontaneous fermentation	81	*Saccharomyces cerevisiae 99%* *Hanseniaspora vinae 1%*
V	Controlled fermentation	32	*Saccharomyces cerevisiae 91%* *Pichia membranifaciens 9%*
	Spontaneous fermentation	69	*Saccharomyces cerevisiae 100%*
BVT	Controlled fermentation	76	*Saccharomyces cerevisiae 100%*

Table 3.
Genetic identification of the yeast isolates.

Furthermore, since in winemaking process many yeast *Saccharomyces* species can interact, De Melo Pereira et al. (2010) has proposed a PCR protocol that is able to unambiguously discriminate *S. bayanus*, *S. cerevisiae* and *S. pastorianus* species by a simple and rapid DNA amplification. A PCR was realized to test this method and to clearly identify those isolates presumptively classified as *S. cerevisiae* from *RFLP-ITS*.. Figure 2 showed an amplification example for some isolates of *HO S. cerevisiae* genes. From this investigation, 319 isolates produced an amplicon of 400 bp characteristic for *S. cerevisiae*, according to the result from *RFLP-ITS* analysis.

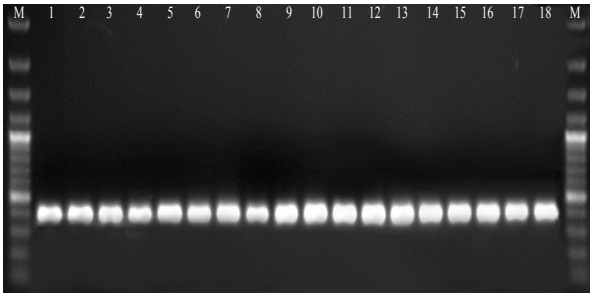

Figure 2.
Agarose gel 0,8% of the PCR amplification using *ScHO-F / ScHO-R* specific primers of the *HO* genes of *Saccharomyces cerevisiae.* **M:** DNA Molecular Weight (GeneRuler 100 bp DNA Ladder marker Plus Fermentas, Italy) **1:** *Saccharomyces cerevisiae* (*(Institut Oenologique de Champagne; Collection de Levures d'Intérêt Biotechnologique IOC 18-2007,* ***2-18:*** *S. cerevisiae* isolates

3.3.3 Genetic typing

Regarding the strain typing, the Interdelta PCR profiles elaboration involved 319 isolates of *S. cerevisiae* collected during the monitoring of controlled and spontaneous fermentation in three *Franciacorta* wineries. The aim of this finger-printing analysis was to detect the presence of different strains and to check their diversity from the starters in both spontaneous and inoculated fermentations. Actually, during the spontaneous process the commercial starters may be present in the winery environment, whereas, in the controlled one, it's in order to verify the starter dominance during the fermentation. Interdelta PCR profiles were compared each other through a software (Gel compare II, Bionumerics Applied Maths, Belgium). Dendrograms were constructed by the un-weighted pair group method using arithmetic averages (UPGMA) and a repeatability of 88% was detected in the experimental conditions (figure 3).

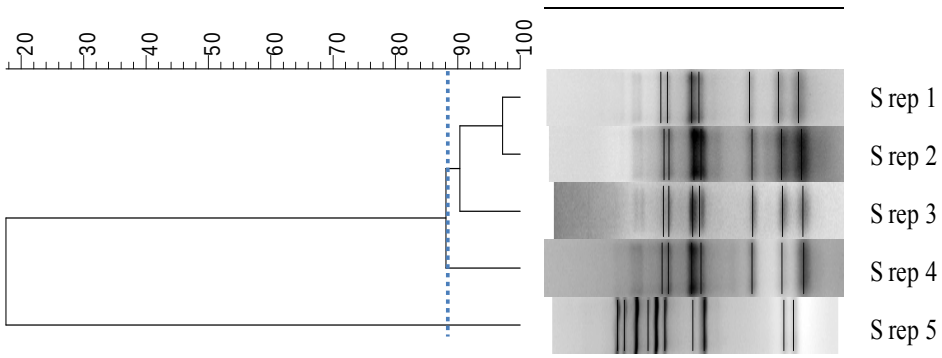

Figure 3.
Reproducibility of the interdelta PCR technique: UPGMA dendrogram from the same strain (S) patterns amplified in four independent PCR reactions and visualized in different electrophoretic runs. These were grouped at a level of similarity = 88%.. This percentage was the discrimination threshold among the 319 isolates. The amplified DNA profiles that were placed over this level were considered clones of the same strain.

3.3.3.1 Controlled fermentations

Controlled fermentation winery VZ

In this winery, it was possible to collect 65 isolates, all of them belonging to *S. cerevisiae* species. From the typing analysis (figure 4) 19 isolates were considered clones of the same strain since these were grouped in 9 different electrophoretic patterns at 88% of the discrimination threshold.

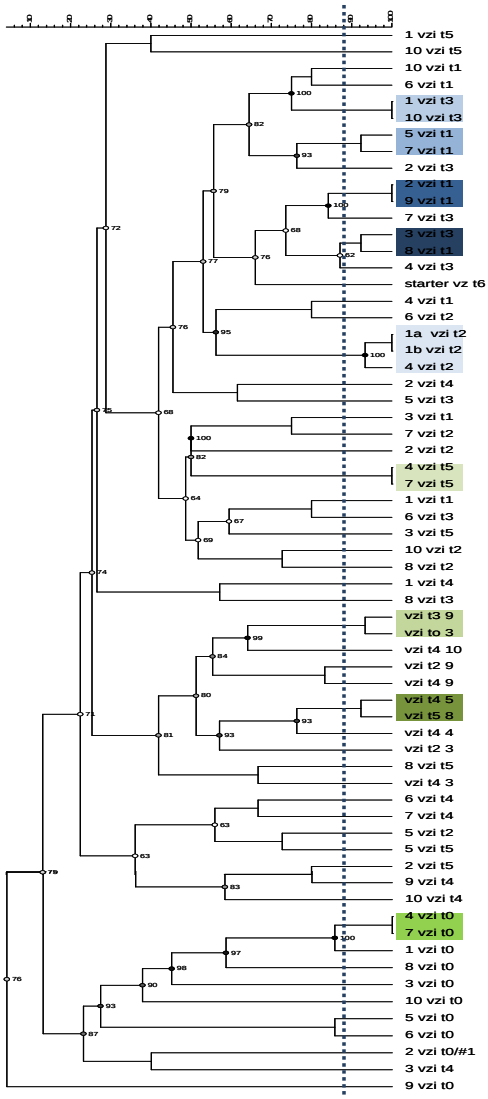

Figure 4.
UPGMA dendrogram of Interdelta PCR patterns from yeast isolates during the monitoring of controlled alcoholic fermentation in winery *VZ.*

It is possible to observe (figures 4 and 5) that the starter used in this winery did not show a high genetic similarity with the other isolates collected during the fermentation process. The isolates at times t1 and t3 are the most similar to it with a percentage of 65%. From the results obtained with this finger-printing analysis, 53 different genetic patterns were detected and a dominant strain within the process did not appear.

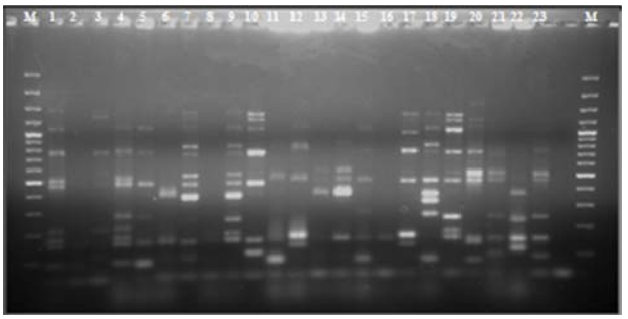

Figure 5.
Agarose gel 2.0% of the PCR amplification from Interdelta PCR analysis. Yeasts isolated from controlled fermentation in winery *VZ* . **M:** DNA Molecular Weight (GeneRuler 100 bp DNA Ladder marker Plus Fermentas, Italy), **1:** Starter strain, **2-23:** yeast isolates at the end of controlled fermentation.

Controlled fermentation winery V

32 isolates were collected during monitoring of controlled fermentation in winery *V*. 29 of them belonged to *S.cerevisiae* species. From dendrogram (figure 6), 27 isolates were considered clones of the same strain since these were grouped in 4 different electrophoretic patterns at 88% of the discrimination threshold. Regarding the starter strain, it presented a lower genetic similarity with the others isolates collected during the fermentation process (46%). From the results of the molecular typing, 8 different genetic patterns were detected among all yeast isolates.

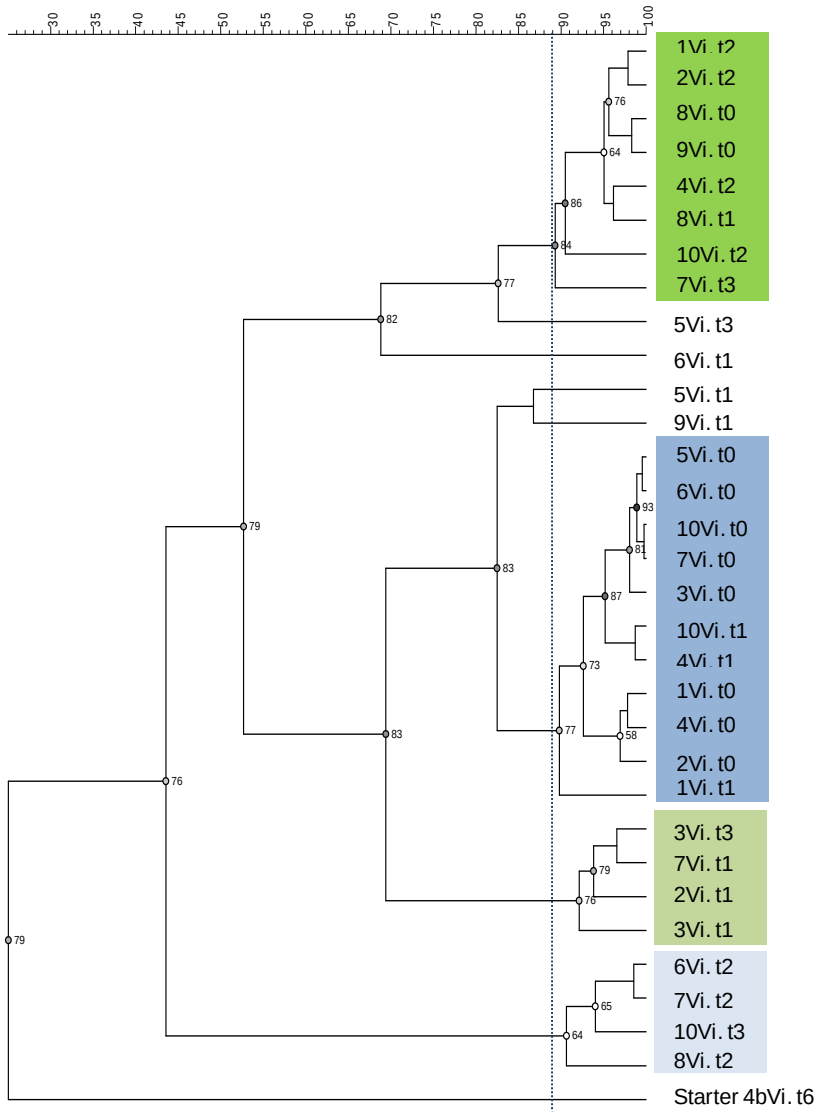

Figure 6.
UPGMA dendrogram of Interdelta PCR patterns from yeast isolates during the monitoring of controlled alcoholic fermentation in winery *V.*

Controlled fermentation winery BV

In this winery 76 isolates were collected, all of them belonging to *S.cerevisiae* species. From the results of the Interdelta PCR analysis (figure 7), 54 isolates were considered clones of the same strain since these were grouped in 2 different electrophoretic patterns at 88% of the discrimination threshold. 14 different genetic patterns among all yeast isolates were detected.

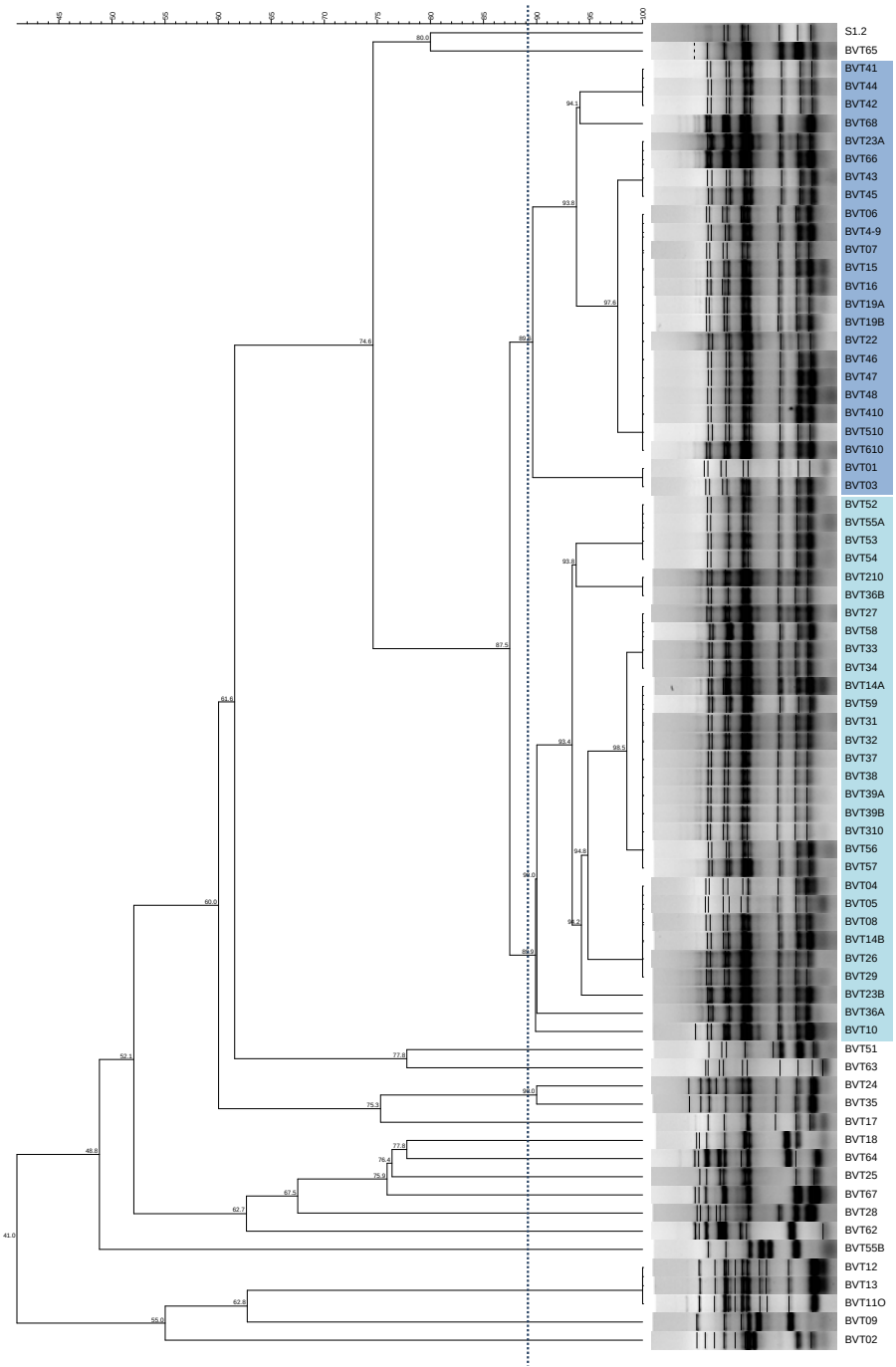

Figure 7.
UPGMA dendrogram of Interdelta PCR patterns from yeast isolates during the monitoring of controlled alcoholic fermentation in winery *BV*

Compared to the wineries *VZ* and *V*, the starter used in this winery was genetically closer (80%) to the other isolates. In fact in figure 8 it is possible to see how this strain is dominant in almost all the intervals of the fermentation. In particular, the strain dominance was observed at *t2* and *t5*. In this interval, the starter represented 91% of the population but in a later sampling at *t6* an incidence of 50% was observed. These data could indicate that *S. cerevisiae* strains different to the starter, could be present during the fermentation process.

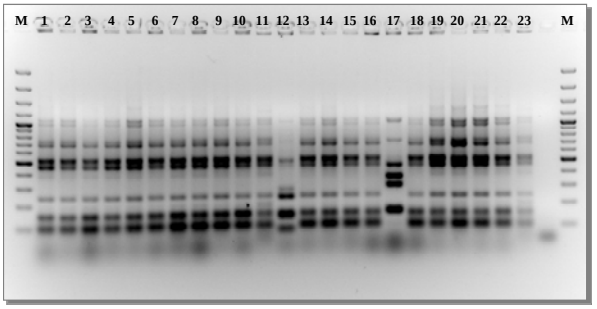

Figure 8.
Agarose gel 2,0% of the PCR amplification from Interdelta PCR analysis of *Saccharomyces cerevisiae.* Yeast isolated from controlled fermentation in winery *BV* . **M:** DNA Molecular Weight (GeneRuler 100 bp DNA Ladder marker Plus Fermentas, Italy), **1-10:** isolates at 193 hours of fermentation, **11:** Starter strain.**12-22:** isolates at 10 days of fermentation**, 22:** Starter strain.

3.3.3.2 Spontaneous fermentations

Spontaneous fermentation winery VZ

The monitoring of spontaeneous fermentation in winery *VZ* involved 81 yeast isolates, of which one isolate (*VZNT0-2*) belonged to *Hanseniaspora vinae* species. From the typing analysis (figure 9), 26 isolates were considered clones of the same strain since these were grouped in 12 different electrophoretic patterns at 88% of the discrimination threshold. A total of 67 different Interdelta PCR patterns among all yeast isolates were detected.

Spontaneous fermentation winery V

In this winery 69 isolates were collected, all of them belonging to *S. cerevisiae* species. From the results obtained with the elaboration of interdelta PCR patterns (figure 10), 59 isolates were considered clones of the same strain since these were grouped in 15 different electrophoretic patterns at 88% of the discrimination threshold. A total of 22 different genetic profiles were discriminated.

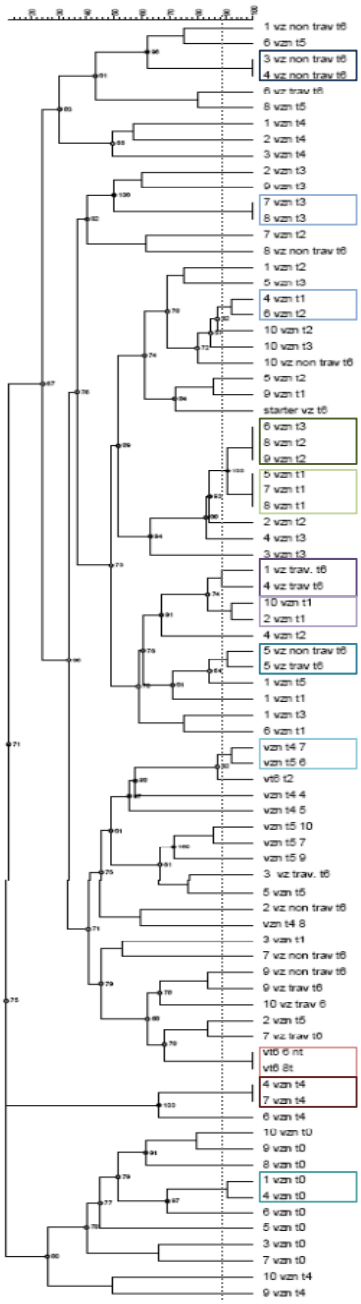

Figure 9.
UPGMA dendrogram of Interdelta PCR patterns from yeast isolates during the monitoring of spontaneous alcoholic fermentation in winery *VZ*

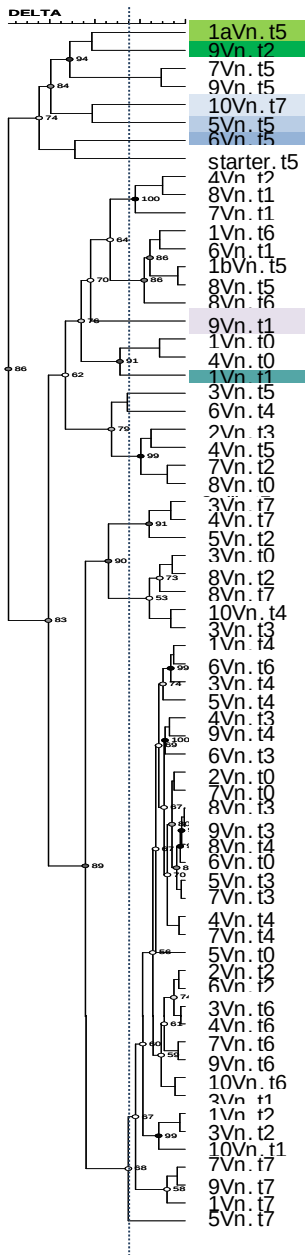

Figure 10.
UPGMA dendrogram of Interdelta PCR patterns from yeast isolates during the monitoring of spontaneous alcoholic fermentation in winery *V*.

Tables 4 and 5 show a comparison between the strains collected in both controlled and spontaneous fermentation from winery *VZ* and Winery *V* respectively. Since these two wineries are relatively far away from each other and the geographical area is different enough to compare the yeast distribution in *Franciacorta* territory, another genetic similarity evaluation was carried out. The results are showed in table 6.

Strains from controlled fermentation	Sampling time	Strains from spontaneous fermentation	Sampling time	Genetic similarity (%)
10 vzi	T4	4 vzn	T4	100%
8 vzi	T0	10 vzn	T0	100%
6 vzi	T0	7 vzn	T0	100%
7 vzi	T1	5 vzn,7 vzn, 8 vzn	T1	100%
2 vzi	T3	2 vzn	T2	100%
4 vzi	T3	10 vzn	T3	100%
5 vzi	T4	6 vzn	T5	100%
9 vzi	T4	7 vzn	T5	100%
3 vzi	T0	8vznt, 6 vzn nt	T6	100%

Table 4.
Strains comparison between the isolates from both controlled fermentation and spontaneous fermentation from winery *VZ*.

Strains from spontaneous fermentation	Sampling time	Strains from controlled fermentation	Sampling time	Genetic similarity (%)
5vn	T0	9vi, 8vi	T0	98%
10vn	T1	8vi, 4vi	T1,T2	97%
8vn, 9vn, 5vn, 7vn, 5vn, 6vn	T3, T0	7vi	T3	96%
2vn	T0	10vi	T2	98%
1vn, 9vn	T6	4vi, 10vi	T1	98%
6vn	T1	9vi	T1	99%
8vn	T1	2vi	T1	96%

Table 5.
Strains comparison between the isolates from both controlled fermentation and spontaneous fermentation from winery *V*.

Winery *V*	Sampling time	Winery *VZ*	Sampling time	Genetic similarity (%)
9vi	T7	2vzi, 9vzi, 8vzi	T1	95%
3vn	T1	7vzi	T3	96%
10vn	T1	3vzi	T3	98%
1bvi, 8vi	T5	1vzn	T3	96%
6vi, 7vi, 10vi	T2-T3	1vzi	T4	97%
2vn	T3	8vzi	T3	97%
4vn	T2	1vzi	T3	98%
3vi	T3	3vzn, 4vzn, 10vzi	T4	98%
9vn	T1	9vzi	T4	97%

Table 6.
Comparison between the strains from both: controlled fermentation and spontaneous fermentation from wineries *V*. and *VZ*.

Through the *S. cerevisiae* genetic typing, 164 electrophoretic profiles were identified. This genetic heterogeneity was found in the wineries *VZ* and *V* where, out of the 243 isolates, 150 different electrophoretic profiles among controlled and spontaneous fermentation were observed (table 7).

Winery	*S. cerevisiae* interdelta profiles analized (n)	Different genetic patterns found (n)	Patterns of genetic variation (%)
Controlled fermentations			
VZ	65	53	82%
V	29	8	28%
BVT	76	14	18%
Spontaneous fermentations			
VZ	80	67	84%
V	69	22	32%

Table 7.
Summary of *S. cerevisiae* strain typing during monitoring of alcoholic fermentations in three *Franciacorta* wineries.

3.4 Discussion and Conclusion

The identity of the yeast population was studied during spontaneous and controlled alcoholic fermentation in three *Franciacorta* wineries during 2009 vintage. Through the monitoring, 323 isolates were collected, of which ninety-eight percent were presumptively classified as *Saccharomyces cerevisiae* by the analysis of RFLP-ITS of rDNA region. Only four isolates were belonging to other species identified as *Pichia membranifaciens* (winery *V*; controlled fermentation) and one isolate identified as *Hanseniaspora vinae* (winery *VZ;* spontaneous fermentation*).* In fact, at the beginning of the process it is common to found apiculate yeasts species (Valero et al., 2007). Nevertheless the finding of *Pichia membranifaciens* could be considered a contamination because the sample was collected from a controlled fermentation in a later stage of fermentation. The genetic diversity of the isolates identified as *S cerevisiae* was studied through the interdelta PCR analysis. A total of 319 genetic profiles were obtained and analyzed. From the elaboration of the results, 164 different strains among controlled and spontaneous alcoholic fermentation were distinguished (corresponding to 51% of the population investigated). The highest *S. cerevisiae* biodiversity was found in winery *VZ* from controlled fermentation with 53 different genetic patterns corresponding to 82% of the yeast population. In winery *V* was found a less biodiversity (28%), even if it remains a high percentage case for a controlled fermentation. In these wineries was carried out a comparison between the strains from both controlled fermentation and spontaneous fermentation. As result, genetic profiles were almost identical (ranged from 95% and 98%, table 6). Though great care is taken during the winemaking process, the high level of biodiversity found in controlled fermentations represents an index of "contamination", which shows how yeasts are transferred easily from one vat to another under working conditions. Anyway in these wineries, the same strains were not found to lead the fermentation, but it was observed that some of them were dominant in an initial period of the process and replaced by other ones that carried out the fermentation until the end. A similar result was obtained by Mercato et al. (2007) who observed that some strains, different to the starter, conducted the inoculated fermentation. Moreover, Frezier and Dubourdieu. (1992), Schutz and Gafner (1993) and Egli et al. (1998) realized genetic studies of identification that allowed them to observe the succession of *Saccharomyces* yeasts strains in the inoculated and non-inoculated fermentations. On the other hand, even if in the winery *BV* the operation was made only by a controlled fermentation, the 18% of the population was constituted by indigenous strains; in this case the starter led the fermentation until the end, instead. This result is in agreement with those of Esteve-Zarzoso et al. (2001); it is generally assumed that indigenous yeasts are suppressed by the starter, however studies show that indigenous yeasts can still participate in the fermentation, or that only 50% of implantation starter is achieved when fermentation is performed with some commercial strains. This was similar to the results of Egli et al. (1998) who observed a decrease in the number of indigenous strains during controlled fermentations. However, this study confirms that inoculated *Saccharomyces* starter cultures do not always completely dominate the fermentative process. In fact starter cultures can take over to different degrees, allowing a different number of indigenous *Saccharomyces* strains to grow as well. It is therefore speculated that nutritional and chemical competition plays an important role in inhibiting some strains and allowing for growth of others.

In this study Interdelta PCR analysis, which sets the amplification of DNA fragments between two delta elements, was used as finger-printing technique. Le Jeune et al. (2006) also used this protocol to determine the evolution of the population of *S. cerevisiae* from grape to wine in a spontaneous fermentation. A library of 1600 clones was analyzed with a basic set of primers and about 20% of the library was further analyzed with an improved set of primers (Legras and Karst, 2003). Delta

elements are direct repeated elements that flank the *Ty1* retrotrasposon which are dispersed on *Saccharomyces* nuclear genome at an amplifiable distance; their number and position in the genome is strain-specific and stable in about 50 generations (Mercado et al., 2007). Each *Ty1* element is about 6 kb in length, including long terminal repeats or delta sequences of about 340 bp. However, these repetitive sequences in the yeast are thought to be a major source of genome instability.

During the fermentations, genetic changes occur spontaneously but certain environmental factors, such ethanol and acethaldehyde, can induce this modification (Sipiczki, 2011). In fact, due to the propensity for their genomic alteration, the wine yeast strains (*S. cereveisiae*) are very diverse. Then, yeast biodiversity that was found during this study in inoculated fermentations mainly, should be treated cautiously. One possible hypothesis to explain the diversity observed in *S. cerevisiae* strains in the present work is the increased speed of retrotrasposition of *Ty1* as result to the possible mutagenic effects of the various chemical compounds produced during alcoholic fermentation.

This study represents a first approach to population dynamics of oenological yeasts in an important viticulture region that was never characterized before. The obtained results have an important significance for the local industry, showing for the first time the microbial ecology of alcoholic fermentation in a vine growing area such as the *Franciacorta* (BS), Italy. However, further studies are necessary to improve knowledge about the behavior of indigenous yeasts considering the ability of strains to remain from year to year and to participate in the spontaneous fermentation. Nowadays, it is widely known that the quality of wine is a direct consequence of the evolution of the yeast population during fermentation. Furthermore, it is also known that the succession of *S. cerevisiae* strains during the process can contribute to the final sensory properties and wine quality could be affected by the yeast strain leading the fermentation.

3.5 References

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Chapter 4

Selection and evaluation of indigenous *Saccharomyces cerevisiae* strains for Sparkling wine production

4.1 Background

In the last twenty years the research in oenological field has been aimed towards the isolation of autochthonous strains to select starter cultures that are potentially better adapted to a particular vine growing area or wine production (Martini and Vaughan-Martini., 1990; Khan et al., 2000; Lopes et al., 2002; Pretorius et al., 1999; Regodon et al., 1997; Sabate et al., 1998; Torija et al., 2001; van der Westhuizen et al., 2000; Versavaud et al., 1995). They are intended to provide chemical properties and organoleptic profiles characteristic of each region.
A large number of studies have been focused on isolation of indigenous yeasts, reporting a great diversity of genetic patterns among the oenological fermentative microbial communities. *Saccharomyces cerevisiae* strains seem to be widely distributed in a determinate oenological region, and they can be found in consecutive years (Vezinhet et al., 1992; Torija et al., 2001).
In order to select microorganisms, the required technological characteristics may vary depending on the musts and on the used winemaking techniques (Giudici and Zambonelli, 1992). However, some of most important oenological criteria are: tolerance and high ethanol production, exhaustion of potential sugar and high fermentation activity, good glycerol production, low foam production, resistance and low sulphur dioxide (SO_2) production, low hydrogen sulfide (H_2S) production, and low volatile acidity production (Perez-Coello et al., 1999; Esteve-Zarzoso et al., 2000). In fact, after a molecular characterization, the first step to select news starters is based on the evaluation of these features at laboratory level, where microvinification tests are carried out. A following estimation at winery level on a smaller scale is necessary to determinate the "implantation" capability of the indigenous strains, i.e. the ability to dominate during the fermentation process and to determine the expression of sensorial characteristicsin wines (Capece et al., 2010; Guerra et al., 1999; Lopes et al., 2002).
Franciacorta and *Oltrepò pavese* are leaders in the sparkling wine DOCG production by *champenoise* method in Italy. However, the starters used till now are not representative of yeast communities present in these areas, since they have been isolated in other countries. This could reduce the wine "typicality" since, as mentioned above, the organoleptic and chemical features of the final product are the result of the action of inoculated yeast. Although the interest for the use of indigenous strains is great, the potential use of them for sparkling wine production in these wine-producing areas has not been investigated. As described in chapter 2, molecular characterization of indigenous *S. cerevisiae* and other yeasts of oenological interest isolated from *Franciacorta* and *Oltrepò Pavese* areas was carried out in order to study their genetic biodiversity and to create a collection to preserve it. The present study was focused on exploring the potential use as new starters of some *S. cerevisiae* strains for sparkling wine production (*champenoise* method). Technological and qualitative characterizations were performed through microfermentation tests in laboratory with *tirage* proves at pilot scale in different *Franciacorta* and *Oltrepò pavese* wineries.

4.2 Materials and Methods

4.2.1 Identification and typing of yeasts

As described in chapter 2 (section 2.2.1) samples of vineyard air, must and base wine were collected in different *Franciacorta* and *Oltrepò* territories during the vintages of 2009, 2010 and 2011 in *Franciacorta* (province of *Brescia*) and *Oltrepò Pavese* (province of *Pavia*) in Lombardy, Italy. Furthermore, as described in chapter 3 (section 3.2.1), base wine was sampled during spontaneous and controlled alcoholic fermentation from three *Franciacorta* wineries during 2009 vintage. Through these experimentation phases, it was possible to set up a large yeast collection which was molecularly characterized. From this collection sixteen indigenous *S. cerevisiae* strains were selected and their technological and qualitative characterizations were performed.

4.2.2 Technological and qualitative characterization of *S. cerevisiae* strains

4.2.2.1 Strains and media

S. cerevisiae strains used in this study are listed in table 1. The indigenous strains were selected for on the basis of typing and geographical origin.

Strain	Origin
VC2-1	Bw- winery 6 - Fcr
V43-1	Bw- winery 7- Fcr
V2-1	Bw- winery 4- Fcr
MIR-12	Maf- winery 3- Fcr
BVT0-9	Maf- winery 3- Fcr
BVT1-3	Maf- winery 3- Fcr
BVT1-8	Maf- winery 3- Fcr
BVT6-5	Maf- winery 3- Fcr
ANT-VB2	Bw- winery A- Olt
CSO-VB2	Bw- winery B- Olt
CSO-VB6	Bw- winery B- Olt
QUA-VB1	Bw- winery F- Olt
QUA-VB7	Bw- winery F- Olt
TRV-VB1	Bw- winery I- Olt
VRD-VB1	Bw- winery L- Olt
VST-VB3	Bw- winery M- Olt
S1	IOC[1] (strain control)
S2	Lalvin[2] (strain control)

Table 1.
S. cerevisiae used in microfermentation test. Bw = isolated from base wine, Fcr = *Franciacorta* vintage 2009, Olt = *Oltrepò Pavese* vintage 2009, Maf = isolated during monitoring of alcoholic fermentation

[1] *Institut Oenologique De Champagne- Collection de Levures d'Intérêt Biotechnologique.*
http://www.institut-oenologique.com/documents/ft/FT%20LEVURE%20IOC%2018-2007%20(EN).pdf
[2] http://www.lalvinyeast.com/images/library/EC1118_Yeast.pdf

Two different media were used in fermentations at laboratory-scale. YEPD medium [1% (w/v) yeast extract, 2% (w/v) peptone and pH 5.5] was modified adding 25% (w/v) of glucose and used as "synthetic must" and sterile *Pignoletto* white must .[3]

4.2.2.2 Microfermentation test

Inoculated fermentation assay was performed in 250-ml-Erlenmeyer flasks containing 100 ml of "synthetic must"/ sterile white must. Each yeast culture was inoculated at a concentration of 10^6 cell/ml from a pre-culture grown for 48 h in the same type of must. The Erlenmeyer flask was sealed with special valves to maintain the sterility and to facilitate the gas exchange. The fermentation was performed at 18°C.

4.2.2.3 Determination of technological parameters

To determine the fermentative power and fermentation vigour of the strains, the weight loss from Erlenmeyer flask was measured every day until obtaining a constant weight for two consecutive days (end of fermentation). Fermentation vigour was expressed as grams of CO_2 produced in the first 48 hours following the inoculation of the must and fermentative power was expressed as % v/v of ethanol produced using the following formula:

$$\{ [(\Delta\text{weight (g)} / 44 \text{ (g/mol}_{CO2}) \times 46{,}7 \text{ (g/mol}_{EtOH})] / 0.789 \text{ (g/ml}_{EtOH}) \}$$

4.2.2.4 Determination of qualitative parameters

Glycerol and Acetic Acid production

At the end of fermentation, aliquots of wine samples were centrifuged (Hettich Zentrifugen, Rotina 380r, Germany) at 4500 *g* and the supernatants were analyzed. For each sample, glycerol and acetic acid were determined in triplicate by using specific enzymatic kits (kits 148270 and 148261, respectively; Boehringer-Mannheim, Germany). Purity of fermentation was expressed as g of acetic acid/100 ml of ethanol.

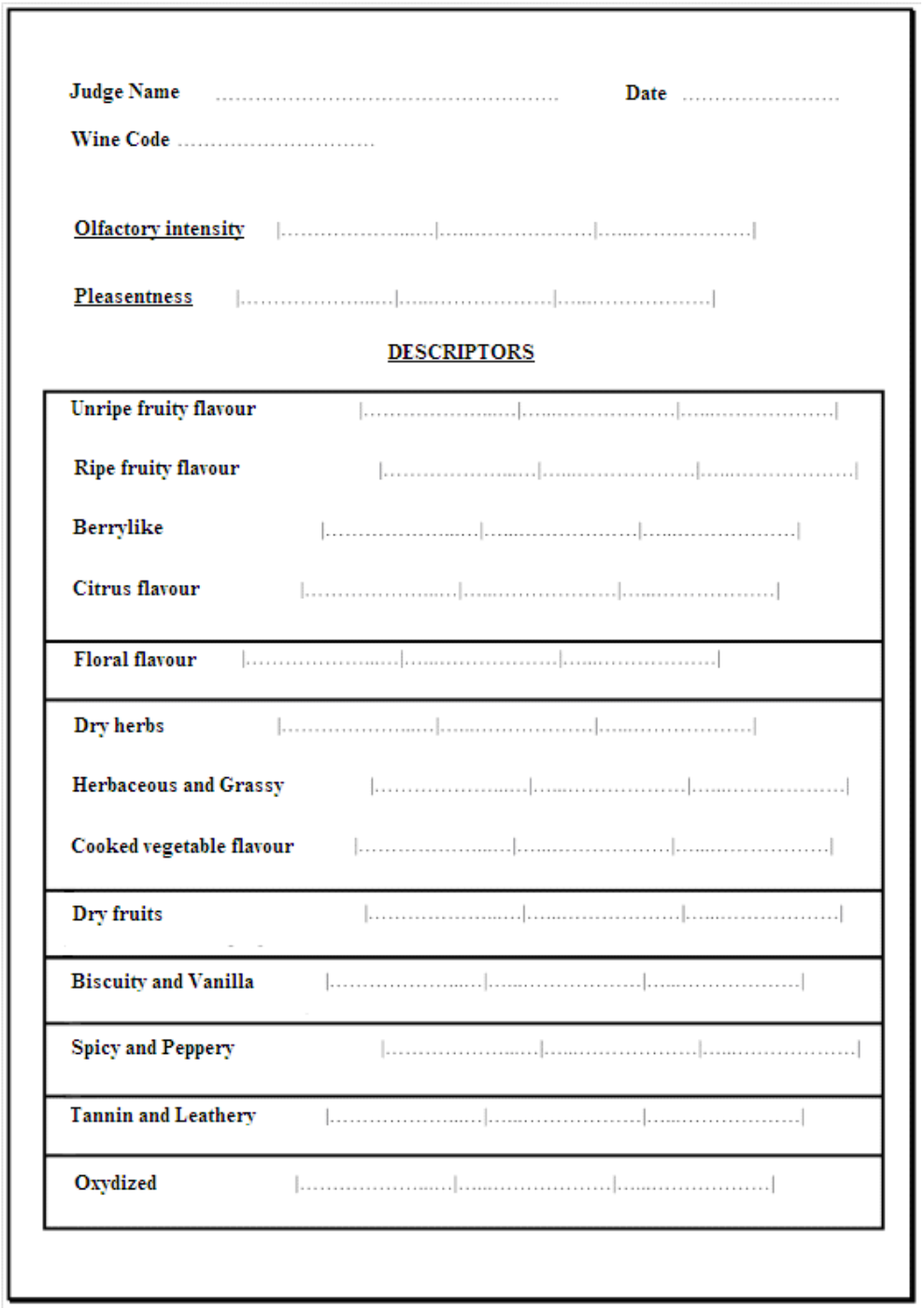
H_2S production

In order to evaluate the H2S production, all strains were plated on Bismuth Sulfite Glucose Glycine Yeast agar-BiGGY (BD, France). In this medium the color of the colonies is determined by the reduction of bismuth sulfite to bismuth sulfide that forms brown to black colonies. According to Redzepovic et al.(2002) the degree of browning associated with yeast growth on the BiGGY agar plates was scored using the following scale: 1=white, 2=cream, 3=light brown, 4= brown, 5 =dark-brown, 6 = black.

4.2.3 Sniffing test

Sniffing tests of wines from proves of microvinification were realized with a panel of tasters using the form reported in the figure 1. Only the intensity and pleasantness (scores from 0 to 10) were considered for statistical analysis. A multifactor ANOVA (Statgraphics Centurion Plus 5.1, United States) was realized to determine which factors (samples of wine and/or Judges) had a significant effect on value.

[3] Indigenous grape variety from Emilia-Romagna region.



Judge Name ..... Date .....

Wine Code .....

Olfactory intensity |.....|.....|.....|

Pleasentness |.....|.....|.....|

DESCRIPTORS

Unripe fruity flavour	
Ripe fruity flavour	
Berrylike	
Citrus flavour	
Floral flavour	
Dry herbs	
Herbaceous and Grassy	
Cooked vegetable flavour	
Dry fruits	
Biscuity and Vanilla	
Spicy and Peppery	
Tannin and Leathery	
Oxydized	

Figure 1.
Form used for wine evaluation during sniffing test.

4.2.4 Inoculated fermentations at pilot scale

On the basis of the results of sniffing test and the technological and qualitative characters, four different indigenous *S. cerevisiae* strains were chosen for each production area to be used as starters in *tirage* proves during vintages of 2010 (strain 1 and strain 2) and 2011 (strain X and strain Y). The wineries participating in the tests and the commercial starters used for sparkling wine production are listed in table 2.

Winery	Starter used	Location	Area
W1	SC1	*Rodengo Saiano (BS)*	*Franciacorta*
W2	SC3	*Erbusco (BS)*	*Franciacorta*
W3	SC1	*Erbusco (BS)*	*Franciacorta*
W4	"not determined[4]"	*Erbusco (BS)*	*Franciacorta*
W5	SC1	*Erbusco (BS)*	*Franciacorta*
W6	SC1	*Rocca de' Giorgi (PV)*	*Oltrepò Pavese*
W7	SC1	*Pietra de' Giorgi (PV)*	*Oltrepò Pavese*
W8	SC2	*Santa Giuletta (PV)*	*Oltrepò Pavese*

Table 2.
Wineries producing sparkling wine included in the study. *BS= Brescia,* Lombardy, *PV= Pavia,* Lombardy.

4.2.4.1 Assembly of the test in laboratory conditions

Cell biomass production

A culture of each indigenous strain was grown overnight in 20 ml of YEPD medium with shaking. Then, cellular concentration was determined measuring the optical density (OD) at A_{660nm} and 2 ml of this culture were transferred in Erlenmeyer flasks containing 200 ml of YEPD. These were incubated with shaking during 48h at 25°C. After incubation, a second measure of OD was carried out and a volume containing 5.10^9 cells was centrifuged (Hettich zentrifugen, rotina 380r, Germany) at 3500 *g* for 10 min. The pellet was suspended in 25 ml of YEPD. This procedure was carried out also for biomass production of the commercial starter used in each winery for sparkling wine production, since those starters were used as control strain. In *tirage* test 2010 the starter strain was denominated "strain 3" and in *tirage* test 2011, starter strain was denominated "strain S".

Preparation of the kits for pied de cuvée *protocol*

For wineries, three kits with the necessary components for *pied de cuvée* were prepared, each one containing:

- 25 ml of concentrated cell culture of the strain (1;2;3/X;Y;S)
- 7 grams of *probios*[5] (Bioenologia s.r.l., Italy) of which 1g, 2g and 4g were distributed in 3 test tubes respectively,
- 3 ml of *Adjuvant 83*[6](*Station Oenotechnique de Champagne*, France)

The kits were transported in the wineries at 4°C and maintained at this temperature to be used not later than 5 days after the preparation.

[4] Winery 4 not discloses the starter strain used, in accordance with their privacy policies
[5] Probiotic food for yeast activation
[6] Bentonite for clarifying of wine in bottle after the secondary fermentation

4.2.4.2 Assembly of *tirage* test in winery conditions

In order to produce cell biomass and to activate the fermentation, a protocol for *pied de cuvée* production was carried out for each strain. The necessary quantity of sugar to produce 6 atm (atmosphere) of pressure was added to 50 L of base wine. This was filtered after pouring the sugar and an aliquot was taken to be used during protocol (figure 1).

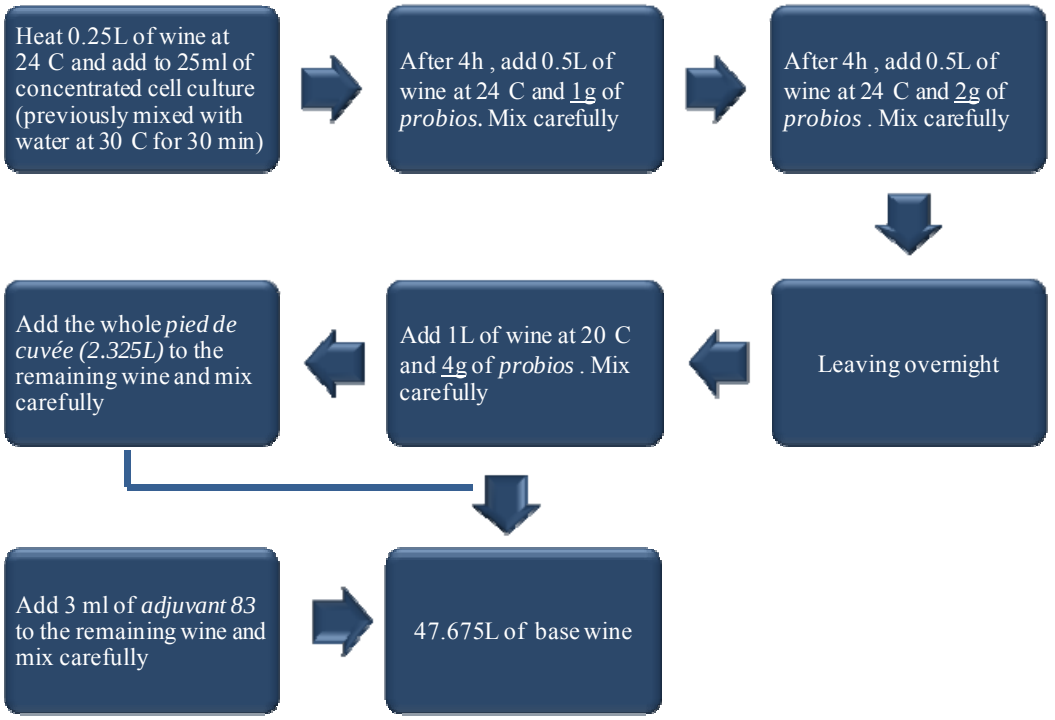


Figure 2.
Protocol for *pied de cuvée* production in winery.

The wine was bottling and then sealed with an interim crown seal and the second alcoholic fermentation (also called "*prise de mousse*") started.

4.2.4.3 Monitoring of *tirage* test

Fermentation was monitored at different intervals. For each winery, 2 bottles for each test were taken. The following analyses were carried out.

Determination of viable counts

The sampling for viable counts was determined in duplicate. Each sample was adequately diluted in sterile water and spread on plates of WL (Merck, Germany) nutrient agar. The plates were incubated at 25 °C for 3 days. After viable yeast counting, 3 colonies showing *Saccharomyces* morphology were randomly selected and isolated on YEPD for further characterization.

Cellular vitality evaluation

As described in Delfini and Formica (2001), vitality was measured by direct microscopic count of cellular material previously treated with vital stains. Briefly, 1ml of wine sample was suspended in 1 ml of Methylene Blue solution. After 5 minutes, a drop of cell suspension was placing on a Thoma - Counting Chamber (ref. 0640711 Marienfeld GmbH & Co, Germany) and viewed at microscope (CH-2, Olympus Optical Co. LTD, Japan). Living cells (uncolored) and dead cells (blue colored) were determined.

Yeast genetic identification

In order to determine the indigenous strain dominance and its permanence during fermentation, yeasts isolated from the bottles were identified and typified using PCR delta amplification technique, as described in section 2.2.2.4 of chapter 2.

4.2.4.4 Wine tasting

Samples of wine were subjected to tasting, six months after *tirage* proves. Intensity and pleasantness were evaluated through the form showed in figure 1 and statistical analysis of data was carried out as described in paragraph 4.2.3 of this chapter.

4.3 Results

4.3.1 Microvinification test

4.3.1.1 Technological parameters

Sixteen indigenous *S. cerevisiae* strains were subjected to a preliminary screening for selection of potential starters. *S1* and *S2* are commercial starters widely used for sparkling wine production that were used as controls. A microfermentation test was performed on "synthetic must" and *Pignoletto* white must. The two technological parameters taken into consideration were the fermentation vigor which expresses the capability of each strain to start the fermentation process promptly (Giudici and Zambonelli, 1992), and the total amount of CO2 produced during the fermentation, which is an indirect measure of the ethanol production (Ciani and Rosini, 1987). Figure 3 shows the trend of fermentation process (as a function of weight loss by CO_2 production).

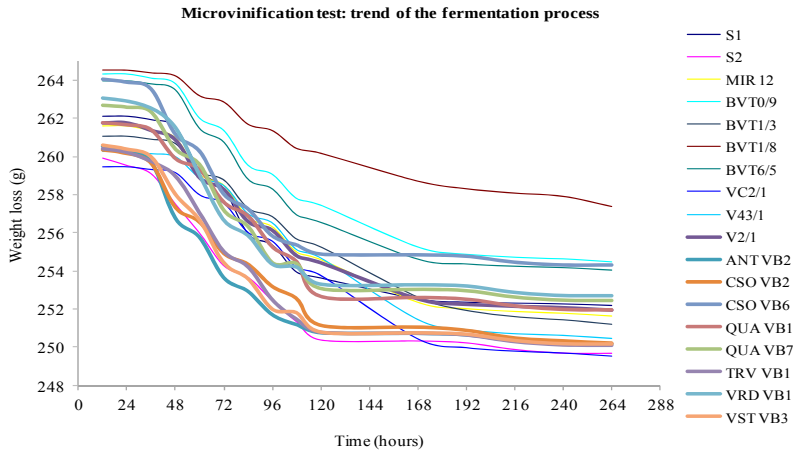


Figure 3.
Trend of fermentation on synthetic must.

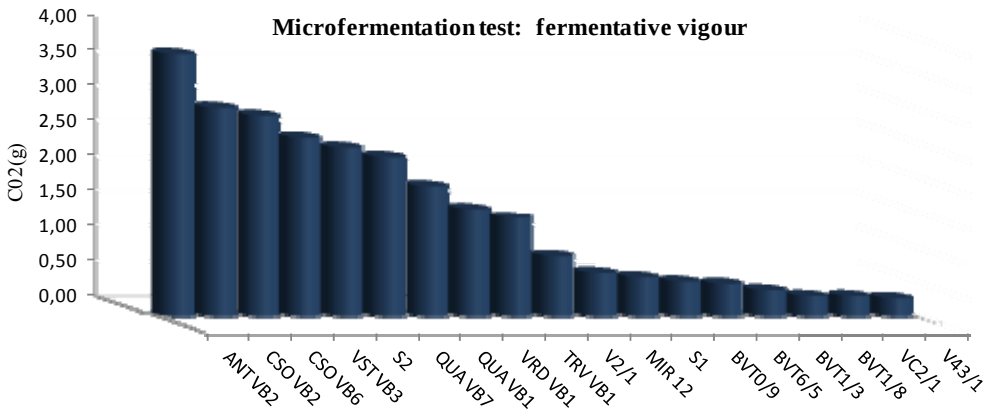


Figure 4.
Fermentative vigor of the strains on synthetic must.

The fermentative vigor (expressed as g of CO_2 produced in the first 48 hours following the inoculation of the must) showed the highest values for CSO-VB2 and ANT-VB2 strains (Oltrepò pavese) with 3. 02 g and 3.79g of CO_2 produced in the first 48 hours respectively (figure 4).

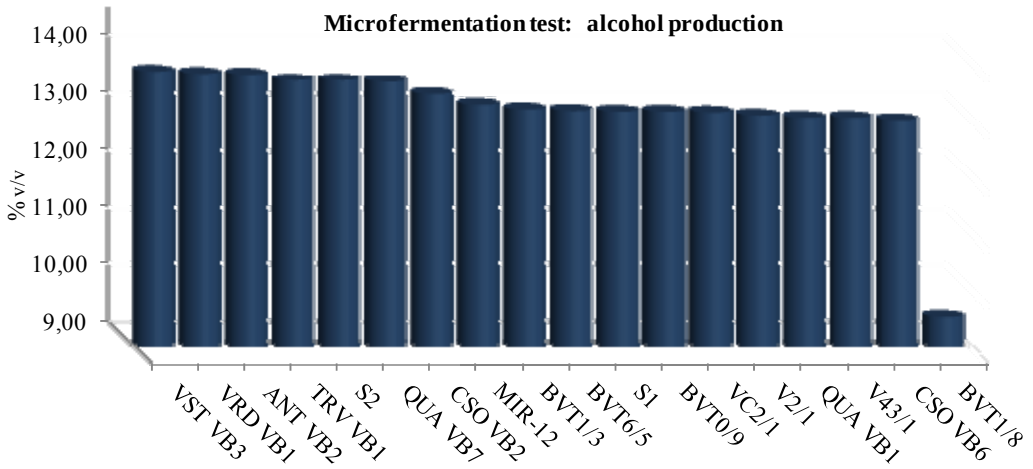


Figure 5.
Ethanol production on synthetic must.

With regard to ethanol production (figure 5), all indigenous strains showed an alcohol production comparable with the starters. Only one strain BVT1-8, was not able to produce it at an accurate level. Particularly in synthetic must ethanol ranged from 9.5 to 13.2 % v/v for *Franciacorta* strains (MIR-12, BVT0-9, BVT1-3, BVT1-8, BVT6-5, VC2-1, V43-1, V2-1) whereas from 13.0 to 13.8 % v/v for those isolated in *Oltrepò pavese* (ANT-VB2, CSO-VB2, CSO-VB6, QUA-VB1, QUA-VB7, TRV-VB1, VRD-VB1,VST-VB3).
In *Pignoletto* must the alcohol production varied from 9.7 to 10.9 % v/v for *Franciacorta* strains, and from 9.8 to 12.0 % v/v for those isolated in *Oltrepò pavese*. This could be caused by nutritional deficiencies, given the characteristics of *Pignoletto* must, which affected the metabolism of yeast.

4.3.1.2 Qualitative parameters

Glycerol and Acetic Acid production

As qualitative parameters of the fermentation process, glycerol, acetic acid and H_2S production were determinated. As regard the two first quality characters, all strains proved to produce glycerol in quantity comparable to starters, however at a lower level (0.08 to 0.25 g/L) (figure 6). Acetic acid production was limited for all strains (0.17 to 0.37 g/l) according to the maximum limit allowed by law[7] (1g/L) (figure 7).

[7] *Decreto Ministeriale* 29/12/1986, published in the *Gazzetta Ufficiale* N$_o$ 13 of 17th January 1987

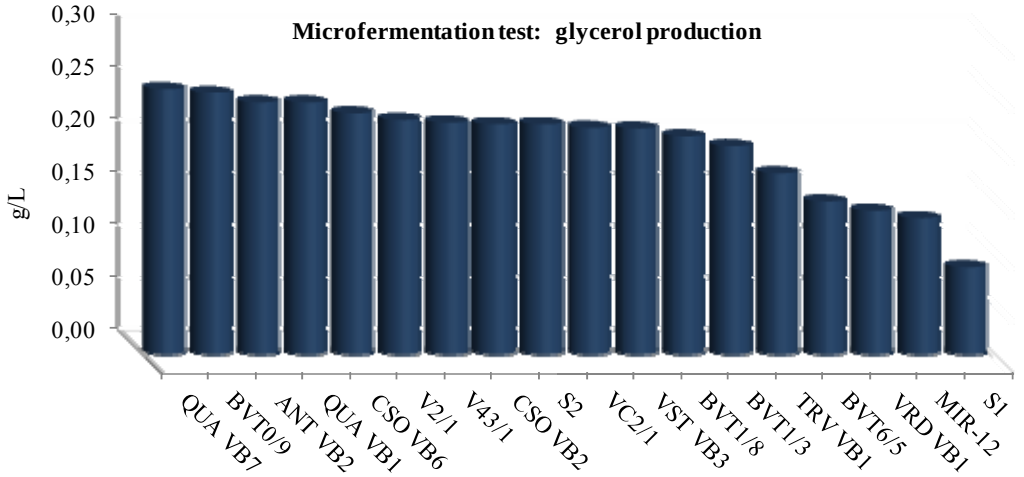


Figure 6.
Glycerol production on synthetic must.

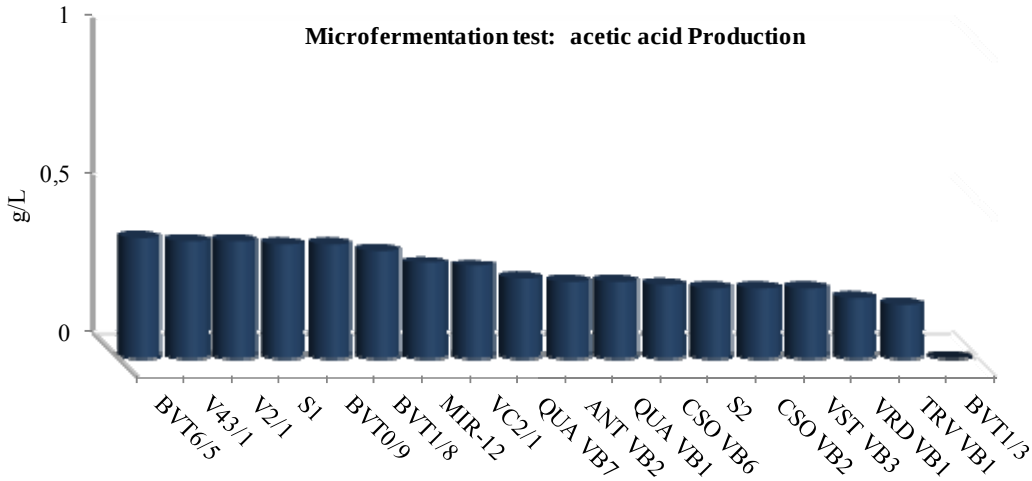


Figure 7.
Acetic acid production on synthetic must.

H2S production

H_2S production was assessed on BiGGY medium (BD, France). The degree of sulphide production was associated on the basis of color of the colonies using the scale previously reported 1, where 1 and 2 belong to lower production; 3 belong to medium production; 4, 5 and 6 belong to high production. Results are shown in table 3. All tested strains were composed by lower producers (75%) and medium producers (25 %) of H_2S.

H_2S production	Strain
Lower producer	MIR-12, BVT6-5, ANT-VB2, CSO-VB2, VRD-VB1, VST-VB3, CSO-VB6, QUA-VB1, QUA-VB7, TRV-VB1, BVT0-9, BVT1-3
Medium producer	BVT1-8, VC2-1, V43-1, V2-1

Table 3
H_2S production test.

4.3.2 Sniffing test

Samples of wine from microvinification test with indigenous *S. cerevisiae* strains isolated in *Franciacorta* and *Oltrepò pavese* were subjected to sniffing test. The statistical analysis of data was carried out for intensity (data not shown) and pleasantness of wines through a multifactor ANOVA (Statgraphics Centurion Plus 5.1, United States). the multifactor ANOVA constructs a multiple range test to determine which means are significantly different from which others, through LSD (Least Significant Difference) method [95% confidence level, ($p<0,05$].

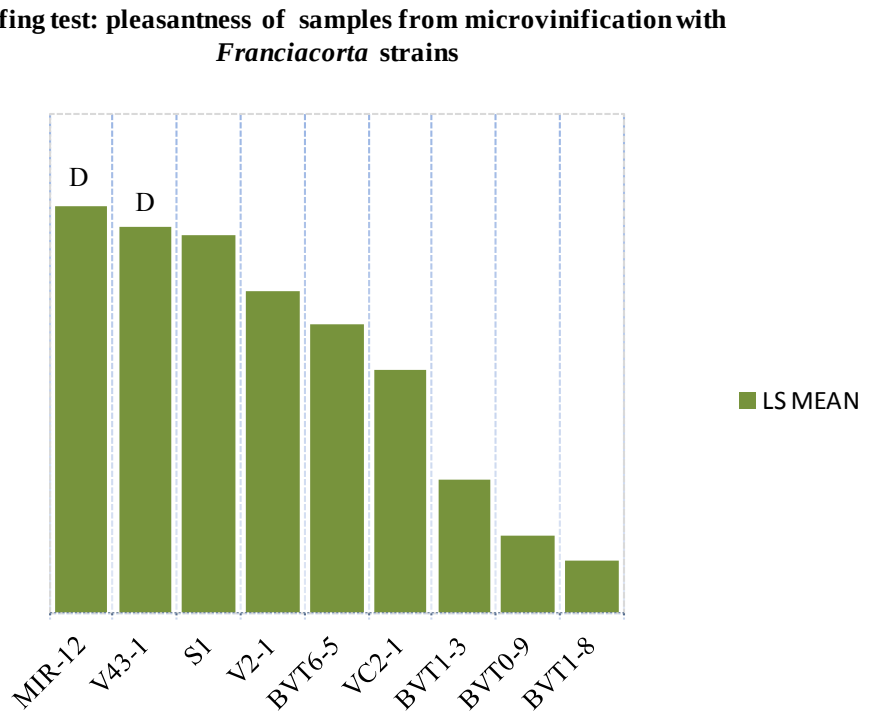


Figure 8.
Pleasantness (LS mean) of wine samples *Franciacorta* strains.

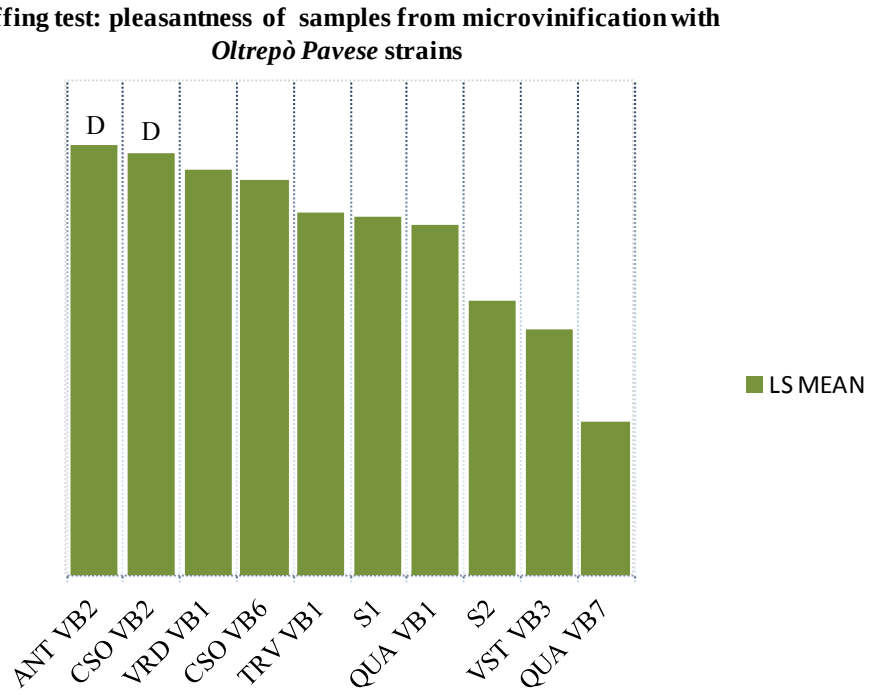


Figure 9.
Pleasantness (LS mean) of wine samples *Oltrepò pavese* strains

Figures 8 and 9 shown the LS mean (means of pleasantness values for both *Franciacorta* and *Oltrepò Pavese* strains) calculated from the multiple range test through LSD (Least Significant Difference) method [95% confidence level, ($p<0.05$)] which evidence the significant difference among the wines. Regarding the pleasantness value, wines samples among *Franciacorta* strains (figure 8) two of them (MIR-12 and V43-1) shown to be significantly different (group D). On the other hand, strains ANT-VB2 and CSO-VB2 from *Oltrepò Pavese* showed to be significantly different compared to the others (group D) (figure 9). The statistical analysis allowed foreseeing significant differences among the indigenous strains through two parameters: intensity and pleasantness. In particular, some indigenous strains scored higher than the commercial starters, which was very interesting.
Not only the sniffing test results were taken as reference to select the strains for *tirage* proves, since the most pleasent wines made by those strains were not always the best for technological and quality parameters. so, also these parameters were taken into consideration; in particular, the attention was focused on the alcohol production, especially. On the base of these data, four strains for each area (Franciacorta and Oltrepò Pavese) were chosen to be used as starters in *tirage* proves at pilot scale in different wineries of the areas.

4.3.3 Inoculated fermentations at pilot scale

4.3.3.1 *Tirage* proves during vintages 2010

Two *S. cerevisiae* strains denominated **strain 1** and **strain 2** were used as starters in four wineries of *Franciacorta* and three wineries of *Oltrepò pavese* areas. The commercial starter commonly used for each winery for the sparkling wine production was use as control (**strain 3**). To monitoring the test, determination of viable counts and cellular vitality at different times was carried out (data not shown). In addition, in order to determine the indigenous strain dominance and permanence during fermentation, yeasts isolated from the bottles at T30 (end of fermentation in 100% of the wineries) were identified and typified using the PCR interdelta amplification technique. The results evidence the dominance of the starters in all *Oltrepò pavese* wineries and in 90% of the cases in *Franciacorta* wineries (data not shown). Interestingly in *Oltrepò pavese*, the commercial starter SC1 was isolated even in the winery 3 where commercial starter SC2 (figure 10) was used.

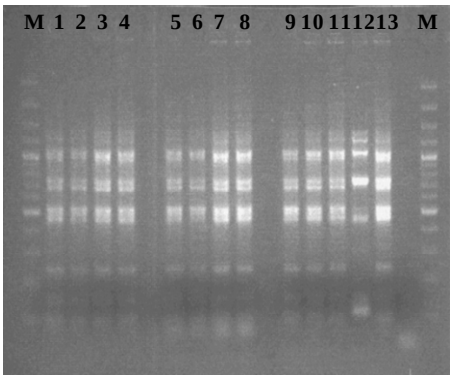


Figure 10
Interdelta profiles of yeast isolated from bottles at T30 (end of fermentation) during 2010 *tirage* proves in *Oltrepò pavese*. **1**: control strain 1. **2-4**: SC1 starter strain from bottles inoculated with strain 1 in wineries 1,2 and 3 respectively. **5:** control strain 2. **6-8**: SC1 starter strain from bottles inoculated with strain 2 in wineries 1,2 and 3 respectively. **9:** control starter strain SC1. **10-11:** SC1 starter strain from bottles inoculated with it (strain 3) in wineries 1 and 2 respetively.**12:** control starter strain SC2. **13:** SC1 starter strain from bottles inoculated whit strain 3 in winery 3 (in this winery, strain 3 corresponds at the commercial starter SC2 strain).

4.3.3.2 *Tirage* proves during vintages 2011

Based on the previous results, two news indigenous strains denominated strain X (MIR-12 *Franciacorta* strain ; CSO-VB2 *Oltrepò pavese* strain) and strain Y (BVT6-5 *Franciacorta* strain; ANT-VB2 *Oltrepò pavese* strain) were used as starters in five wineries of *Franciacorta* and three wineries of *Oltrepò pavese* areas. The commercial starter commonly used for each winery for the sparkling wine production was used as control (strain S). The results were positively different and are shown below.

Monitoring of* tirage *test in Franciacorta area

The fermentation was monitored at different intervals, starting from T0 which corresponds to the first day of wine bottling. For each winery, 2 bottles for each test strain were taken. The following analyses were carried out.

Determination of viable counts at different times

The sampling for viable counts was done in duplicate. Each sample was adequately diluted in sterile water and spread on plates of WL (Merck, Germany) nutrient agar. The trend of cell concentration for each *Franciacorta* winery is shown in figures 11a,11b,11c,11d and 11e.

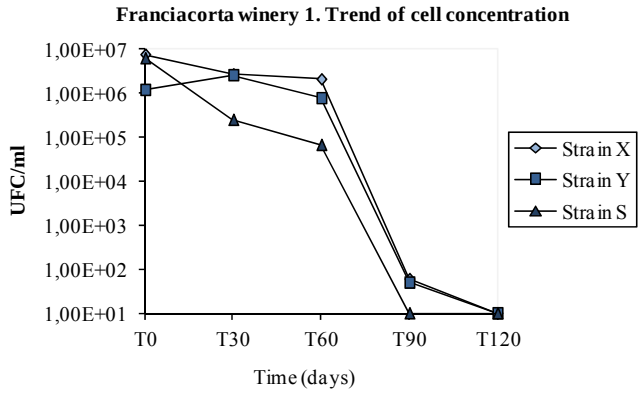


Figure 11a.
Trend of cell concentration during *tirage* test 2011 in *Franciacorta* winery 1.

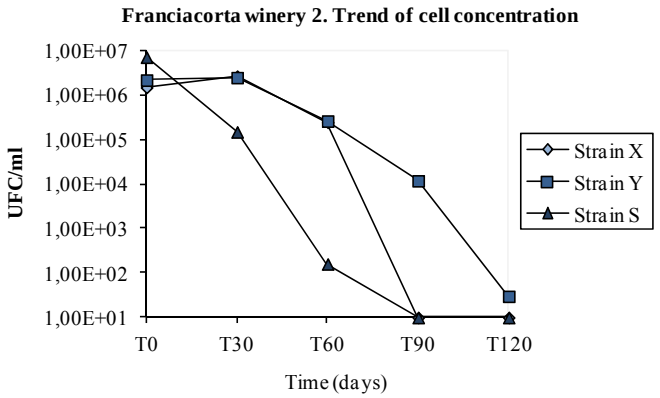


Figure 11b.
Trend of cell concentration during *tirage* test 2011 in *Franciacorta* winery 2.

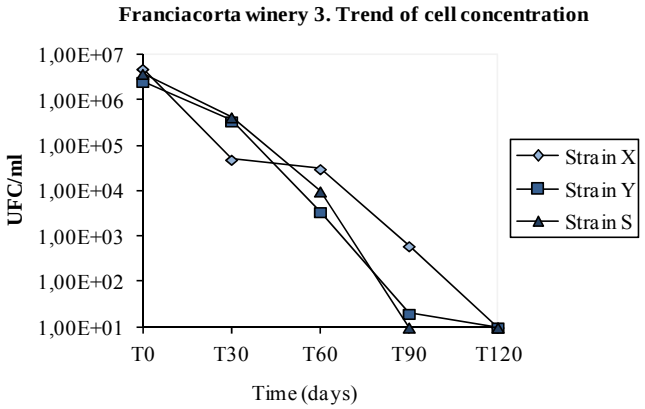


Figure 11c.
Trend of cell concentration during *tirage* test 2011 in *Franciacorta* winery 3.

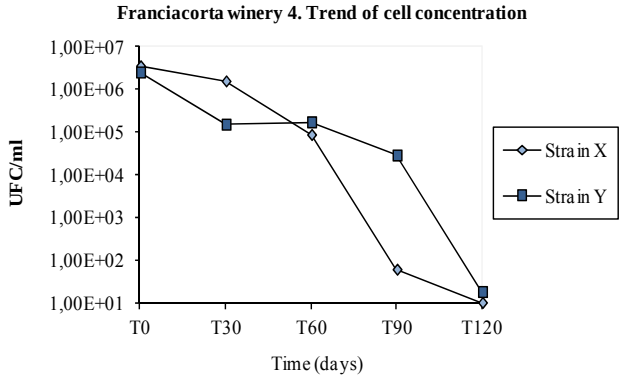


Figure 11d.
Trend of cell concentration during *tirage* test 2011 in *Franciacorta* winery 4.

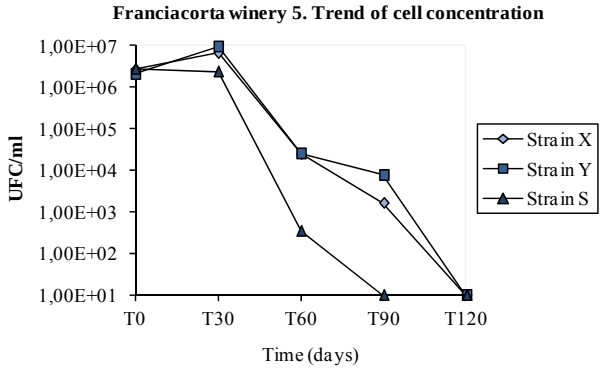


Figure 11e.

Trend of cell concentration during *tirage* test 2011 in *Franciacorta* winery 5.

The viable count determination showed an overall similar trend of the indigenous strains (strain X and strain Y). However, in most cases the commercial starter (Strain s) evidences a faster decline through the time, probably due to greater fermentative vigor. During the "*prise de mousse"* step is generally possible to observe 3 phases:

Phase 1: after a short latency period (which can be due to the inversion of sugars), a yeast multiplication is observed. During this phase, the activity of yeasts is at their maximum.

Phase 2: phase of stabilization. The pressure increases almost linearly, showing that the activity of yeasts is constant (an example is shown for the winery 2 in figure 12).

Phase 3: phase of decline. At the end of the second fermentation (past 5 g/L of sugar) yeast population and its activity decline. The conditions at the end of the process is always more unfavorable to yeasts (nutrients, toxic parameters such as CO_2 and alcohol represent the main factors for cellular death).

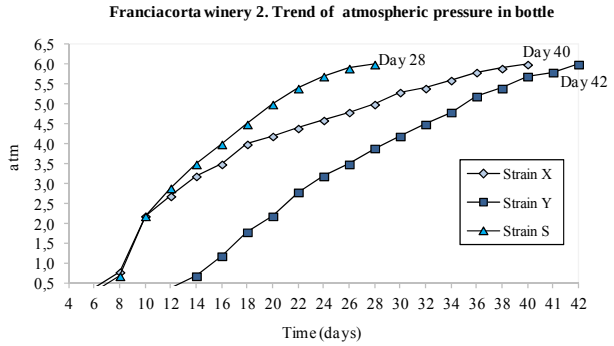


Figure 12.
Trend of atmospheric pressure (atm) in bottle for *Franciacorta* winery 2.

In *Franciacorta* wineries, the indigenous and commercial strains did not show an exponential growth phase but a constant maintenance and a rapid population decline over the time. However, the indigenous starters presented a lower fermentative vigor (promptness to begin the fermentation) than the commercial starter strain, starting later the fermentation process. This is probably the reason why viable cells were found in most cases until the T120 for the indigenous starters and only until T90 for starter strain. Regarding the cellular vitality (data not shown) the 100% of live cells were corresponding to viable counts at the different times. Two results were verified: the viable count reached its maximum approximately at the end of the fermentation, and, in the meanwhile, the pressure arrived at 6 atmospheres. Then it is possible to infer that the number of the yeast population is an indirect description of the fermentation process: as the sugar is fermented, pressure and alcohol are produced, reducing the viable and vital cells.

Yeast genetic identification

In order to verify the results from trend of fermentation process (viable count), both indigenous and commercials starters were identified and typified using the PCR interdelta amplification technique. The figure 13 shows the results at T60 (time on which the latest indigenous starter has already finished the fermentation and all the viable and vital cells were still detected)

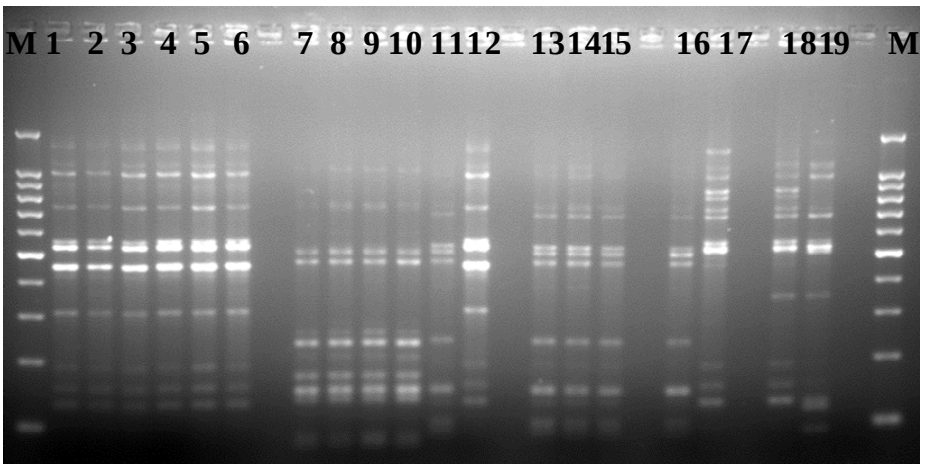


Figure 13.
Interdelta profiles of yeast isolated from bottles at T60 during 2011 *Tirage* proves in *Franciacorta.* **M:** DNA Molecular Weight Ladder 100bp XL (5 Prime, Milan, Italy). **1**: control starter strain X (MIR-12). **2-6**: starters strain X from bottles inoculated in all the wineries. **7:** control strain Y (BVT6/5). **8-10:** starters strain Y from bottles inoculated in wineries 1, 2 and 3 respectively. **11:** SC1 Commercial starter strain from bottles inoculated with strain Y in winery 5. **12:** starter strain X from bottles inoculated with strain Y in winery 4.**13:** control starter strain SC1. **14-16:** SC1 starters strain from bottles inoculated by strain S (as nominated in *tirage* prove) in wineries 1 and 3 and 5 respectively. **17:** control starter strain SC3.**18:** SC3 starter from bottles inoculated by strain S in winery 2.**19:** Electrophoretical pattern from yeast strain isolated from bottles inoculated by strain Y in winery 4.

The results from PCR interdelta amplification for the indigenous and commercial starters evidenced:
Indigenous starter X (MIR-12): 100% of electrophoretical patterns were corresponding to the inoculated X strain starter in all *Franciacorta* wineries.
Indigenous starter Y (BVT6-5): 60% of electrophoretical patterns were corresponding to the inoculated strain Y from 3 wineries; 20% was corresponding to electrophoretical pattern belonging to commercial starter SC1, isolated from winery 5; remaining 20% was corresponding to strain X which is a contamination index but however confirms the capability of implantation of this strain.
The commercial starter group SC1, used in the wineries 1, 3 and 5, was dominating in all the tests, as well as the commercial starter SC3 used in the winery 2. Only one electrophoretical profile belonging to bottles for winery 4 did not correspond to any of the inoculated strains, nor indigenous, nor commercial. However its profile resulted similar to commercial starter SC3.

Wine tasting

Samples of wine were subjected to tasting six months after *tirage* proves. Intensity and pleasantness were evaluated through the form shown in figure 1. The sensorial analyses were done for wines samples inoculated with strain X, strain Y and Strain S. A panel of 11 tasters including the oenologist of each winery participated to the test. The results for intensity and pleasantness are shown in figure 14.

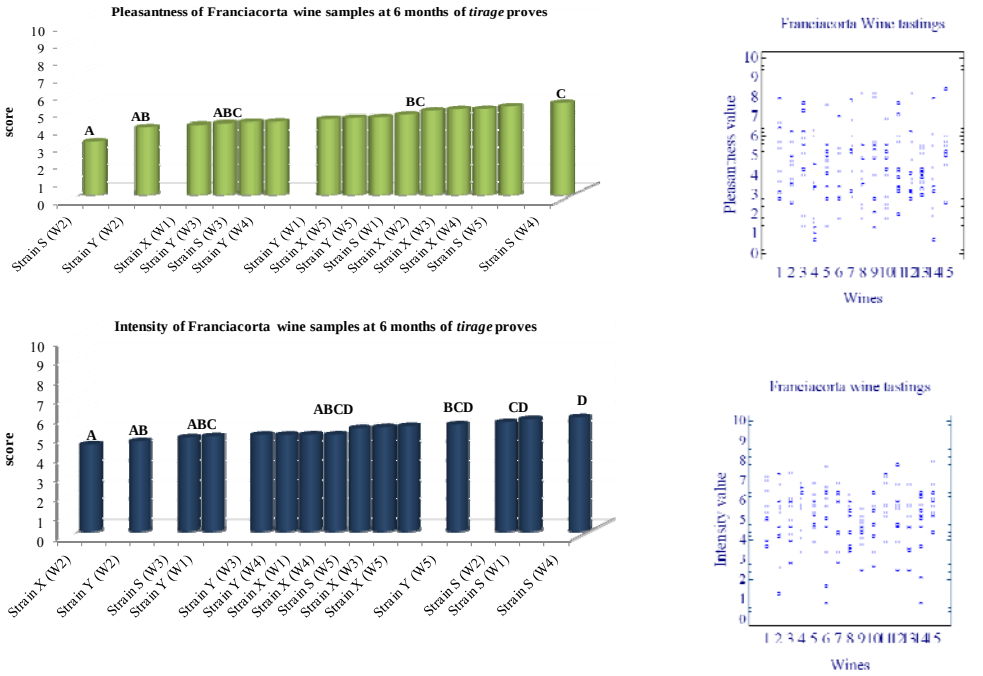


Figure 14 .
Pleasantness and intensity of sparkling wine samples after six months from *tirage* proves with indigenous starters from *Franciacorta*.

With regard to the pleasantness evaluation, three homogeneous groups (A B C) were evidenced, but only the strain S belonging to winery 4, was significantly different compared to the others. On the other hand, the intensity evaluation evidenced 4 homogeneous groups (A B C D) and, in accordance to the pleasantness result, the strain S belonging to winery 4 was significantly different again. In addition, the strain X belonging to winery 2 resulted significantly less intense than the others. Anyway, the 80% of the strains X were ranked on BC group that contains the highest means for pleasantness value and in the same group was ranked the 40% of the strains Y. In particular, some indigenous starters obtained higher means than commercial starters for both pleasantness and intensity value.

Monitoring of tirage *test in Oltrepò Pavese area*

Strain X (CSO-VB2) and strain Y (ANT-VB2) were used as starters in three wineries of *Oltrepò Pavese* area. Also in this case, the commercial starter commonly used for each winery for the sparkling wine production was used as control (strain S).

Determination of viable counts at different times

The viable count determination showed a similar trend of the indigenous strains and the starter strain in winery 7 (figures 15a,15b and 15c). In the remaining two wineries, the trend of concentration showed to be similar among the indigenous starters, but different to commercial starter. In fact, as it happened in *Franciacorta*, apparently the indigenous starters have a lower fermentative vigor than the commercial strain, starting the fermentation process later. Therefore, viable cells were found until the T120 for the indigenous starters of the wineries 6 and 8.

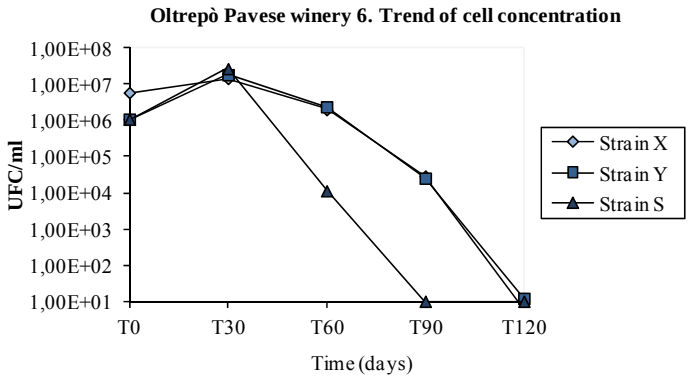


Figure 15a.
Trend of cell concentration during *tirage* test 2011 in *Oltrepò Pavese* winery 6.

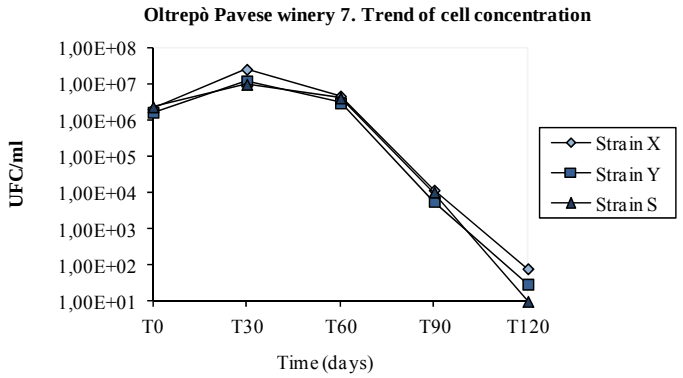


Figure 15b.
Trend of cell concentration during *tirage* test 2011 in *Oltrepò Pavese* winery 7.

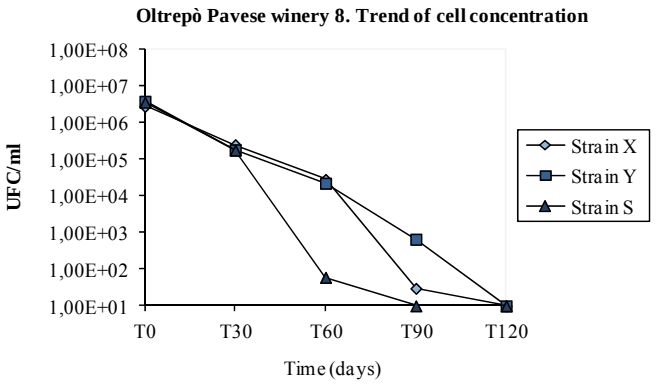


Figure 15c.
Trend of cell concentration during *tirage* test 2011 in *Oltrepò Pavese* winery 8.

In *Oltrepò Pavese* wineries, the fermentation process carried out by indigenous starters and commercial starters presented a similar behavior to those in *Franciacorta*, where the yeast population did not show an exponential growth phase but a constant maintenance and a rapid population decline over the time. Also in this case, the percent of live cells were corresponding to viable counts at the different times (data not shown).

Yeast genetic identification

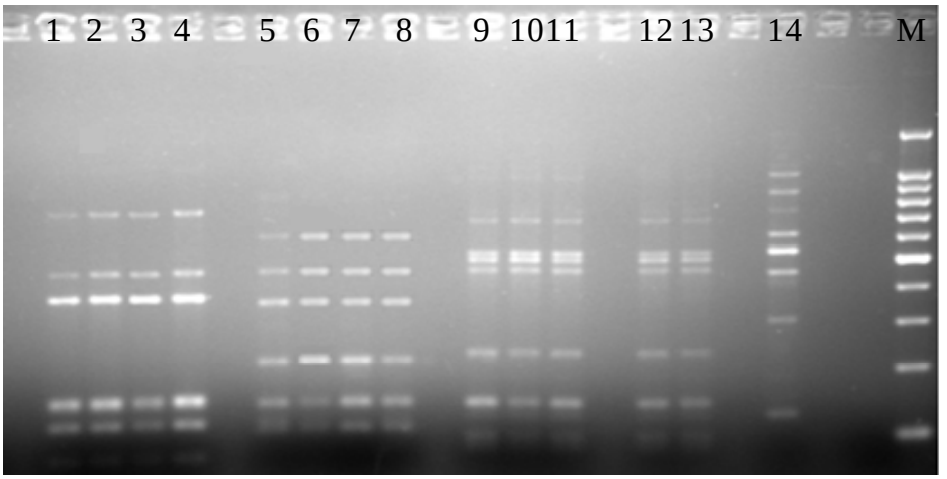


Figure 16.
Interdelta profiles of yeast isolated from bottles at T60 during 2011 *Tirage* proves in *Oltrepò Pavese*. **1**: control starter strain X (CSO-VB2). **2-4**: starters strain X from bottles inoculated in all the wineries. **5:** control strain Y (ANT-VB2). **6-8:** starters strain Y from bottles inoculated in all the wineries.**9:** control starter strain SC1. **11-10:** SC1 Commercial starter strain from bottles inoculated in winery 6 and 7 respectively. **12:** control starter strain SC3. **13:** SC3 starters strain from bottles inoculated by strain S (as nominated in *tirage* proves) in winery 8. **14:** .Electrophoretical pattern from yeast strain isolated from bottles inoculated with strain X in winery 6. **M:** DNA Molecular Weight Ladder 100bp XL (5 Prime, Milan, Italy)

From electrophoretical patterns of PCR interdelta amplification, it was possible to confirm that both indigenous Strain X and Y, were dominating in 100% of the wineries. This result was the same for the group of the commercial starters. The success of the test confirms also the effectiveness of the procedures in the wineries, since in most of the cases no contamination was present. It is interesting to note how interdelta genetic profiles are very similar in some commercial starters. It is highly possible that SC1 and SC3 are the same strain.

Wine tasting

The sensorial analyses of wine samples were carried out six months after *tirage* proves. Also in this case, intensity and pleasantness were evaluated through the form shown in figure 1, with a panel of 13 tasters. The results for intensity and pleasantness are shown in figure 17.

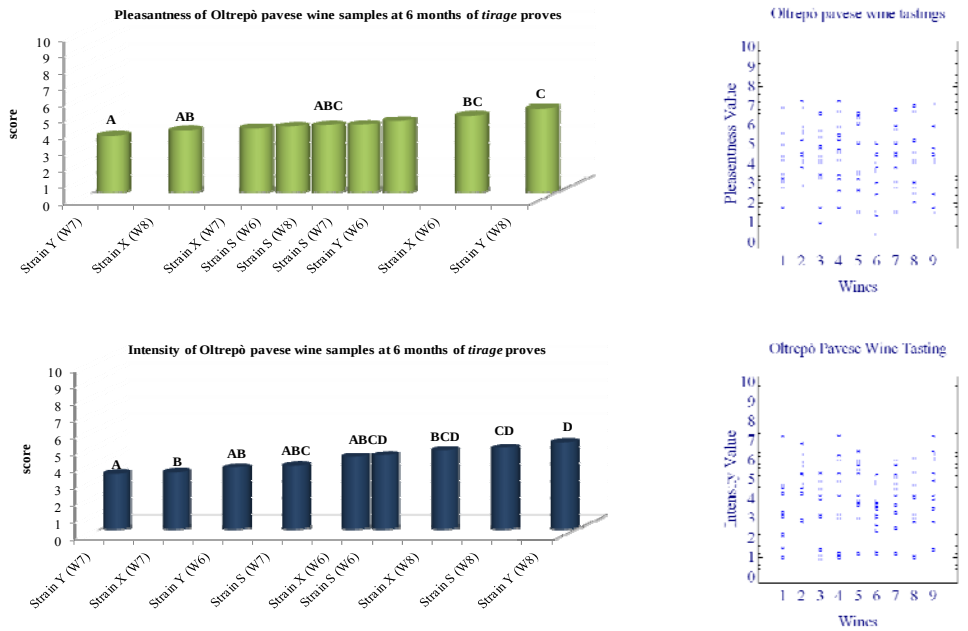


Figure 17 .
Pleasantness and intensity of sparkling wine samples after six months from *tirage* proves with indigenous starters from *Oltrepò pavese*

Regarding to the pleasantness, 3 homogeneous groups (A B C) were evidenced, of which only the strain Y belonging to winery 8, was significantly more pleasant than others ($p<0.05$). On the other hand, the intensity evaluation evidenced 4 homogeneous groups (A B C D) and, in accordance with the pleasantness result, also in this case the strain Y belonging to winery 8, was significantly more intense. In the winery 7, instead, the same Y strain resulted significantly less pleasant and less intense than the others (group A). Timing of the test is probably the reason why after 6 months only, the judges' opinions were more focused on evaluating the effect of the wine bases (the *cuvèe*) rather than evaluating the boquet of the strains, since more time for the wine aging is required.
Anyway, the strain X of the winery 6 was ranked on BC group that contains the highest means for pleasantness value, higher than the commercial starters.

4.4 Discussion and Conclusion

The screening of *S. cerevisiae* strains for parameters of technological and quality characteristics revealed a similar behaviour among the analyzed yeasts and the commercial starters used as controls. In some cases, indigenous strains showed better performances than the starters. In addition to the primary role played by any wine yeast starter (to efficiently catalyse the complete conversion of grape sugars into alcohol), it is also important that they do not produce off-flavours. Due to their high volatility, reactivity and potency at very low threshold levels, sulphur-containing compounds, such as H_2S, have a profound effect on the flavor of wine (Pretorius, 2000). The major volatile products of yeast metabolism, ethanol and carbon dioxide, make a relatively small, but, nonetheless, fundamental contribution to wine flavor. The main groups of compounds that form the "fermentation bouquet" are the organic acids, higher alcohols and esters and, to a lesser extent, aldehydes (Rapp and Versini, 1991). When present in excess concentrations, some fermentation bouquet compounds may also be regarded as undesirable, such as acetaldehyde, acetic acid, ethyl acetate, higher alcohols and diacetyl. The most "negative" aroma compounds are the reduced sulfur compounds, hydrogen sulfide, organic sulfides and thiols.
Fermentative flavor is not only brought about by the conversion of directly fermentable substances, but also by the long-chain fatty acids, organic nitrogen-containing compounds, sulfur-containing compounds and many others. These substances are able to penetrate from the grape juice through the yeast cell wall membrane, where they participate in biochemical reactions producing numerous volatile substances as by-products (Boulton et al., 1995).
Different works comparing the effects of different starter cultures and indigenous yeasts have shown that there are significant differences in the chemical composition of the resulting wines (Mora *et al*. 1990; Longo *et al*. 1992; Gafner *et al*. 1993; Lema *et al*. 1996). However, few of them have been focused in measuring the sensory differences using taste panels. Studies which simply measure concentrations of aroma-active compounds are not satisfactory without considering sensory thresholds and without considering sensory responses by tasters (Egli et al., 1998). Sensorial analysis should be considered when comparing differences in chemical concentrations. Some results on the effect of yeast strains have been contradictory, possibly due to the use of grape cultivars with neutral flavor characteristics and because the yeast populations were not stringently analyzed (Lorenzini 1994; Kunkee and Vilas, 1994). In fact, together to technological and qualitative characterization, an organoleptic evaluation should determine the complete profile of studied strains.
Regarding to general result from 2010 *tirage* prove, several factors (such as those related to the wineries operations, grape must type, geographical characteristics among others) can affect the diversity of the indigenous yeast population, also influencing the implantation capacity of a wine yeast starter (Barrajón et al., 2009). The fermentation monitoring showed that implantation was lower for all indigenous starters in the wineries of both areas. The results obtained in this study underline that the inoculation of fermentation tanks with both commercial and indigenous selected starters does not always guarantee their implantation during alcoholic fermentation. This is because the commercial starter has contaminated the tanks containing the indigenous starters tests and then, it took at the upper hand. These results agree with the work of Capece et al. (2010).
Regarding the result from 2011 *tirage* prove, during the fermentation process in both *Franciacorta* and *Oltrepò Pavese* areas it is important to take into consideration some extrinsic factors that could affect the biochemical reactions of the yeasts. The temperature of fermentation is one of these factors (Fleet and Heard, 1993). Several authors have suggested that some species of non-*Saccharomyces* have a better capability of growing at low temperatures than *Saccharomyces* (Sharf and Margalith, 1983; Heard and Fleet, 1988) because they can increase their tolerance to ethanol (Gao and Fleet, 1988). The number of different species, as well as their endurance during alcoholic

fermentation, is also conditioned by both the temperature of the must and the temperature during fermentation. These changes determine the chemical and organoleptic qualities of the wine (Fleet and Heard, 1993). Temperature is also known to affect yeast metabolism and its result could be the formation of secondary metabolites such as glycerol, acetic acid, succinic acid, etc. (Lafon-Lafourcade, 1983). Since the importance of temperature factor, another point to be considered is the possibility of hybridization within the *Saccharomyces* genus (Marinoni et al., 1999; Barnett, 1992) as a result of evolutionary adaptation to different industrial environments (Matzke et al., 1999). An example of a well-known industrial interspecies hybrid is the lager yeast *S. pastorianus*, formed by the union of *S. cerevisiae* and *S. bayanus*-related yeasts in response to selective pressures from brewing at low temperatures (Kodama et al., 2005). Although interspecies hybrids are quite common among beer and wine-making *Saccharomyces* strains (Vaughan Martini & Martini, 1987; Groth et al., 1999; Masneuf et al., 1998; Kielland-Brandt et al., 1995; González et al., 2007), it remains unclear how the hybridization occurs in indigenous *Saccharomyces* populations (Landry et al., 2006).

On the other hand, in the wineries of *Franciacorta* and *Oltrepò pavese* the winemaking temperatures vary between 12°C (rather low) and 16°C. For this, in order to verify the "genetic purity" of indigenous starters, a *MET2* PCR-RFLP analysis was carried out according to Masneuf et al. (1998). In this analysis, the four indigenous starters were tested and *S. cerevisiae, S. paradoxus, S.partorianus, and S. bayanus* from the lab collection yeast were used as controls (data not shown). The results were in accordance with Masneuf et al. (1998), and the four indigenous starters were confirmed as genetically pure *S. cerevisiae*. This confirms that temperature of winery might be a factor of competition affecting the rate of fermentation if carried out in presence of hybrid starters that could be formed by the union of *S. cerevisiae* and *S. bayanus*-related yeasts responding better to low temperatures. This could be the case of *Franciacorta* winery 4, where the winemaking temperature of cellar is 12°C. Unfortunately, the data corresponding to their starter strain were not available due to privacy policies.

Regarding to yeast genetic identification during *tirage* 2011 in both *Franciacorta* and *Oltrepò Pavese* strains, it was possible to verify that genetic properties of *S. cerevisiae* strains may be reflected in their phenotypic features (Nadal et al., 1996; Antonelli et al., 1999; Fleet, 2003; Romano et al., 2003), since the 4 indigenous starters were chosen also on the base of an interesting interdelta genetic profile. During alcoholic fermentation a number of genetically distinct *S. cerevisiae* strains release various aroma compounds which influence the organoleptic quality of wines. Moreover, the possibility to associate a molecular pattern to each of the new starters can be useful in controlling their ability to lead the fermentation process and to dominate over the spontaneous yeast microflora of musts (de Barros Lopes et al., 1996). The correlation of genetic patterns of strains with oenologically useful characteristics (Nadal et al., 1996) in yeast characterization at strain level can facilitate the selection of wild yeasts with the desired metabolic and genetic traits. Though there are commercial yeasts to accomplish must fermentation, the use of local selected yeasts is believed to be much more effective, in respect of their competitiveness and their ability to contribute to the maintenance of the typical sensory properties of the wines produced in any given region, if selected strains are used as starters (Degré, 1993; Querol et al., 1992).

Concerning to wine tasting of the wine samples from *Franciacorta* and *Oltrepò Pavese* wineries in general, the indigenous starters were sensorially accepted at 6 months of aging.

As an result of this study, the indigenous starters representative of each area demonstrated a high potential to successfully implant and carry out the fermentation. In addition, this potential was supported through tasting-wine proves, demonstrating their high potential to be considered as future new indigenous starters for sparkling wine production by *Champenoise* method in *Franciacorta* and *Oltrepò Pavese* .

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Chapter 5
Development of a protocol for recovery of yeast DNA from sparkling wines

5.1 Background

Sparkling wine produced by *champenoise* is performed through two phases. The first step is obtaining the *cuvée* (base wine) by a spontaneous or inoculated fermentation. For the second stage, yeasts (*Saccharomyces cerevisiae* or *Saccharomyces bayanus* alone or mixed), sucrose (usually 25 g/liter asfinal concentration) and coadjuvants agents (*écorces*, aminoacids, alginates and bentonite) are added to the base wine to start the second fermentation. This mixture is bottled (*tirage*) and stored for long periods (at least 9 months for *Cava* in Spain, 12 months for *Champagne* in France and 18 and 15 months for *Franciacorta* and *Oltrepò Pavese* sparkling wine respectively) to allow the re-fermentation and the aging of the wine. After a time, when sugars are exhausted and CO_2 pressure achieves 6 atmopheres, the yeast population die and autolysis occurs inside of the bottle. This fact implies the release of intracellular material such as amino acids, peptides, proteins, polysaccharides, nucleic acids, and phospholipids generating a positive effect on the aroma quality, flavor and foam of the wine (Charpentier and Feuillat, 1992).
A positive correlation between the autolytic capacity of yeast strains and the quality of the sparkling wines obtained has been shown by several studies (Lacueva et al., 1997; Escot et al., 2001; Martinez-Rodriguez et al., 2002; Leroy et al., 1990). Indeed, "aging on lees" is becoming increasingly popular due to its positive effect on several properties, including the chemical stability of white wines and the color stability and mouth feel, among other properties, of red wines (Fornairon-Bonnefond et al., 2002).
On the other hand, as mentioned in section 1.4.1 of chapter 1, Italian quality sparkling wines are identified with two hallmarks of Italian origin: the *DOC* (*Denominazione di origine controllata*) to certify the particular grape origin area and process practices where the wine is produced; and *DOCG* (*Denominazione di origine controllata e garantita*) which is the label assigned to the *DOC* wines, which are also subjected to a quality control test before the sale and they have a "particular quality value" of a recognized national and international prestige. Both *Franciacorta* sparkling wine and *Oltrepò Pavese Metodo Classico* sparkling wine have the *DOCG* mark. *DOCG* is obtained after chemical and sensorial tests and it is strictly related with typicality/authenticity concepts. In the case of wine, "typicality" is a concept not easily definable. It could be described as the combination of certain recognizable characteristics derived from grape type, soil, climate or even the microclimate and the production technology. However some of these features could become ambiguous and not always guaranteed. During the last decade, the wine sector has implemented programs to encourage the development of biotechnology based testing, since these analyses are not affected by different environmental conditions and are thus more reliable (İşçi et al., 2009). Therefore, different molecular approaches have been development toward the wine authentication (Leopold et al., 2003; Savazzini and Martinelli, 2006; Pereira et al., 2011; Spaniolas et al., 2008). In these studies DNA extraction methods from grapes, must and wine have been studied (Baleiras-Couto and Eiras-Dias, 2006; Faria et al., 2008; Garcia-Beneytez et al., 2002; Rodríguez-Plaza et al., 2006; Siret et al., 2002). Researchers have demonstrated that DNA is present in wines at the end of the fermentation, on solid particles in some cases, as well as in solution. As it was previously described, in the case of sparkling wines made by *champenoise*

method, the cellular debris (including DNA of yeast used as starter during second fermentation) is probably released after autolysis and remains in the bottle until consumption. This suggests that it could be possible to apply molecular techniques for DNA amplification to control wine quality and origin certification, as well as in detecting wine falsification. The purpose of this study was the assessment of a protocol for extraction and amplification of yeast DNA from sparkling wine, aimed to a development of a molecular test that enables the verification of sparkling wines authenticity as a requisite to obtain *DOCG* status. This objective is closely related to the results obtained during the study presented in the previous chapter, where it was demonstrated that 4 indigenous strains (two from *Franciacorta* and two from *Oltrepò Pavese* areas) of *S. cerevisiae* showed aptitude as potential starters (either in laboratory than in small-scale level in 8 wineries) in the sparkling wine production made by *Champenoise* method. The researches pursue the same objective of improving the typical characteristics and traceability of the product.

5.2 Materials and Method

5.2.1 Treatment of samples for DNA extraction

Sparkling wine bottles from both *Oltrepò Pavese* and *Franciacorta* territories, obtained during 2010 *tirage* test, were used in this study. As described in section 4.3.3.1 of previously chapter the fermentation into the tested bottles was carried out by the starter yeasts (not indigenous). So, 125ml of wine sample (in triplicate) were filtered through membranes with pores of 0.45µm and stored at -20°C. In particular, bottles from 4 wineries in *Franciacorta* and 3 wineries of *Oltrepò Pavese* were analysed. The sampling was carried out every 30 days starting from T0 (day of *tirage*). Sampling intervals are shown in table 1.

FRANCIACORTA	
Winery*	sampling interval times
5	0 – 30 – 60 – 90 – 120 – 180 – 210 – 240 – 300
4	0 – 30 – 60 – 90 – 120 – 180 – 240 – 300 – 420
3	0 – 30 – 60 – 90 – 120 – 180 – 270 – 330 – 450
1	0 – 30 – 60 – 90 – 120 – 180 – 240 – 300 – 360 – 420
OLTREPO' PAVESE	
Winery*	sampling interval times
6	0 – 30 – 60 – 90 – 120 – 180 – 270 – 330
7	0 – 30 – 60 – 90 – 120 – 180 – 240 – 300 – 360 – 420 – 480
8	0 – 30 – 60 – 90 – 120 – 180 – 240 – 300 – 360 – 420

Table 1.
Interval times of sampling for sparking wines.*the wineries code in this study is the same given in previously chapter (table 2 in section 4.1.5). *Franciacorta* winery 2 did not participate in *tirage* 2010.

5.2.2 DNA extraction protocols from sparkling wine

The DNA extraction protocols from sparkling wine are listed below. For any protocol, a preliminary test was performed with a wine sample added with a specific quantity of DNA, as control, in order to evaluate the efficiency of the method.

5.2.2.1 DNA extraction through *Nucleospin* kit

DNA protocol through *Nucleospin* kit (Machery-Nagel, Germany) was performed as follows:
in order to binding DNA to the silica membrane inside the colomn, 400 µl of wine sample were mixed up with a volume of 96% ethanol and a volume of C4 buffer. The mixture was shaken during 30 s and then 750 µl of this were centrifuged at 11000 *g* for 1 minute (Hettich zentrifugen,

mikro 200, Germany). The liquid phase which is deposited at the bottom of the eppendorf was eliminated and then 3 separated washing steps were performed:

- 400 µl of CQW buffer (guanidine hydrochloride, ethanol) were added at the silica colomn and centrifuged at 11,000 *g* for 11 min. The liquid phase deposited at the bottom of the eppendorf was eliminate;
- 700 µl of C5 buffer were added at the silica colomn and centrifuged at 11,000 *g* for 1 min. The liquid phase deposited at the bottom of the eppendorf was eliminate;
- Additional, 200 µl of C5 buffer were added at the silica colomn and centrifuged at 11,000 g for 1 min.

Subsequently, the silica column was transferred into a new eppendorf and then 100 µl of CE elution buffer (5mM Tris/HCl, pH8) (previously warmed at 70°C) was added. The mixture was incubated for 5 min at room temperature. After incubation, the mixture was centrifuged at 11000 *g* for 1 min. Finally, DNA was store at -20°C and concentrations were determined by measuring the A_{260nm}.

5.2.2.2 DNA extraction through Na-acetate

28 ml of Na-acetate 3M (pH5,2) and 60 ml of ethanol 96% were added to 172 ml of wine samples. The samples were allowed to precipitate at -20°C for 15 days and, after that, were centrifuged at 13,000 *g* for 10 min at room temperature (Hettich zentrifugen, rotina 380r, Germany). The supernatant was eliminated and the pellet was re-suspend in 750 µl of CTAB buffer (25 mM of EDTA, 1 M Tris-HCl, pH 8, 2 M of NaCl and 3% p/v of CTAB). 0.,2 % v/v of β-mercaptoethanol and 1% p/v of polyvinylpyrrolidone were added at the moment. The mixture was incubated at 65°C for 60 min and after purified with a volume of phenol: chloroform: isoamyl alcohol (25:24:1). A centrifugation a 13,000 *g* for 5 min was performed immediately. The liquid phase was then transferred in a new Eppendorf and 0.6 volumes of isopropanol were added. The mixture was leave overnight at -20°C. DNA was recovered through centrifugation at 13,000 *g* for 30 min at 10°C. Supernatant was then eliminated and DNA was washed with 70% ethanol. A centrifugation at 13,000 *g* for 30 min was done immediately. The pellet was dried and dissolved in 50 µl of sterile and filtered *mQ* water (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). Finally, DNA was stored at -20°C.

5.2.2.3 DNA extraction through filtering

In order to verify if microbial DNA was enclosed into residual died cells, a protocol to recover cellular debris from sample was evaluated. In this case the whole content of a wine bottle was filtered with Sartorius membranes (Millipore, Bedford), using a pore filter of 0.45 µm of diameter. The membrane was then transferred into a sterile tube and treated with 3 ml of a solution containing 0.9 M sorbitol-0.1 M EDTA, pH 7.5 to which 500 µg/ml of zymolyase 100T (USBiological, USA) and 1 µl/ml of 14 mM β-mercaptoethanol were added at the moment. From this point onwards the DNA extraction was carried out according to Querol et al. (1992) as described in section 2.3.1 of chapter 2.

5.2.2.4 DNA extraction through *MagMAX* Multi-Sample kit

The following procedure is divided in two separated steps: the former consists in DNA extraction through organic solvents; in the latter, DNA purification was performed through magnetic beads using the MagMAX DNA Multi-sample kit (Applied Biosystems – Life Technologies, United States).

DNA extraction through organic solvents

The first phase was performed as follows. The pH value of wine was adjusted at 7.0. A volume of isopropanol was added and mixed gently. The mixture was precipitated at -20°C overnight. A

centrifugation was done at 15000 *g* for 30 min at 10°C. The supernatant was eliminated and then the pellet was re-suspended in 1 ml of TE (Tris 10 mM, EDTA 1mM) and solubilised for 4 hours. After this time, 10 µl of 20 mg/ml K proteinase (Sigma-Aldrich, Germany) were added at 500 µl of suspension. It was incubated at 37°C for 1 hour and half. Following, 500 µl of phenol (saturated with 10 mM of Tris –HCl, pH 8.1 mM EDTA) were added and a centrifugation was done at 14,000 *g* for 10 min. The supernatant was taken and transferred into a new tube. 250 µl of phenol and 250 µl of chloroform/alcohol isoamylic 24:1were added. A centrifugation was done at 14,000 *g* for 10 min. The supernatant was taken and transferred into a new tube. 500 µl of chloroform/alcohol isoamylic 24:1were added. A centrifugation was done at 14,000 *g* for 10 min. Finally, the supernatant was taken and transferred into a new tube.

DNA extraction through MagMAX DNA Multi-sample kit

The second step is made by the following procedure. 200 µl of PK Buffer (containing K proteinase 100 mg/ml) were added to 200 µl of the wine sample. Incubation at 60°C for 20 min was done. After incubation, 600 µl of lytic buffer were added. The suspension was mixed up and then 40 µl of magnetic beads and 800 µl of isoprapanol 100% were added. The mixture was shaken for some minutes. The tubes were placed onto the magnetic support for 5 min. Once the magnetic beads adhered to the tubes, the supernatant was eliminated without removing the tube from the magnetic support. Following, the pellet was washed with 300 µl of the first washing solution and re-place onto the magnetic support for 1 min. The supernatant was eliminated and again washed with 300 µl of the second washing solution. The supernatant was eliminated and the pellet was dried in an incubator for about 10 min. 100 µl of elution buffer 1 were added and the mixture was incubated at 70°C for 5 min. After, 100 µl of elution buffer 2 were added. The mixture was mixed up, and the tubes were placed onto the magnetic support for 5 min. Finally, the supernatant was transferred into a new tube and then stored at -20°C.
The principle underlying magnetic bead procedures involves attracting DNA to magnetic beads, holding the beads in place using a magnetized source, such as a rack or tube holder, and washing away other components of the sample. Figure 1 shows Schematic process of magnetic beads principle.

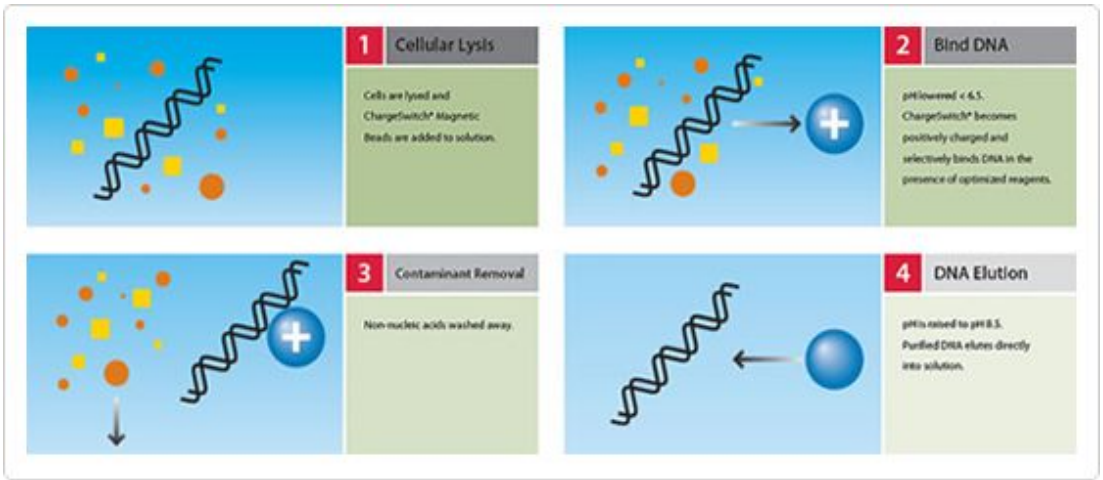

Figure 1.
Schematic process of magnetic beads principle.

5.2.3 Qualitative PCR

Extracted DNA of each protocol was subjected to internal transcribed spacers (ITS) amplification as described in section 2.2.2.2 of chapter 2.

5.2.4 Real-Time PCR

Extracted DNA of each protocol was subjected to internal transcribed spacers (ITS) amplification in Real-Time PCR. Primers were designed from ITS region of *S. cerevisiae.* Amplification was performed in a 20 µl reaction mixture containing 1X *SYBR GREEN* (Applied Biosystems – Life Technologies, Unites States), 0.25 µM of primers VF1 *(*5' GGGCCCAGAGGTAACAAACAC 3') and VF3(5' CCAGTTACGAAAATTCTTGTTTTTG 3'), 3% of *DMSO* (Sigma chemical, United States), and 2 µl of DNA. The temperature profile was: denaturation step at 95°C for 30 seconds, annealing step at 62 °C for 30 seconds, extension step at 72°C for 30 seconds. This was repeated for 40 cycles. In order to evaluate the specificity of the amplification products, at the end of the amplification reaction a thermal profile was added for dissociation curve (melting curve).

5.2.4.1 Primers design

ITS sequences from *S. cerevisiae, Dekkera bruxellensis, Hanseniaspora uvarum, Zygosaccharomyces rouxii, Issatchenkia orientalis, Saccharomyces bayanus, Saccharomyces pastorianus and Saccharomyces paradoxus* were downloaded from Gene Bank. The sequences were then aligned through (ClustalX 2.0.12). Furthermore, the program Annhyb allowed the design of most appropriate primer for the region not conserved and specific of *S. cerevisiae.*

5.3 Results

5.3.1 DNA protocols extraction

5.3.1.1 DNA extraction through Nucleospin Kit

The basis of this protocol is the cell-lysis by chemical, enzymatic methods (denaturing agents, detergents and proteinase K) and silica membrane in a spin column format. The samples used for this analysis were previously contaminated with *DNA* of *Candida* and *M. pulcherrina*. After amplification of ITS regions, the protocol did not provide reproducible results, since only once was possible to obtain DNA amplified. In addition, when wines samples were contaminated with DNA from *S. cerevisiae* UV quantification was carried out. The protocol showed a lower extraction yield, highlighted by the fact that the maximum amount of wine that could be loaded on silica column is 2 ml of sample, only.

5.3.1.2 DNA extraction through Na-acetate

DNA extraction through Na-acetate, in agreement with Savazzini et al. (2006) did not evidence any amplification result. This protocol was focused on finding free DNA in the wine. There are some problems with the recover of nucleic acids in this matrix. Limiting factors for the analysis of wine include the low quantity of DNA present in solution and/or the possible degradation of DNA during the fermentation and aging process. Another limiting factor is the complexity of the wine, as it contains potential inhibitors such as tannins and polyphenols or polysaccharides.

5.3.1.3 DNA extraction through filtering

On the base of the autolysis did not occur for some yeast during fermentation and so a number of cells can remain intact into the bottle of sparkling wine; a filtration method was tested. For this, specials membranes were used and the cells possibly retained in them were treated with a protocol for DNA extraction as described for Querol et al. (1992). Also in this case, the results were not reproducible. In fact, DNA amplified (evidenced through the ITS regions amplification) was obtained in few cases (figure 2).

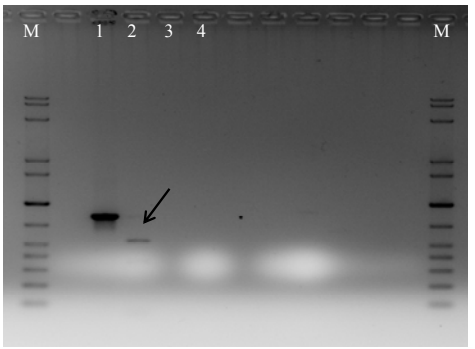

Figure 2
ITS amplification from DNA extracted through filtration protocol. **M:** Marker ladder 100 bp xl (5 Prime, Milan, Italy), **1:** *S. cerevisiae* positive control, **2-3:** ITS from DNA extracted from bottled 1 and 2 respectively, **4:** negative control.

The low repeatability of the protocol could be due to cell number variability in wine bottles. In fact when wines from different bottles were plated on WL agar, the number of CFU was very low or growth was completely absent.

5.3.1.4 DNA extraction through MagMAX Multi-Sample kit

To carry out this protocol, preliminary tests were conducted on samples of wine. 10µl of *S. cerevisiae* genomic DNA (previously quantified to UV) were added to 15 ml of wine. In addition, to evaluate the protocol sensitivity, decimal dilutions (until 10^{-3}) of this DNA were realized. Then, to extract DNA from wine both a protocol using organic solvents and a protocol using magnetic beads (MagMAX Multi-Sample kit) were evaluated. DNA extracted through both methods was subjected to ITS regions amplification. The results are shown in figure 3.

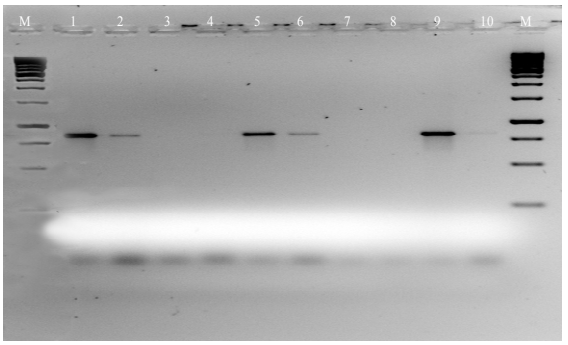

Figure 3.
ITS amplification from DNA extracted through organic solvents and Magnetic beads. **M:** Marker ladder 1Kb (Fermentas, Lithuania), **1:** DNA not diluted (magnetic beads method), **2:** DNA diluted 10^{-1} (magnetic beads method), **3:** DNA diluted 10^{-2} (magnetic beads method), **4:** DNA diluted 10^{-3} (magnetic beads method), **5:** DNA not diluted (organic solvents method), **6:** DNA diluted 10^{-1} (organic solvents method), **7:** DNA diluted 10^{-2}(organic solvents method), **8:** DNA diluted 10^{-3}(organic solvents method), **9:** *S. cerevisiae* positive control, **10:** negative control.

Both extraction methods allowed to obtained an amplification signal. In particular, an amplification fragment was obtained for DNA diluted until 10^{-1}. On the base of these results, the following step to optimize the protocol was to combine the two techniques in order to obtain a higher yield of amplified DNA. The result of these methods combination is shown in figure 4

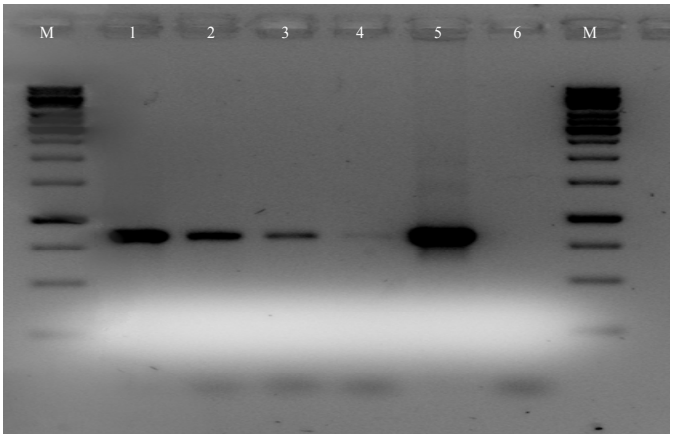

Figure 4
ITS amplification from DNA extracted through combination of organic solvents and Magnetic bead. **M:** Marker ladder 1Kb (Fermentas, Lithuania), **1:** DNA not diluted, **2:** DNA diluted 10^{-1} **3:** DNA diluted 10^{-2}, **4:** DNA diluted 10^{-3}, **5:** *S. cerevisiae* positive control, **6:** negative control.

The results show that throughout a combination of both techniques a probably greater amount of DNA was extracted, compared to that obtained when the two techniques were used separately. In fact, in this case it was possible to achieve amplification of DNA subjected to dilution equal to 10^{-3}. In order to determine the repeatability of the methods, proves were carried out for double tests. Repeatability equal to 50% was obtained for magnetic beads method (10^{-1} as maximum dilution); 100% for organic solvents method (10^{-1} as maximum dilution) and 100% for the combination of both methods (10^{-3} as maximum dilution).

5.3.2 Real-Time PCR for DNA traceability

In order to improve the DNA recovery, the extraction was performed using the combination of techniques "magnetic beads and organic solvents". Then, Real-Time PCR was used for DNA amplification. This technique shows a sensitivity higher than the qualitative PCR, since it is able to detect only 10 copies of DNA target, while the second requires 100-1000. The test was prepared adding to wine samples different dilutions of yeast DNA. Initially, primers SC1/SC2 according to Zott et al. (2010) were used. These were designed on the ITS regions and are specific for the genus *Saccharomyces.* To set up the amplification reaction, both a negative control basis and a negative control of *Dekkera bruxellensis* were included in order to evaluate the specificity of primers. The results of amplification are shown in figure 5 The results shown in figure 6 evidence that amplification signal occur up to 10^{-10} DNA dilution in which the quantity of DNA is too low to be detected by Real-Time PCR. Further, as shown in figure 5, an amplification signal for *Dekkera bruxellensis* was evidenced. The presence of this false positive signal led to change the primers.

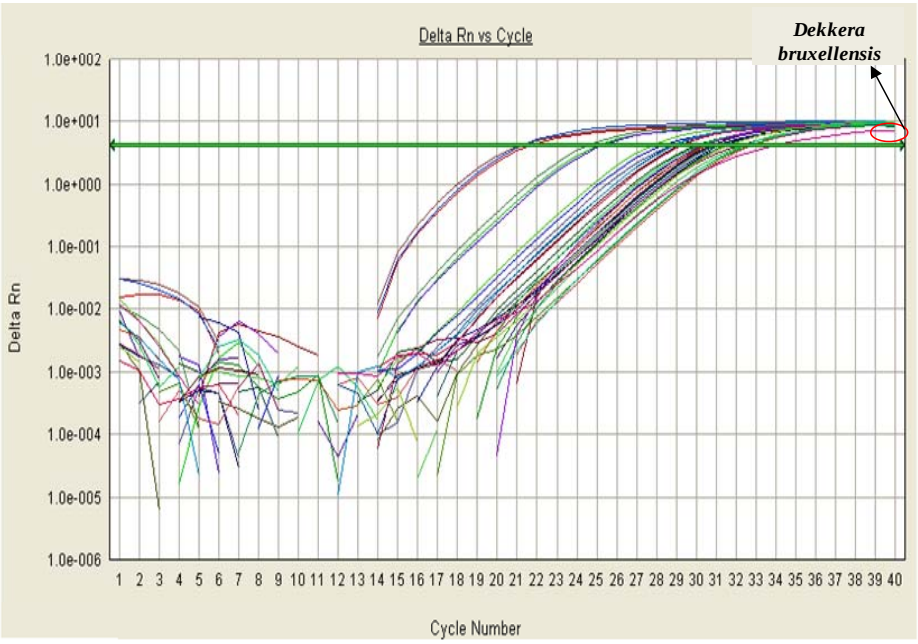

Figure 5.
Real-time PCR ITS amplification using SC1/SC2 according to Zott et al. (2010)

Well	Sample Name	Detector	Task	Ct	StdDev Ct	Qty	Mean Qty	StdDev Qty	Filtered	Tm
A1	tq	SyBR	Standard	21.88	0.307	1.00e+007				76.8 °C
A2	-1	SyBR	Standard	25.24	0.394	1000000.00				76.8 °C
A3	-2	SyBR	Standard	28.48	0.509	100000.00				76.6 °C
A4	-3	SyBR	Standard	30.53	0.152	10000.00				76.6 °C
A5	-4	SyBR	Standard	31.15	0.947	1000.00				76.8 °C
A6	-5	SyBR	Standard	31.35	0.436	100.00				76.8 °C
A7	-6	SyBR	Standard	29.27	0.190	10.00				76.8 °C
A8	-7	SyBR	Standard	30.17	0.387	1.00				76.8 °C
A9	-8	SyBR	Standard	31.58	0.065	1.00e-001				76.6 °C
A10	-9	SyBR	Standard	32.93	0.173	1.00e-002				76.3 °C
A11	-10	SyBR	Standard	32.43	1.029	1.00e-003				76.0 °C
B1	tq	SyBR	Standard	21.32	0.307	1.00e+007				77.1 °C
B2	-1	SyBR	Standard	24.70	0.394	1000000.00				77.3 °C
B3	-2	SyBR	Standard	28.88	0.509	100000.00				76.6 °C
B4	-3	SyBR	Standard	30.44	0.152	10000.00				76.8 °C
B5	-4	SyBR	Standard	30.83	0.947	1000.00				77.1 °C
B6	-5	SyBR	Standard	30.59	0.436	100.00				77.1 °C
B7	-6	SyBR	Standard	28.93	0.190	10.00				76.8 °C
B8	-7	SyBR	Standard	30.93	0.387	1.00				76.8 °C
B9	-8	SyBR	Standard	31.64	0.065	1.00e-001				76.8 °C
B10	-9	SyBR	Standard	32.81	0.173	1.00e-002				76.6 °C
B11	-10	SyBR	Standard	32.42	1.029	1.00e-003				76.6 °C
C1	tq	SyBR	Standard	21.39	0.307	1.00e+007				77.1 °C
C2	-1	SyBR	Standard	25.47	0.394	1000000.00				76.8 °C
C3	-2	SyBR	Standard	27.87	0.509	100000.00				77.3 °C
C4	-3	SyBR	Standard	30.23	0.152	10000.00				76.8 °C
C5	-4	SyBR	Standard	29.38	0.947	1000.00				77.3 °C

Figure 6.
Real-time PCR report from ITS amplification using SC1/SC2 according to Zott et al. (2010)

5.3.2.1 Primers optimization

In order to evaluate the possible dimers formation, primers SC1 and SC2 were subjected to temperature gradient test, using annealing between 45.2 °C and 65°C. Dimers formation can result in false-positives during the analysis. The result of this test is shown in figure 7.

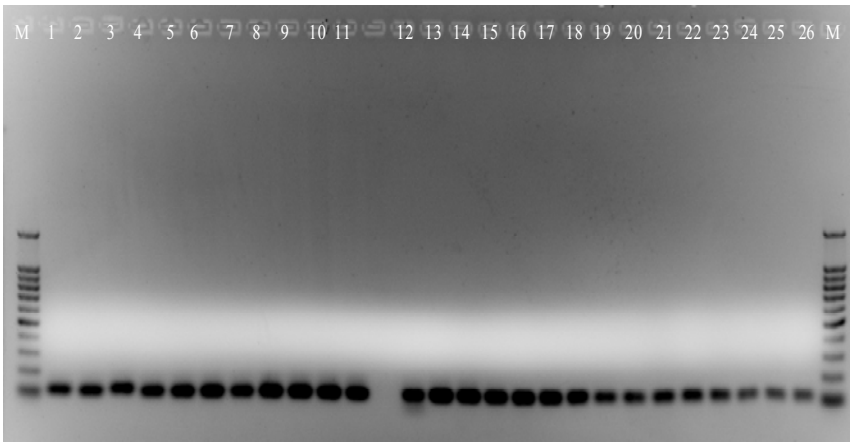

Figure 7
Agarose gel control of temperature gradient test for primers SC1 and SC2. **M:** Marker ladder 100 bp xl (5 Prime, Milan, Italy). **1-11:** 45.2 °C to 65°C annealing temperature, **13-15:** negative controls, **16-26:** DNA extracted through "magnetic beads more organic solvents" from not diluted until 10^{-10}.

As shown in figure 7, primers formed dimers. Moreover, a positive signal of amplification was observed for *Dekkera bruxellensis*, which proved the lower specificity. On the basis of this result, specific primers from the *S. cerevisiae* ITS region were designed. The news primers, VF1 and VF3 were tested in the same experimental conditions. The results shown in figure 8 evidenced a non-amplification signal for *Dekkera bruxellensis.* It was also seen that amplification was stopped with a 10^{-8} DNA dilution.

Plate | Spectra | Component | Amplification Plot | Standard Curve | Dissociation | Report

Well	Sample Name	Detector	Task	Ct	StdDev Ct	Qty	Mean Qty	StdDev Qty	Filtered	Tm
A1	tq	SyBR	Standard	21.95	2.160					71.8 °C
A2	-1	SyBR	Standard	25.34	1.598					71.8 °C
A3	-2	SyBR	Standard	30.42	5.003					71.8 °C
A4	-3	SyBR	Standard	29.86	2.746					71.8 °C
A6	-5	SyBR	Standard	31.12	1.479					71.8 °C
A7	-6	SyBR	Standard	33.54	5.015					73.2 °C
A8	-7	SyBR	Standard	31.03	2.902					71.8 °C
A9	-8	SyBR	Standard	32.72	0.292					71.8 °C
B1	tq	SyBR	Standard	21.23	2.160					71.8 °C
B2	-1	SyBR	Standard	24.67	1.598					71.8 °C
B4	-3	SyBR	Standard	29.51	2.746					71.8 °C
B5	-4	SyBR	Standard	30.67	3.452					71.8 °C
B6	-5	SyBR	Standard	30.63	1.479					71.8 °C
B8	-7	SyBR	Standard	30.48	2.902					71.8 °C
B9	-8	SyBR	Standard	32.37	0.292					71.8 °C
C1	tq	SyBR	Standard	22.21	2.160					71.8 °C
C2	-1	SyBR	Standard	24.22	1.598					71.8 °C
C3	-2	SyBR	Standard	31.48	5.003					71.8 °C
C4	-3	SyBR	Standard	29.13	2.746					71.8 °C
C5	-4	SyBR	Standard	30.10	3.452					71.8 °C
C6	-5	SyBR	Standard	30.86	1.479					71.8 °C
C7	-6	SyBR	Standard	28.70	5.015					71.8 °C
C9	-8	SyBR	Standard	32.12	0.292					71.8 °C
G1	d	SyBR	Standard	Undet.						71.8 °C
G2	d	SyBR	Standard	Undet.						76.4 °C
G3	d	SyBR	Standard	Undet.						81.5 °C
H1	-	SyBR	Standard	Undet.						80.6 °C

Figure 8.
Real-time PCR report from ITS amplification using VF1/VF3

5.3.2.2 Reproducibility of technique

The trial that showed the best performance was repeated several times in order to verify the repeatability of amplification protocol. Each experiment showed always identical results for both minimum dilution of DNA amplified and absence of the signal amplification for the *D. bruxellensis*, demonstrating the primers specificity for *Saccharomyces* genus. Furthermore, since the *SYBR-GREEN* was used as intercalanting of nucleic bases during amplification in Real-Time PCR, at the end of the amplification reaction a temperature profile for the analysis of the melting curve was investigated. This step was necessary because *SYBR-GREEN* does not allow for distinguishing the specific products of amplification than non-specific ones. So, the quality and specificity of the amplification products obtained were assessed. Figures 8 and 9 show an example of the Real-Time PCR amplification, on which the melting temperature actually reflects the one obtained from samples in which *S. cerevisiae* DNA was added, where the peak of dissociation curve is equal to 71.8 ± 0.3 °C.

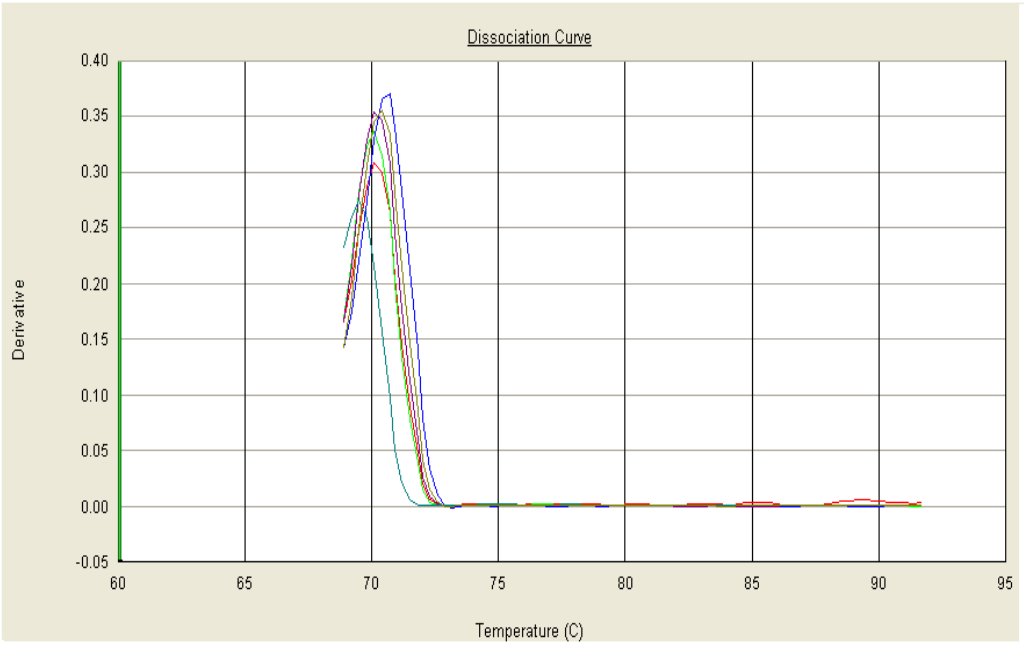

Figure 9.
DNA dissociation curve.

Setup | Instrument | Results

Plate | Spectra | Component | Amplification Plot | Standard Curve | Dissociation | Report

Well	Sample Name	Detector	Task	Ct	StdDev Ct	Qty	Mean Qty	StdDev Qty	Filtered	Tm
A1	mir 1 s	SyBR	Unknown	32.44	1.492					71.7 °C
A2	mir 2 s	SyBR	Unknown	39.03	1.231					71.7 °C
B1	mir 1 s	SyBR	Unknown	31.82	1.492					71.7 °C
B2	mir 2 s	SyBR	Unknown	37.47	1.231					71.7 °C
C1	mir 1 s	SyBR	Unknown	34.66	1.492					71.7 °C
C2	mir 2 s	SyBR	Unknown	36.60	1.231					71.7 °C

Figure 10.
Real-Time PCR amplification report on which the melting temperature is observed.

5.3.3 DNA monitoring during sparkling wine aging

In both *Oltrepò Pavese* and *Franciacorta* areas, two wineries were chosen (wineries 7 and 8 for *Oltrepò Pavese* and 1 and 4 for *Franciacorta*). To monitor the DNA presence during the aging of sparkling wine, Real-Time PCRs were conducted in triplicate for each sample. The results of these amplifications are reported in the table 2 as a function of the aging.

Winery Sample	Time of sparkling wine aging (months)	ITS Real-Time PCR amplification using VF1/VF3		
		Replica 1	Replica 2	Replica 3
1	6	+	+	+
4	6	+	+	+
8	6	+	+	+
7	6	+	+	+
1	8	+	+	+
4	8	+	+	+
8	8	+	+	+
7	8	+	+	+
1	10	+	+	+
4	10	+	+	+
8	10	+	+	+
7	10	+	+	+
1	14	-	+	+
4	14	+	+	-
8	14	+	+	+
7	16	+	+	+

Table 2.
Results from DNA monitoring during sparkling wine aging (*tirage* 2010 *Oltrepò Pavese* and *Franciacorta*). "magnetic beads and organic solvents" extraction method and ITS Real-Time PCR amplification using VF1/VF3

Figures 11 and 12 show an example of ITS Real-Time PCR amplification conducted on filtered samples of the winery 1 of *Franciacorta* territory at 8 months of aging, and winery 8 of *Oltrepò Pavese* territory at 10 months of aging.

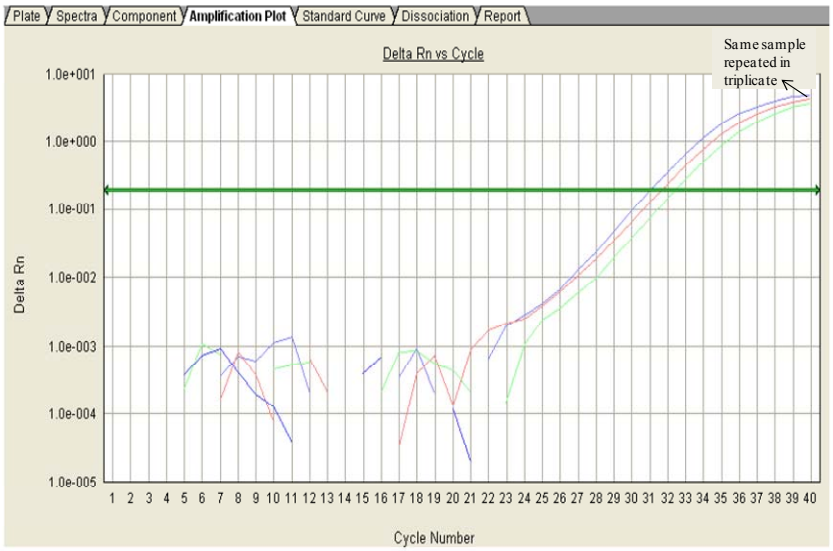

Figure 11.
ITS Real-Time PCR amplification using VF1/VF3 primers. DNA of sparkling wine at 8 months of aging in winery 1 of *Franciacorta* territory.

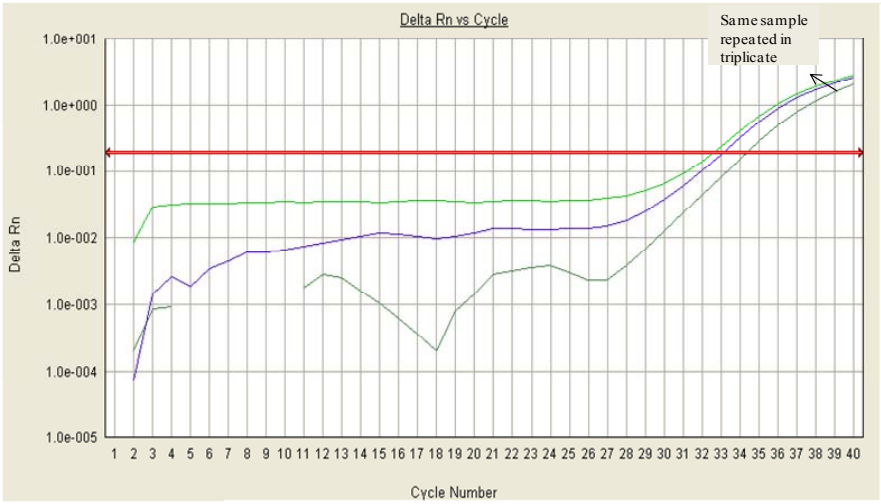

Figure 12.
ITS Real-Time PCR amplification using VF1/VF3 primers. DNA of sparkling wine at 10 months of aging in winery 8 of Oltrepò Pavese territory.

Sensitivity of the DNA extraction protocol was estimated. To obtain a result of amplification, 5,000 DNA molecules should be present in the bottle.

5.4 Discussion and Conclusion

This study was focused on the assessment of a protocol for extraction and amplification of yeast DNA from sparkling wine, aimed to a future development of a molecular test that allows the use of *S. cerevisiae* as "tracking" element during the process. This could be a suitable tool to certify the authenticity of the product as a prerequisite to obtain *DOCG* status, as well as in the wine-making chain. In the specific case of the sparkling wine made by *champenoise* method this could be due to those cellular residues staying in the bottle after the re-fermentation process.
Currently, very few studies have been focused on extracting DNA from wine. One of these was done in Portugal (Faria et al., 2000). Its protocol to extract DNA consisted on a concentration of the must through centrifuges (or for vine leaves, on breaking the vegetal structure by a sterilised pestle)and extraction through CTAB (Hexadecyl trimethyl-ammonium bromide) , ß-mercaptoethanol and several purification steps. This research obtained very good results after RAPD amplification, since it started from a high concentration of microorganisms as the susbtrate was very favourable for growth. This protocol was improved later in a further analysis by the same researchers and obtained better results than the former protocol (Faria et al., 2008). Clearly, big DNA quantities have been obtained through this method since the starting substrate was even richer in microorganism than a normal commercial wine. Another study in *Trento* (Italy) (Savazzini et al., 2006) was focused on comparing three different DNA extraction methods from final wine: the three cases have been distinguished by how the DNA was initially precipitated. The DNA precipitation was performed with 5 M NaCl, with isopropanol or with Na-acetate after the precipitation. The three methods converged to the same process where DNA was extracted by using CTAB buffer, including ß-mercaptoethanol and polivynilpirrolidone. DNA extracted was then amplified with Real-Time PCR.
The Na-acetate solution protocol achieved better results. On the basis of these results, in the present study, Na-acetate was also used as a first attempt to extract DNA from sparkling wine; the result was not satisfactory anyway, probably because a lower quantity of DNA was present into the bottles. In a recent research (Jara et al., 2008) other DNA extraction protocols have been compared through the analysis from acetic acid bacteria in wine and vinegar. In particular, four different methods were analysed: *Wizard*, buffer CTAB method, *Nucleospin* kit and *Mo-Bio* kit. The better results were obtained with *Nucleospin* kit. In fact, the *Nucleospin* kit was used in DNA extraction from sparkling wine, but with still not satisfactory results, probably due to columns with a too-small capacity (2 ml) which forced at a small quantity of recovered DNA.
The bottled wine was plated and growth of yeast colonies was obtained; in fact, cells in suspensions were still present at a following analysis and confirmed by a microscope observation, despite a *remuage* and disgorgement process. This result was useful to define a new extraction protocol where these cells were separated from the liquid phase at an initial stage. In order to reach this result, cells filtering was performed on a membrane which, in a second stage, was subjected to DNA extraction according to Querol et al (1992). Anyway these results were not repeatable, probably because some bottles had some cells in suspension, whereas other did not; this is probably due to the different processes used in the wineries, in particular with disgorgement. The last attempt was extracting DNA through magnetic beads performed with organic solvents which could improve the total process of DNA extraction from wine. Wine samples were initially precipitated with isopropanol and treated with proteinase K in order to degrade both polysaccharides and proteins which could inhibit PCR reaction (Nakamura et al., 2007), purified with organic solvents as phenol, chloroform and isoamylic alcohol and finally subjected to magnetic beads process. protocols using magnetic beads for DNA extraction from wines are very unusual. Nonetheless, in the case of other food matrices is widely diffused and it has demonstrated to be effective.

For example, a research in *Fano* (region of *Marche,* Italy) was focused in extracting *Lysteria monocytogenes* DNA from the milk through magnetic beads. The final results showed that the method was successful and specific, allowing also for eliminating some components able to interfere with *Taq* polymerase during the DNA amplification (Amagliani et al., 2005). Another research was carried out in Spain (Cepeda et al., 2000) and the magnetic beads were used to extract DNA from *Flavobacterium psychrophilum,* a pathogen bacterium, able to contaminate especially the fish products. Using the magnetic beads method on the product after a homogenization, great results were obtained with a very quick process of identification.
Taken this into account, magnetic beads method was carried out for this research and associated to an extraction protocol using organic solvents. In order to evaluate the accuracy of the method, some wine samples were tested adding DNA at a certain quantity; specific primers for *Saccharomyces* were initially used to amplify the DNA according to Zott et al. (2010). After some tests in Real-time PCR, these primers were producing false-positive results, due to both the dimers formation and the non-specific amplification. Actually, they produced positive results also in case of addition of a non-*Saccharomyces* DNA (i.e. *D. bruxellensis*). Due to these results, special primers, named VF1 and VF3, were design on the region ITS of *S. cerevisieae.* The results were satisfactory. This new extracting process was applied to the filtered wine sample, from *Oltrepò Pavese* and *Franciacorta*
after the *tirage* prove of 2010. In particular, the sparkling wine aging period was monitored during different intervals of time; two wineries from each area were taken into consideration and the monitoring was done from T180 (6 months of aging) until T420 (14 months of aging) or T480 (16 months of aging) in the case of *Oltrepò Pavese* winery 7. The results showed how the DNA extraction occurred smoothly during all the monitoring timing.
Future developments of the protocol will be focused on applying the same DNA extraction protocol to commercial bottles ready for the consumer market. If the second fermentation was conducted by an indigenous strain, the residual DNA could be an element of traceability to ensure the quality and authenticity of the wine, representing a benefit for both producers and consumers.

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Chapter 6
Conclusions and future perspectives

6.1 Final conclusion

The main purposes of this PhD thesis were focused on:

- Isolation and genetic identification of yeasts involved in winemaking process in *Franciacorta* and *Oltrepò Pavese* areas in order to evaluate the population biodiversity;
- Determination of technological characteristic of indigenous *S. cerevisiae* strain to be used as new starters in the sparkling wine production made by *champenoise* method;
- Development of a protocol for recovery of DNA from commercial sparkling wines, aimed to improve the traceability of *DOCG* Lombardy sparkling wines.

The study was conducted during 2009, 2010 and 2011 vintages in *Franciacorta* and *Oltrepò Pavese* areas where a great yeast species biodiversity were found, confirming the vast potential of these areas as a source of isolation of a large number of interesting oenological yeasts. A remarkable biodiversity was observed for both *Saccharomyces* and Non-*Saccaromyces* species which support the study significance since it has been widely demonstrated that wine quality is strongly correlated to the yeasts involved in the fermentation process. Assessment yeasts biodiversity in both the various stages of vintages and monitoring of yeast evolution during controlled and spontaneous alcoholic fermentation, was relevant for understanding the evolution in winemaking process. Consequently, these results allow for increasing the control capacity on such processes. Regarding *S. cerevisiae* species, a large collection of strains was set up from isolates involved in winemaking process (chapter 1) as well as from alcoholic fermentations (chapter 3). Through intraspecific genetic diversity analysis, it was possible to observe that in *Franciacorta* and *Oltrepò Pavese* territories there is a great opportunity to select autochthonous *S. cerevisiae* strains with typical oenological characters which could be representative of these oenological areas. In fact another important result of this research was obtained through the technological and qualitative characterizations of some indigenous *S. cerevisiae* strains. From microfermentation tests in laboratory with *tirage* proves at pilot scale in different *Franciacorta* and *Oltrepò Pavese* wineries, two indigenous starters representative of each area demonstrated a high potential to implant and carry out the fermentation successfully. In addition, this potential was supported through tasting-wine proves on which wines produced by these indigenous starters were sensorially accepted at 6 months of aging. The use of indigenous starters for sparkling wine production in *Franciacorta* and *Oltrepò Pavese* areas translates into the real opportunity to improve the *DOCG Franciacorta* and *DOCG Oltrepò Pavese Metodo Classico* by enhancing the 'local value' (typicality), thus promoting the diversification of wine products. The latter contribution of this research was establishing a novel protocol for extraction and amplification of yeast DNA from sparkling wine. The result was significant and as consequence, this protocol could be used to improve the traceability for the *DOCG* sparkling wines in Lombardy, allowing the use of *S. cerevisiae* as "trace element" during the process of sparkling wine authentication and as prerequisite to obtain *DOCG* status as well as in the wine-making production chain.

6.2 Future perspectives

The future development of this research will address the understanding of some aspects regarding to sensory characteristics of sparkling wines made by using four indigenous strains selected in this study as indigenous starters during 2011 *tirage* test in *Franciacorta* and *Oltrepò Pavese*. When the wine aging period in bottle will be completed, a second wine-tasting is required to a more precise evaluation. In addition, efforts will be emphasized in determining chemical compounds produced by these yeasts and their impact in wine quality. Another perspective will be focused on evaluating the behavior of these indigenous strains as a culture starter mixture. The combination of more than one starter strain could optimize the results of implantation capacity and fermentation process. In addition to this assessment, it is essential to determine whether these strains will be able to pass the test of large-scale production at industrial level because, although many strains have good quality and technological characters, unfortunately not all of them pass this barrier. After that, an attempt to replace the commercial starters that are currently used in *Franciacorta* and *Oltrepò Pavese* wineries for sparkling wine production would be possible. It is a long process but the obtained results so far are more than promising. This objective is closely related with setting up a final protocol for the yeast DNA extraction and its future employment on large scale; the optimisation of the protocol described in this study as a bimolecular test could be a starting point and finally could permit the verification of the commercial sparkling wine authenticity as a prerequisite to obtain *DOCG* status; if the second fermentation was conducted by a *Franciacorta* or *Oltrepò Pavese* indigenous starter, its DNA could be a trace element that would ensure the quality and authenticity of the wine. Although isolating DNA from commercial sparkling wine is difficult, the results of this study show that DNA was successfully amplified from wine with 16 months of aging. Therefore, a molecular analysis which is currently under development (coinciding with the minimum aging time required to release *Franciacorta* and *Oltrepò Pavese* DOCG) could be included as a requirement to *DOCG* release, together to chemical and sensory analysis.
Hopefully, the results of this research will be disclosed in a convention for *Franciacorta* and *Oltrepò Pavese* wine sector and discussed with the local wineries in order to evaluate the impact of this study on the development and improvement of wine production in Lombardy region.

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APPENDIX A

Isolate yeasts collection from *Franciacorta* and *Oltrepò Pavese* areas
2009-2010-2011
vintages

VINTAGE	ORIGIN*	WINERY CODE	SAMPLE	GENETIC IDENTIFICATION	ISOLATE
2009	FCR	BVT	AIR	*Aureobasidium pullulans*	1FA-11Z500
2009	FCR	UBT	AIR	*Aureobasidium pullulans*	1FA-123X100
2009	FCR	VLA	AIR	*Cryptococcus laurentii*	1FA-5X500
2009	FCR	MTR	AIR	*Aureobasidium pullulans*	1FA-124Z100A
2009	FCR	MTR	AIR	*Aureobasidium pullulans*	1FA-124Z100B
2009	FCR	MTR	AIR	*Cryptococcus laurentii*	1FA-124Z500
2009	FCR	MTR	AIR	*Cryptococcus laurentii*	1FA-124Z1001
2009	FCR	MTR	AIR	*Cryptococcus laurentii*	1FA-124Z1002
2009	FCR	BVT	MUST	*Saccharomyces cerevisiae*	1FM-11A
2009	FCR	BVT	MUST	*Saccharomyces cerevisiae*	1FM-11B
2009	FCR	BVT	MUST	*Saccharomyces cerevisiae*	1FM-11C
2009	FCR	BVT	MUST	*Saccharomyces cerevisiae*	1FM-40A
2009	FCR	BVT	MUST	*Saccharomyces cerevisiae*	1FM-40B
2009	FCR	BVT	MUST	*Saccharomyces cerevisiae*	1FM-40D
2009	FCR	BVT	MUST	*Saccharomyces cerevisiae*	1FM-19C
2009	FCR	UBT	MUST	*Saccharomyces cerevisiae*	1FM-123A
2009	FCR	VLA	MUST	*Saccharomyces cerevisiae*	1FM-2A
2009	FCR	VLA	MUST	*Saccharomyces cerevisiae*	1FM-2B
2009	FCR	FGT	MUST	*Saccharomyces cerevisiae*	1FM-43B
2009	FCR	FGT	MUST	*Saccharomyces cerevisiae*	1FM-43C
2009	FCR	MJL	MUST	*Saccharomyces cerevisiae*	1FM-59B
2009	FCR	MJL	MUST	*Saccharomyces cerevisiae*	1FM-59C
2009	FCR	MJL	MUST	*Saccharomyces cerevisiae*	1FM-59D
2009	FCR	CBC	MUST	*Saccharomyces cerevisiae*	1FM-C2C2
2009	FCR	CBC	MUST	*Saccharomyces cerevisiae*	1FM-C2D1
2009	FCR	CBC	MUST	*Saccharomyces cerevisiae*	1FM-C2D2
2009	FCR	CBC	MUST	*Saccharomyces cerevisiae*	1FM-C2B
2009	FCR	CBC	MUST	*Saccharomyces cerevisiae*	1FM-T1B
2009	FCR	VLA	MUST	*Saccharomyces cerevisiae*	1FM-5A
2009	FCR	VLA	MUST	*Saccharomyces cerevisiae*	1FM-5B
2009	FCR	BVT	MUST	*Issatchenkia occidentalis*	1FM-19A
2009	FCR	BVT	MUST	*Issatchenkia occidentalis*	1FM-19B
2009	FCR	BVT	MUST	*Issatchenkia occidentalis*	1FM-40C
2009	FCR	BVT	MUST	*Issatchenkia occidentalis*	1FM-40E
2009	FCR	FGT	MUST	*Issatchenkia occidentalis*	1FM-43A
2009	FCR	FGT	MUST	*Issatchenkia occidentalis*	1FM-122
2009	FCR	MTR	MUST	*Zygosaccharomyces bailii*	1FM-124A

VINTAGE	ORIGIN	WINERY CODE	SAMPLE	GENETIC IDENTIFICATION	ISOLATE
2009	FCR	MTR	MUST	*Zygosaccharomyces bailii*	1FM-124B
2009	FCR	MTR	MUST	*Zygosaccharomyces bailii*	1FM-124C
2009	FCR	MTR	MUST	*Zygosaccharomyces bailii*	1FM-124D
2009	FCR	VLA	MUST	*Zygosaccharomyces bailii*	1FM-C2C1
2009	FCR	VLA	MUST	*Zygosaccharomyces bailii*	1FM-C2E
2009	FCR	VLA	MUST	*Zygosaccharomyces bailii*	1FM-5C
2009	FCR	BVT	MUST	*Zygosaccharomyces bailii*	1FM-11D
2009	FCR	VLA	MUST	*Zygosaccharomyces bailii*	1FM-C2A
2009	FCR	MJL	MUST	*Zygosaccharomyces bailii*	1FM-59A
2009	FCR	CBC	MUST	*Zygosaccharomyces bailii*	1FM-T1A
2009	FCR	VLA	BASE WINE	*Saccharomyces cerevisiae*	1F-V2-1
2009	FCR	BVT	BASE WINE	*Saccharomyces cerevisiae*	1F-V19-1
2009	FCR	BVT	BASE WINE	*Saccharomyces cerevisiae*	1F-V19-2
2009	FCR	BVT	BASE WINE	*Saccharomyces cerevisiae*	1F-V19-3
2009	FCR	BVT	BASE WINE	*Saccharomyces cerevisiae*	1F-V19-4
2009	FCR	BVT	BASE WINE	*Saccharomyces cerevisiae*	1F-V19-5
2009	FCR	BVT	BASE WINE	*Saccharomyces cerevisiae*	1F-V40-5
2009	FCR	BVT	BASE WINE	*Saccharomyces cerevisiae*	1F-V40-6
2009	FCR	BVT	BASE WINE	*Saccharomyces cerevisiae*	1F-V40-9
2009	FCR	BVT	BASE WINE	*Saccharomyces cerevisiae*	1F-V40-10
2009	FCR	FGT	BASE WINE	*Saccharomyces cerevisiae*	1F-V43-1
2009	FCR	FGT	BASE WINE	*Saccharomyces cerevisiae*	1F-V43-2
2009	FCR	FGT	BASE WINE	*Saccharomyces cerevisiae*	1F-V43-3
2009	FCR	FGT	BASE WINE	*Saccharomyces cerevisiae*	1F-V43-4
2009	FCR	UBT	BASE WINE	*Saccharomyces cerevisiae*	1F-V120-1
2009	FCR	UBT	BASE WINE	*Saccharomyces cerevisiae*	1F-V120-5
2009	FCR	UBT	BASE WINE	*Saccharomyces cerevisiae*	1F-V123-2
2009	FCR	UBT	BASE WINE	*Saccharomyces cerevisiae*	1F-V123-3
2009	FCR	UBT	BASE WINE	*Saccharomyces cerevisiae*	1F-V123-4
2009	FCR	UBT	BASE WINE	*Saccharomyces cerevisiae*	1F-V123-5
2009	FCR	UBT	BASE WINE	*Saccharomyces cerevisiae*	1F-V123-6
2009	FCR	MTR	BASE WINE	*Saccharomyces cerevisiae*	1F-V124-1
2009	FCR	MTR	BASE WINE	*Saccharomyces cerevisiae*	1F-V124-2
2009	FCR	MTR	BASE WINE	*Saccharomyces cerevisiae*	1F-V124-3
2009	FCR	MTR	BASE WINE	*Saccharomyces cerevisiae*	1F-V124-4
2009	FCR	MTR	BASE WINE	*Saccharomyces cerevisiae*	1F-V124-6
2009	FCR	CBC	BASE WINE	*Saccharomyces cerevisiae*	1F-VC2-1

VINTAGE	ORIGIN	WINERY CODE	SAMPLE	GENETIC IDENTIFICATION	ISOLATE
2009	FCR	CBC	BASE WINE	*Saccharomyces cerevisiae*	1F-VC2-2
2009	FCR	CBC	BASE WINE	*Saccharomyces cerevisiae*	1F-VC2-3
2009	FCR	CBC	BASE WINE	*Saccharomyces cerevisiae*	1F-VC2-4
2009	FCR	UBT	BASE WINE	*Saccharomyces cerevisiae*	1F-VSU-2
2009	FCR	VLA	BASE WINE	*Saccharomyces cerevisiae*	1F-V5-1
2009	FCR	VLA	BASE WINE	*Pichia membranifaciens*	1F-V2-3
2009	FCR	VLA	BASE WINE	*Pichia membranifaciens*	1F-V2-4
2009	FCR	UBT	BASE WINE	*Pichia membranifaciens*	1F-V123-5
2009	FCR	BVT	BASE WINE	*Pichia membranifaciens*	1F-V40-7
2009	FCR	BVT	BASE WINE	*Pichia membranifaciens*	1F-V40-8
2009	FCR	UBT	BASE WINE	*Pichia membranifaciens*	1F-V120-2
2009	FCR	UBT	BASE WINE	*Pichia membranifaciens*	1F-V123-1
2009	FCR	CBC	BASE WINE	*Pichia membranifaciens*	1F-VT1-2
2009	FCR	MTR	BASE WINE	*Pichia membranifaciens*	1F-V124-5
2009	FCR	VLA	BASE WINE	Zygosaccharomyces bailii	1F-V2-2
2009	FCR	VLA	BASE WINE	Zygosaccharomyces bailii	1F-V2-6
2009	FCR	UBT	BASE WINE	*Issatchenkia occidentalis*	1F-V120-3
2009	FCR	CBC	BASE WINE	*Pichia fluxum*	1F-VC2-5
2009	FCR	FGT	BASE WINE	*Pichia fluxum*	1F-V122-1
2009	FCR	FGT	BASE WINE	*Pichia fluxum*	1F-V122-2
2009	FCR	UBT	BASE WINE	*Torulaspora delbrueckii*	1F-VSU-1
2009	OLT	SGG	AIR	*Saccharomyces cerevisiae*	1OA-SG-IIA
2009	OLT	SGG	AIR	*Saccharomyces cerevisiae*	1OA-SG-VS
2009	OLT	MLN	AIR	*Saccharomyces cerevisiae*	1OA-MZZ-I
2009	OLT	MLN	AIR	*Saccharomyces cerevisiae*	1OA-MZZ
2009	OLT	MLN	AIR	*Saccharomyces cerevisiae*	1OA-MZZ-II
2009	OLT	MLN	AIR	*Saccharomyces cerevisiae*	1OA-MZZ-IIA
2009	OLT	TVN	AIR	*Saccharomyces cerevisiae*	1OA-TRV-IA
2009	OLT	QRN	AIR	*Aureobasidium pullulans*	1OA-QUA-1
2009	OLT	VRD	AIR	*Issatchenkia spp*	1OA-VRD-B
2009	OLT	ATO	MUST	*Saccharomyces cerevisiae*	1OM-ANT-BB
2009	OLT	ATO	MUST	*Saccharomyces cerevisiae*	1OM-ANT-VA
2009	OLT	ATO	MUST	*Saccharomyces cerevisiae*	1OM-ANT-VB
2009	OLT	CSO	MUST	*Saccharomyces cerevisiae*	1OM-CSO-BA
2009	OLT	MSL	MUST	*Saccharomyces cerevisiae*	1OM-MNS-BA
2009	OLT	MSL	MUST	*Saccharomyces cerevisiae*	1OM-MNS-BB
2009	OLT	MSL	MUST	*Saccharomyces cerevisiae*	1OM-MNS-VB

VINTAGE	ORIGIN	WINERY CODE	SAMPLE	GENETIC IDENTIFICATION	ISOLATE
2009	OLT	SGG	MUST	*Saccharomyces cerevisiae*	1OM-SG-BP
2009	OLT	SGG	MUST	*Saccharomyces cerevisiae*	1OM-SG-VA
2009	OLT	MLN	MUST	*Saccharomyces cerevisiae*	1OM-MZZ-BA
2009	OLT	MLN	MUST	*Saccharomyces cerevisiae*	1OM-MZZ-BB
2009	OLT	MLN	MUST	*Saccharomyces cerevisiae*	1OM-MZZ-BC
2009	OLT	VTN	MUST	*Saccharomyces cerevisiae*	1OM-VST-BA
2009	OLT	VTN	MUST	*Saccharomyces cerevisiae*	1OM-VST-BB
2009	OLT	VRD	MUST	*Zygosaccharomyces bailii*	1OM-VRD-BB
2009	OLT	SGG	MUST	*Zygosaccharomyces bailii*	1OM-SG-BA
2009	OLT	ATO	MUST	*Zygosaccharomyces bailii*	1OM-ANT-BA
2009	OLT	TVN	MUST	*Zygosaccharomyces bailii*	1OM-TRV-BA
2009	OLT	CSO	MUST	*Issatchenkia spp*	1OM-CSO-BB
2009	OLT	CSO	MUST	*Hanseniaspora uvarum*	1OM-CSO-VA
2009	OLT	QRN	MUST	*Candida zemplinina*	1OM-QUA-V
2009	OLT	TVN	MUST	*Candida diversa*	1OM-TRV-VBB
2009	OLT	ATO	BASE WINE	*Saccharomyces cerevisiae*	1OV-ANT-VB4
2009	OLT	ATO	BASE WINE	*Saccharomyces cerevisiae*	1OV-ANT-VB5
2009	OLT	ATO	BASE WINE	*Saccharomyces cerevisiae*	1OV-ANT-VB6
2009	OLT	ATO	BASE WINE	*Saccharomyces cerevisiae*	1OV-ANT-VB7
2009	OLT	VTN	BASE WINE	*Saccharomyces cerevisiae*	1OV-VST-VB6
2009	OLT	VTN	BASE WINE	*Saccharomyces cerevisiae*	1OV-VST-VB4
2009	OLT	VTN	BASE WINE	*Saccharomyces cerevisiae*	1OV-VST-VB5
2009	OLT	IBC	BASE WINE	*Saccharomyces cerevisiae*	1OV-ILB-VB1
2009	OLT	IBC	BASE WINE	*Saccharomyces cerevisiae*	1OV-ILB-VB5
2009	OLT	IBC	BASE WINE	*Saccharomyces cerevisiae*	1OV-ILB-VB3
2009	OLT	IBC	BASE WINE	*Saccharomyces cerevisiae*	1OV-ILB-VB4
2009	OLT	VRD	BASE WINE	*Saccharomyces cerevisiae*	1OV-VRD-VB6
2009	OLT	MSL	BASE WINE	*Saccharomyces cerevisiae*	1OV-MNS-VB3
2009	OLT	IBC	BASE WINE	*Saccharomyces cerevisiae*	1OV-ILB-VB2
2009	OLT	CSO	BASE WINE	*Saccharomyces cerevisiae*	1OV-CSO-VB3
2009	OLT	CSO	BASE WINE	*Saccharomyces cerevisiae*	1OV-CSO-VB6
2009	OLT	CSO	BASE WINE	*Saccharomyces cerevisiae*	1OV-CSO-VB5
2009	OLT	QRN	BASE WINE	*Saccharomyces cerevisiae*	1OV-QUA-VB7
2009	OLT	QRN	BASE WINE	*Saccharomyces cerevisiae*	1OV-QUA-VB5
2009	OLT	QRN	BASE WINE	*Saccharomyces cerevisiae*	1OV-QUA-VB2
2009	OLT	TVN	BASE WINE	*Saccharomyces cerevisiae*	1OV-TRV-VB5
2009	OLT	TVN	BASE WINE	*Saccharomyces cerevisiae*	1OV-TRV-VB6

VINTAGE	ORIGIN	WINERY CODE	SAMPLE	GENETIC IDENTIFICATION	ISOLATE
2009	OLT	TVN	BASE WINE	*Saccharomyces cerevisiae*	1OV-TRV-VB7
2009	OLT	TVN	BASE WINE	*Saccharomyces cerevisiae*	1OV-TRV-VB4
2009	OLT	MSL	BASE WINE	*Saccharomyces cerevisiae*	1OV-MNS-VB1
2009	OLT	ATO	BASE WINE	*Saccharomyces cerevisiae*	1OV-ANT-VB1
2009	OLT	CSO	BASE WINE	*Saccharomyces cerevisiae*	1OV-CSO-VB7
2009	OLT	VTN	BASE WINE	*Saccharomyces cerevisiae*	1OV-VST-VB2
2009	OLT	VRD	BASE WINE	*Saccharomyces cerevisiae*	1OV-VRD-VB2
2009	OLT	TVN	BASE WINE	*Saccharomyces cerevisiae*	1OV-TRV-VB2
2009	OLT	TVN	BASE WINE	*Saccharomyces cerevisiae*	1OV-TRV-VB1
2009	OLT	IBD	BASE WINE	*Saccharomyces cerevisiae*	1OV-ISM-VB1
2009	OLT	IBD	BASE WINE	*Saccharomyces cerevisiae*	1OV-ISM-VB2
2009	OLT	VRD	BASE WINE	*Saccharomyces cerevisiae*	1OV-VRD-VB3
2009	OLT	VRD	BASE WINE	*Saccharomyces cerevisiae*	1OV-VRD-VB1
2009	OLT	VTN	BASE WINE	*Saccharomyces cerevisiae*	1OV-VST-VB1
2009	OLT	TVN	BASE WINE	*Saccharomyces cerevisiae*	1OV-TRV-VB3
2009	OLT	ATO	BASE WINE	*Saccharomyces cerevisiae*	1OV-ANT-VB2
2009	OLT	MLN	BASE WINE	*Saccharomyces cerevisiae*	1OV-MZZ-VB1
2009	OLT	MSL	BASE WINE	*Saccharomyces cerevisiae*	1OV-MNS-VB2
2009	OLT	ATO	BASE WINE	*Saccharomyces cerevisiae*	1OV-ANT-VB3
2009	OLT	SGG	BASE WINE	*Saccharomyces cerevisiae*	1OV-SG-VB2
2009	OLT	IBD	BASE WINE	*Saccharomyces cerevisiae*	1OV-ISM-VB3
2009	OLT	VTN	BASE WINE	*Saccharomyces cerevisiae*	1OV-VST-VB3
2009	OLT	QRN	BASE WINE	*Saccharomyces cerevisiae*	1OV-QUA-VB1
2009	OLT	QRN	BASE WINE	*Saccharomyces cerevisiae*	1OV-QUA-VB3
2009	OLT	QRN	BASE WINE	*Saccharomyces cerevisiae*	1OV-QUA-VB4
2009	OLT	QRN	BASE WINE	*Saccharomyces cerevisiae*	1OV-QUA-VB6
2009	OLT	CSO	BASE WINE	*Saccharomyces cerevisiae*	1OV-CSO-VB1
2009	OLT	CSO	BASE WINE	*Saccharomyces cerevisiae*	1OV-CSO-VB2
2009	OLT	CSO	BASE WINE	*Saccharomyces cerevisiae*	1OV-CSO-VB4
2009	OLT	CSO	BASE WINE	*Saccharomyces cerevisiae*	1OV-CSO-VB8
2010	FCR	BVT	MUST	*Saccharomyces cerevisiae*	2F-M11-III
2010	FCR	FGT	MUST	*Saccharomyces cerevisiae*	2F-M43-VIII
2010	FCR	MJL	MUST	*Saccharomyces cerevisiae*	2F-M59-I
2010	FCR	MJL	MUST	*Saccharomyces cerevisiae*	2F-M59-III
2010	FCR	UBT	MUST	*Saccharomyces cerevisiae*	2F-M120-III
2010	FCR	UBT	MUST	*Saccharomyces cerevisiae*	2F-M120-IV
2010	FCR	BVT	MUST	*Saccharomyces cerevisiae*	2F-MC19-I

VINTAGE	ORIGIN	WINERY CODE	SAMPLE	GENETIC IDENTIFICATION	ISOLATE
2010	FCR	FGT	MUST	*Saccharomyces cerevisiae*	2F-MC-43-II
2010	FCR	FGT	MUST	*Saccharomyces cerevisiae*	2F-MC43-V
2010	FCR	FGT	MUST	*Saccharomyces cerevisiae*	2F-MC43-VI
2010	FCR	MJL	MUST	*Saccharomyces cerevisiae*	2F-MC59-VI
2010	FCR	UBT	MUST	*Saccharomyces cerevisiae*	2F-MC120-III
2010	FCR	FGT	MUST	*Saccharomyces cerevisiae*	2F-MC122-IV
2010	FCR	FGT	MUST	*Saccharomyces cerevisiae*	2F-M43-IV
2010	FCR	FGT	MUST	*Saccharomyces cerevisiae*	2F-M43-V
2010	FCR	FGT	MUST	*Saccharomyces cerevisiae*	2F-M122-III
2010	FCR	FGT	MUST	*Saccharomyces cerevisiae*	2F-M122-IV
2010	FCR	FGT	MUST	*Saccharomyces cerevisiae*	2F-M122-VI
2010	FCR	UBT	MUST	*Saccharomyces cerevisiae*	2F-M123-II
2010	FCR	MJL	MUST	*Saccharomyces cerevisiae*	2F-MC59-II
2010	FCR	MJL	MUST	*Saccharomyces cerevisiae*	2F-MC59-III
2010	FCR	MJL	MUST	*Saccharomyces cerevisiae*	2F-MC59-IV
2010	FCR	MJL	MUST	*Saccharomyces cerevisiae*	2F-MC59-V
2010	FCR	FGT	MUST	*Saccharomyces cerevisiae*	2F-M43-VI
2010	FCR	FGT	MUST	*Saccharomyces cerevisiae*	2F-M43-VII
2010	FCR	FGT	MUST	*Saccharomyces cerevisiae*	2F-MC122-VI
2010	FCR	UBT	MUST	*Saccharomyces cerevisiae*	2F-MC123-I
2010	FCR	BVT	MUST	*Metschnikowia fructicola*	2F-M11-II
2010	FCR	BVT	MUST	*Metschnikowia fructicola*	2F-M11-VI
2010	FCR	BVT	MUST	*Metschnikowia fructicola*	2F-M19-I
2010	FCR	BVT	MUST	*Metschnikowia fructicola*	2F-M19-V
2010	FCR	CBC	MUST	*Metschnikowia fructicola*	2F-M34-I
2010	FCR	BVT	MUST	*Metschnikowia fructicola*	2F-M40-I
2010	FCR	FGT	MUST	*Metschnikowia fructicola*	2F-M43-I
2010	FCR	MJL	MUST	*Metschnikowia fructicola*	2F-M59-IV
2010	FCR	UBT	MUST	*Metschnikowia fructicola*	2F-M120-II
2010	FCR	FGT	MUST	*Metschnikowia fructicola*	2F-M122-I
2010	FCR	FGT	MUST	*Metschnikowia fructicola*	2F-M122-II
2010	FCR	UBT	MUST	*Metschnikowia fructicola*	2F-M123-III
2010	FCR	UBT	MUST	*Metschnikowia fructicola*	2F-M123-IV
2010	FCR	MTR	MUST	*Metschnikowia fructicola*	2F-M124-I
2010	FCR	BVT	MUST	*Metschnikowia fructicola*	2F-MC11-V
2010	FCR	BVT	MUST	*Hanseniaspora uvarum*	2F-M11-I
2010	FCR	BVT	MUST	*Hanseniaspora uvarum*	2F-M19-II

VINTAGE	ORIGIN	WINERY CODE	SAMPLE	GENETIC IDENTIFICATION	ISOLATE
2010	FCR	CBC	MUST	*Hanseniaspora uvarum*	2F-M34-II
2010	FCR	CBC	MUST	*Hanseniaspora uvarum*	2F-M34-IV
2010	FCR	BVT	MUST	*Hanseniaspora uvarum*	2F-M40-II
2010	FCR	MJL	MUST	*Hanseniaspora uvarum*	2F-M59-II
2010	FCR	UBT	MUST	*Hanseniaspora uvarum*	2F-M120-I
2010	FCR	FGT	MUST	*Hanseniaspora uvarum*	2F-M122-V
2010	FCR	UBT	MUST	*Hanseniaspora uvarum*	2F-M123-I
2010	FCR	BVT	MUST	*Hanseniaspora uvarum*	2F-MC11-III
2010	FCR	CBC	MUST	*Hanseniaspora uvarum*	2F-MC34-I
2010	FCR	FGT	MUST	*Hanseniaspora uvarum*	2F-MC43-IV
2010	FCR	FGT	MUST	*Hanseniaspora uvarum*	2F-MC122-VII
2010	FCR	FGT	MUST	*Pichia kluyveri*	2F-M43-II
2010	FCR	FGT	MUST	*Pichia kluyveri*	2F-M43-III
2010	FCR	MJL	MUST	*Pichia kluyveri*	2F-M59-V
2010	FCR	BVT	MUST	*Pichia kluyveri*	2F-M11-II
2010	FCR	BVT	MUST	*Pichia kluyveri*	2F-MC11-VI
2010	FCR	BVT	MUST	*Pichia kluyveri*	2F-MC40-I
2010	FCR	FGT	MUST	*Pichia kluyveri*	2F-MC43-III
2010	FCR	UBT	MUST	*Pichia kluyveri*	2F-MC120-II
2010	FCR	FGT	MUST	*Pichia kluyveri*	2F-MC122-I
2010	FCR	FGT	MUST	*Pichia kluyveri*	2F-MC122-III
2010	FCR	BVT	MUST	*Issatchenkia occidentalis*	2F-MC19-III
2010	FCR	CBC	MUST	*Issatchenkia occidentalis*	2F-M34-V
2010	FCR	BVT	MUST	*Issatchenkia occidentalis*	2F-M19-III
2010	FCR	CBC	MUST	*Issatchenkia occidentalis*	2F-MC34-II
2010	FCR	CBC	MUST	*Issatchenkia occidentalis*	2F-MC34-III
2010	FCR	BVT	MUST	*Issatchenkia terricola*	2F-MC11-I
2010	FCR	BVT	MUST	*Issatchenkia terricola*	2F-MC19-II
2010	FCR	BVT	MUST	*Issatchenkia terricola*	2F-MC40-II
2010	FCR	FGT	MUST	*Issatchenkia terricola*	2F-MC43-I
2010	FCR	FGT	MUST	*Issatchenkia terricola*	2F-MC122-II
2010	FCR	BVT	MUST	*Torulaspora delbrueckii*	2F-M11-IV
2010	FCR	BVT	MUST	*Torulaspora delbrueckii*	2F-M19-IV
2010	FCR	CBC	MUST	*Torulaspora delbrueckii*	2F-M34-VI
2010	FCR	BVT	MUST	*Torulaspora delbrueckii*	2F-M40-III
2010	FCR	BVT	MUST	*Pichia anomala*	2F-M11-V
2010	FCR	BVT	MUST	*Pichia anomala*	2F-M40-IV

VINTAGE	ORIGIN	WINERY CODE	SAMPLE	GENETIC IDENTIFICATION	ISOLATE
2010	FCR	UBT	MUST	*Pichia anomala*	2F-M120-V
2010	FCR	MJL	MUST	*Pichia kudriavzevii*	2F-MC59-I
2010	FCR	UBT	MUST	*Pichia kudriavzevii*	2F-MC120-I
2010	FCR	MTR	MUST	*Rhodotorula spp*	2F-M124-II
2010	FCR	MTR	MUST	*Rhodotorula spp*	2F-M124-IV
2010	FCR	BVT	MUST	*Candida parapsilosis*	2F-MC11-IV
2010	FCR	CBC	MUST	*Candida zemplinina*	2F-M34-III
2010	FCR	MTR	MUST	*Cryptococcus flavescens*	2F-M124-III
2010	FCR	FGT	MUST	*Pichia guilliermondii*	2F-MC122-V
2010	FCR	UBT	MUST	*Zygoascus spp*	2F-M123-V
2010	FCR	BVT	BASE WINE	*Saccharomyces cerevisiae*	2F-V11-IV
2010	FCR	BVT	BASE WINE	*Saccharomyces cerevisiae*	2F-V11-V
2010	FCR	CBC	BASE WINE	*Saccharomyces cerevisiae*	2F-V34-I
2010	FCR	CBC	BASE WINE	*Saccharomyces cerevisiae*	2F-V34-III
2010	FCR	CBC	BASE WINE	*Saccharomyces cerevisiae*	2F-V34-IV
2010	FCR	FGT	BASE WINE	*Saccharomyces cerevisiae*	2F-V43-II
2010	FCR	UBT	BASE WINE	*Saccharomyces cerevisiae*	2F-V123-I
2010	FCR	UBT	BASE WINE	*Saccharomyces cerevisiae*	2F-V123-II
2010	FCR	BVT	BASE WINE	*Saccharomyces cerevisiae*	2F-V19-I
2010	FCR	BVT	BASE WINE	*Saccharomyces cerevisiae*	2F-V19-II
2010	FCR	BVT	BASE WINE	*Saccharomyces cerevisiae*	2F-V40-I
2010	FCR	FGT	BASE WINE	*Hanseniaspora uvarum*	2F-V43-I
2010	FCR	FGT	BASE WINE	*Hanseniaspora uvarum*	2F-V122-I
2010	FCR	FGT	BASE WINE	*Hanseniaspora uvarum*	2F-V122-II
2010	FCR	CBC	BASE WINE	*Pichia membranifaciens*	2F-V34-II
2010	FCR	CBC	BASE WINE	*Pichia membranifaciens*	2F-V34-V
2010	FCR	BVT	BASE WINE	*Pichia membranifaciens*	2F-V40-II
2010	FCR	BVT	BASE WINE	*Pichia spp*	2F-V11-I
2010	FCR	BVT	BASE WINE	*Pichia spp*	2F-V11-III
2010	FCR	BVT	BASE WINE	*Issatchenkia occidentalis*	2F-V11-II
2010	FCR	FGT	BASE WINE	*Zygosaccharomyces bailii*	2F-V43-I
2010	OLT	VTN	AIR	*Saccharomyces cerevisiae*	2OA-VIS-1
2010	OLT	QRN	AIR	*Issatchenkia terricola*	2OA-QUA-2
2010	OLT	QRN	AIR	*Pichia fermentans*	2OA- QUA-1
2010	OLT	MSL	MUST	*Saccharomyces cerevisiae*	2O-M-MON-1
2010	OLT	MSL	MUST	*Saccharomyces cerevisiae*	2O-M+-MON-4

VINTAGE	ORIGIN	WINERY CODE	SAMPLE	GENETIC IDENTIFICATION	ISOLATE
2010	OLT	IBD	MUST	*Saccharomyces cerevisiae*	2O-M-ISM-4
2010	OLT	CSO	MUST	*Saccharomyces cerevisiae*	2O-M-CAS-2
2010	OLT	MLN	MUST	*Saccharomyces cerevisiae*	2O-M+-MAZ-1
2010	OLT	CSO	MUST	*Saccharomyces cerevisiae*	2O-CAS-3
2010	OLT	IBD	MUST	*Saccharomyces cerevisiae*	2O-M-ISM-3
2010	OLT	MSL	MUST	*Saccharomyces cerevisiae*	2O M+-MON-3
2010	OLT	IBD	MUST	*Saccharomyces cerevisiae*	2O-M-ISM-5
2010	OLT	MSL	MUST	*Saccharomyces cerevisiae*	2O-M+-MON-1
2010	OLT	CSO	MUST	*Saccharomyces cerevisiae*	2O-M-CAS-1
2010	OLT	MLN	MUST	*Saccharomyces cerevisiae*	2O-M-MAZ-2
2010	OLT	MSL	MUST	*Saccharomyces cerevisiae*	2O-M-MON-2
2010	OLT	IBD	MUST	*Saccharomyces cerevisiae*	2O-M-3IB
2010	OLT	MSL	MUST	*Saccharomyces cerevisiae*	2O-M-MON-3a
2010	OLT	IBD	MUST	*Saccharomyces cerevisiae*	2O-M-ISM-2
2010	OLT	IBD	MUST	*Saccharomyces cerevisiae*	2O-M-ISM-6
2010	OLT	ATO	MUST	*Candida railenensis*	2OM-ANT-1
2010	OLT	ATO	MUST	*Issatchenkia terricola*	2OM-ANT-4
2010	OLT	ATO	MUST	*Metschnikowia pulcherrima*	2OM-ANT-3
2010	OLT	CSO	MUST	*Candida railenensis*	2OM-CAS-4
2010	OLT	IBC	MUST	*Candida railenensis*	2OM-CDP-4
2010	OLT	IBC	MUST	*Candida railenensis*	2OM-CDP-2
2010	OLT	IBC	MUST	*Torulaspora delbrueckii*	2OM-CDP-1
2010	OLT	IBD	MUST	*Candida railenensis*	2OM-ISI-1
2010	OLT	IBD	MUST	*Candida railenensis*	2OM-ISI-3A
2010	OLT	MLN	MUST	*Hanseniaspora uvarum*	2OM-MAZ-1
2010	OLT	MSL	MUST	*Candida railenensis*	2OM-MON-3B
2010	OLT	MSL	MUST	*Pichia fermentans*	2OM-MON-4
2010	OLT	SGG	MUST	*Candida railenensis*	2OM-SG-1
2010	OLT	SGG	MUST	*Candida railenensis*	2OM-SG-2
2010	OLT	QRN	MUST	*Candida railenensis*	2OM-QUA-4
2010	OLT	QRN	MUST	*Hanseniaspora uvarum*	2OM-QUA-1B
2010	OLT	QRN	MUST	*Issatchenkia terricola*	2OM-QUA-1A
2010	OLT	QRN	MUST	*Metschnikowia pulcherrima*	2OM-QUA-3
2010	OLT	QRN	MUST	*Metschnikowia pulcherrima*	2OM-QUA-2
2010	OLT	VTN	MUST	*Torulaspora delbrueckii*	2OM-VIS-2
2010	OLT	VTN	MUST	*Torulaspora delbrueckii*	2OM-VIS-1
2010	OLT	ATO	MUST	*Candida railenensis*	2OM-ANT-1

VINTAGE	ORIGIN	WINERY CODE	SAMPLE	GENETIC IDENTIFICATION	ISOLATE
2010	OLT	ATO	MUST	*Candida railenensis*	2OM-ANT-3
2010	OLT	CSO	MUST	*Pichia fermentans*	2OM-CAS-2
2010	OLT	CSO	MUST	*Torulaspora delbrueckii*	2OM-CAS-1
2010	OLT	IBC	MUST	*Hanseniaspora uvarum*	2OM-CDP-3
2010	OLT	IBC	MUST	*Hanseniaspora uvarum*	2OM-CDP-4
2010	OLT	IBC	MUST	*Torulaspora delbrueckii*	2OM-CDP-1
2010	OLT	IBD	MUST	*Issatchenkia terricola*	2OM-ISI-2
2010	OLT	IBD	MUST	*Pichia fermentans*	2OM-ISI-1
2010	OLT	MLN	MUST	*Issatchenkia terricola*	2OM-MAZ-2
2010	OLT	MLN	MUST	*Pichia fermentans*	2OM-MAZ-3
2010	OLT	SGG	MUST	*Candida railenensis*	2OM-SG-3
2010	OLT	SGG	MUST	*Pichia fermentans*	2OM-SG-1
2010	OLT	SGG	MUST	*Torulaspora delbrueckii*	2OM-SG-2
2010	OLT	QRN	MUST	*Issatchenkia terricola*	2OM-QUA-1
2010	OLT	QRN	MUST	*Issatchenkia terricola*	2OM-QUA-2
2010	OLT	VTN	MUST	*Candida railenensis*	2OM-VIS-1
2010	OLT	VTN	MUST	*Pichia membranifaciens*	2OM-VIS-2
2010	OLT	VTN	BASE WINE	*Saccharomyces cerevisiae*	2O-V-VIS-2
2010	OLT	IBC	BASE WINE	*Saccharomyces cerevisiae*	2O-V-CD1
2010	OLT	CSO	BASE WINE	*Saccharomyces cerevisiae*	2O-V-CAS2
2010	OLT	MSL	BASE WINE	*Saccharomyces cerevisiae*	2O-V-MON-2
2010	OLT	CSO	BASE WINE	*Saccharomyces cerevisiae*	2O-V-CAS-1
2010	OLT	ATO	BASE WINE	*Saccharomyces cerevisiae*	2O-V-ANT-1
2010	OLT	ATO	BASE WINE	*Pichia fermentans*	2OV-ANT-3
2010	OLT	ATO	BASE WINE	*Pichia fermentans*	2OV-ANT-2
2010	OLT	IBD	BASE WINE	*Candida railenensis*	2OV-ISI-1
2010	OLT	MLN	BASE WINE	*Candida railenensis*	2OV-MAZ-1
2010	OLT	MSL	BASE WINE	*Hanseniaspora uvarum*	2OV-MON-1
2010	OLT	SGG	BASE WINE	*Candida railenensis*	2OV-SG-2
2010	OLT	SGG	BASE WINE	*Hanseniaspora uvarum*	2OV-SG-1
2010	OLT	VTN	BASE WINE	*Candida railenensis*	2OV-VIS-1
2010	OLT	VTN	BASE WINE	*Pichia fermentans*	2OV-VIS-3
2011	FCR	BVT	MUST	*Saccharomyces cerevisiae*	3FM-11-I
2011	FCR	BVT	MUST	*Saccharomyces cerevisiae*	3FM-19-II
2011	FCR	BVT	MUST	*Saccharomyces cerevisiae*	3FM-19-III
2011	FCR	CBC	MUST	*Saccharomyces cerevisiae*	3FM-34-II
2011	FCR	CBC	MUST	*Saccharomyces cerevisiae*	3FM-34-III

VINTAGE	ORIGIN	WINERY CODE	SAMPLE	GENETIC IDENTIFICATION	ISOLATE
2011	FCR	BVT	MUST	*Saccharomyces cerevisiae*	3FM-40-II
2011	FCR	BVT	MUST	*Saccharomyces cerevisiae*	3FM-40-III
2011	FCR	FGT	MUST	*Saccharomyces cerevisiae*	3FM-43-II
2011	FCR	MJL	MUST	*Saccharomyces cerevisiae*	3FM-59-II
2011	FCR	MJL	MUST	*Saccharomyces cerevisiae*	3FM-59-III
2011	FCR	UBT	MUST	*Saccharomyces cerevisiae*	3FM-120-II
2011	FCR	UBT	MUST	*Saccharomyces cerevisiae*	3FM-120-III
2011	FCR	FGT	MUST	*Saccharomyces cerevisiae*	3FM-122-II
2011	FCR	UBT	MUST	*Saccharomyces cerevisiae*	3FM-123-II
2011	FCR	UBT	MUST	*Saccharomyces cerevisiae*	3FM-123-III
2011	FCR	VZL	MUST	*Saccharomyces cerevisiae*	3FM-137-II
2011	FCR	BVT	MUST	*Torulaspora delbrueckii*	3FM-11-III
2011	FCR	BVT	MUST	*Candida zemplinina*	3FM-11-IV
2011	FCR	BVT	MUST	*Kluyveromyces thermotolerans*	3FM-19-I
2011	FCR	CBC	MUST	*Hanseniaspora uvarum*	3FM-34-I
2011	FCR	BVT	MUST	*Hanseniaspora vineae*	3FM-40-I
2011	FCR	BVT	MUST	*Torulaspora delbrueckii*	3FM-40-IV
2011	FCR	FGT	MUST	*Kluyveromyces thermotolerans*	3FM-43-I
2011	FCR	FGT	MUST	*Torulaspora delbrueckii*	3FM-43-III
2011	FCR	MJL	MUST	*Hanseniaspora vineae*	3FM-59-I
2011	FCR	UBT	MUST	*Hanseniaspora vineae*	3FM-120-I
2011	FCR	FGT	MUST	*Hanseniaspora vineae*	3FM-122-I
2011	FCR	FGT	MUST	*Torulaspora delbrueckii*	3FM-122-III
2011	FCR	UBT	MUST	*Kluyveromyces thermotolerans*	3FM-123-I
2011	FCR	UBT	MUST	*Torulaspora delbrueckii*	3FM-123-IV
2011	FCR	MTR	MUST	*Metschnikowia pulcherrima*	3FM-124-I
2011	FCR	MTR	MUST	*Metschnikowia pulcherrima*	3FM-124-II
2011	FCR	MTR	MUST	*Candida oleophila*	3FM-124-III
2011	FCR	VZL	MUST	*Hanseniaspora vineae*	3FM-137-I
2011	FCR	VZL	MUST	*Torulaspora delbrueckii*	3FM-137-III
2011	FCR	BVT	BASE WINE	*Saccharomyces cerevisiae*	3FV-11-I
2011	FCR	BVT	BASE WINE	*Saccharomyces cerevisiae*	3FV-11-II
2011	FCR	BVT	BASE WINE	*Saccharomyces cerevisiae*	3FV-11-III
2011	FCR	BVT	BASE WINE	*Saccharomyces cerevisiae*	3FV-19-I
2011	FCR	BVT	BASE WINE	*Saccharomyces cerevisiae*	3FV-19-II
2011	FCR	CBC	BASE WINE	*Saccharomyces cerevisiae*	3FV-34-I
2011	FCR	CBC	BASE WINE	*Saccharomyces cerevisiae*	3FV-34-II

VINTAGE	ORIGIN	WINERY CODE	SAMPLE	GENETIC IDENTIFICATION	ISOLATE
2011	FCR	CBC	BASE WINE	*Saccharomyces cerevisiae*	3FV-34-III
2011	FCR	CBC	BASE WINE	*Saccharomyces cerevisiae*	3FV-34-IV
2011	FCR	FGT	BASE WINE	*Saccharomyces cerevisiae*	3FV-43-I
2011	FCR	FGT	BASE WINE	*Saccharomyces cerevisiae*	3FV-43-II
2011	FCR	FGT	BASE WINE	*Saccharomyces cerevisiae*	3FV-43-III
2011	FCR	MJL	BASE WINE	*Saccharomyces cerevisiae*	3FV-59-I
2011	FCR	MJL	BASE WINE	*Saccharomyces cerevisiae*	3FV-59-II
2011	FCR	FGT	BASE WINE	*Saccharomyces cerevisiae*	3FV-122-I
2011	FCR	FGT	BASE WINE	*Saccharomyces cerevisiae*	3FV-122-II
2011	FCR	FGT	BASE WINE	*Saccharomyces cerevisiae*	3FV-122-III
2011	FCR	FGT	BASE WINE	*Saccharomyces cerevisiae*	3FV-122-IV
2011	FCR	FGT	BASE WINE	*Saccharomyces cerevisiae*	3FV-122-V
2011	FCR	FGT	BASE WINE	*Saccharomyces cerevisiae*	3FV-122-VI
2011	FCR	CBC	BASE WINE	*Pichia membranifaciens*	3FV-34-II
2011	FCR	VZL	BASE WINE	*Pichia membranifaciens*	3FV-137-I
2011	FCR	VZL	BASE WINE	*Pichia membranifaciens*	3FV-137-II
2011	OLT	ATO	AIR	currently under identification	3OA-3-2
2011	OLT	CSO	AIR	currently under identification	3OA-5-1
2011	OLT	IBC	AIR	currently under identification	3OA-6-1
2011	OLT	IBC	AIR	currently under identification	3OA-6-2
2011	OLT	MSL	AIR	currently under identification	3OA-8-1
2011	OLT	MSL	AIR	currently under identification	3OA-8-2
2011	OLT	VRD	MUST	currently under identification	3O-1M-1
2011	OLT	VRD	MUST	currently under identification	3O-1M-2
2011	OLT	VRD	MUST	currently under identification	3O-1M-3
2011	OLT	VRD	MUST	currently under identification	3O-1M-4
2011	OLT	VRD	MUST	currently under identification	3O-1M-5
2011	OLT	QRN	MUST	currently under identification	3O-2M-1
2011	OLT	QRN	MUST	currently under identification	3O-2M-2
2011	OLT	QRN	MUST	currently under identification	3O-2M-3
2011	OLT	QRN	MUST	currently under identification	3O-2M-4
2011	OLT	ATO	MUST	currently under identification	3O-3M-2
2011	OLT	ATO	MUST	currently under identification	3O-3M-3
2011	OLT	ATO	MUST	currently under identification	3O-3M-4
2011	OLT	ATO	MUST	currently under identification	3O-3M-5
2011	OLT	VTN	MUST	currently under identification	3O-4M-2
2011	OLT	VTN	MUST	currently under identification	3O-4M-3

VINTAGE	ORIGIN	WINERY CODE	SAMPLE	GENETIC IDENTIFICATION	ISOLATE
2011	OLT	VTN	MUST	currently under identification	3O-4M-4
2011	OLT	VTN	MUST	currently under identification	3O-4M-5
2011	OLT	CSO	MUST	currently under identification	3O-5M-1
2011	OLT	CSO	MUST	currently under identification	3O-5M-2
2011	OLT	CSO	MUST	currently under identification	3O-5M-3
2011	OLT	CSO	MUST	currently under identification	3O-5M-4
2011	OLT	CSO	MUST	currently under identification	3O-5M-5
2011	OLT	IBC	MUST	currently under identification	3O-6M-1
2011	OLT	IBC	MUST	currently under identification	3O-6M-2
2011	OLT	IBC	MUST	currently under identification	3O-6M-3
2011	OLT	IBC	MUST	currently under identification	3O-6M-4
2011	OLT	TVN	MUST	currently under identification	3O-7M-1
2011	OLT	TVN	MUST	currently under identification	3O-7M-2
2011	OLT	TVN	MUST	currently under identification	3O-7M-3
2011	OLT	MSL	MUST	currently under identification	3O-8M-1
2011	OLT	MSL	MUST	currently under identification	3O-8M-2
2011	OLT	MSL	MUST	currently under identification	3O-8M-3
2011	OLT	MSL	MUST	currently under identification	3O-8M-4
2011	OLT	MLN	MUST	currently under identification	3O-9M-1
2011	OLT	MLN	MUST	currently under identification	3O-9M-2
2011	OLT	MLN	MUST	currently under identification	3O-9M-3
2011	OLT	MLN	MUST	currently under identification	3O-9M-4
2011	OLT	IBD	MUST	currently under identification	3O-10M-1
2011	OLT	IBD	MUST	currently under identification	3O-10M-2
2011	OLT	IBD	MUST	currently under identification	3O-10M-3
2011	OLT	SGG	MUST	currently under identification	3O-11M-1
2011	OLT	SGG	MUST	currently under identification	3O-11M-2
2011	OLT	QRN	BASE WINE	currently under identification	3O-2V-1
2011	OLT	ATO	BASE WINE	currently under identification	3O-3V-1
2011	OLT	ATO	BASE WINE	currently under identification	3O-3V-2
2011	OLT	ATO	BASE WINE	currently under identification	3O-3V-3
2011	OLT	ATO	BASE WINE	currently under identification	3O-3V-4
2011	OLT	VTN	BASE WINE	currently under identification	3O-4V-1
2011	OLT	VTN	BASE WINE	currently under identification	3O-4V-2
2011	OLT	VTN	BASE WINE	currently under identification	3O-4V-3
2011	OLT	CSO	BASE WINE	currently under identification	3O-5V-1
2011	OLT	CSO	BASE WINE	currently under identification	3O-5V-2

VINTAGE	ORIGIN	WINERY CODE	SAMPLE	GENETIC IDENTIFICATION	ISOLATE
2011	OLT	IBC	BASE WINE	currently under identification	3O-6V-1
2011	OLT	IBC	BASE WINE	currently under identification	3O-6V-2
2011	OLT	TVN	BASE WINE	currently under identification	3O-7V-1
2011	OLT	TVN	BASE WINE	currently under identification	3O-7V-2
2011	OLT	MSL	BASE WINE	currently under identification	3O-8V-1
2011	OLT	MSL	BASE WINE	currently under identification	3O-8V-2
2011	OLT	MLN	BASE WINE	currently under identification	3O-9V-1
2011	OLT	MLN	BASE WINE	currently under identification	3O-9V-2
2011	OLT	SGG	BASE WINE	currently under identification	3O-10V-1
2011	OLT	SGG	BASE WINE	currently under identification	3O-10V-2
2011	OLT	SGG	BASE WINE	currently under identification	3O-10V-3
2011	OLT	SGG	BASE WINE	currently under identification	3O-10V-4

*FCR= *Franciacorta* area; OLT= *Oltrepò Pavese* area.

APPENDIX B

Scientific products

Barrera-Cárdenas SM. (2010)
Biodiversity analysis of *Saccharomyces cerevisiae* strains isolated from *Franciacorta* and *Oltrepò Pavese* to improve sparkling wine production made by *champenois* method.
15th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, Naples, Sep 15-17
2nd Annual PhD Report

Biodiversity analysis of *Saccharomyces cerevisiae* strains isolated from Franciacorta and Oltrepo' Pavese to improve sparkling wine production made by *champenois* method

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This PhD research project is aimed to the identification, typing and selection of *Saccharomyces cerevisiae* strains to evaluate the interspecific biodiversity and the potential use as new starters in the sparkling wine production made by *Champenois* method. Indigenous yeasts were isolated in Franciacorta (BS) and Oltrepo' Pavese (PV) and some *S. cerevisiae* strains were tested for their proper attitudes for winemaking.

Valutazione della Biodiversità di Ceppi di *Saccharomyces cerevisiae* isolati in Franciacorta e Oltrepo' Pavese per il miglioramento della produzione di vino spumante metodo classico

Questo progetto di tesi di dottorato mira all'identificazione, tipizzazione e selezione di ceppi di *Saccharomyces cerevisiae* per valutare la biodiversità interspecifica e il potenziale uso come nuovi starters nella produzione di vino spumante metodo classico. Ceppi autoctoni di lievito sono stati isolati in Franciacorta e l'Oltrepò Pavese e alcuni appartenenti alla specie *S. cerevisiae* sono stati saggiati per identificare quelli con adeguata attitudine alla vinificazione.

Keywords: *Saccaromyces cerevisiae*, indigenous strains, biodiversity, sparkling wine, typing.

1.Introduction

The study of biodiversity within microbial populations involved in wine-making have recently become an object of growing interest due to the possibility of obtaining new strains with useful capabilities for the wine industry. The first step towards the development of new starters is the clonal selection of the wild yeasts isolated from natural environments associated with the wine-producing areas (Giudici et al.,1992). Starters currently used in Lombardy have been isolated from French territories on the basis of the quality characteristics of Champagne wine. Moreover a biodiversity analysis of yeasts populations in vinery districts of Lombardy has not still carried out.

2. Materials and Methods

2.1. Identification and typing of yeasts

Samples of vineyard air, must before SO_2 addition and wine at the end of spontaneous or controlled fermentation have been collected in different Franciacorta and Oltrepò territories. The following techniques were used for the identification and typing of the isolates: Restriction Fragment Lenght Polymorphism (RFLP) of ITS (Esteve-Zaezoso et al.,1999), D1/D2 of 26S rDNA sequence analysis (White et al., 1990) and Interdelta analysis (δ-PCR) (Legras et al., 2002). Electrophoretical patterns have been analyzed through software Bionumerics (Applied Maths, Belgium). Dendrograms were constructed by the unweighted pair group method using arithmetic averages (UPGMA).

2.2. Fermentation tests of selected *S. cerevisiae* strains

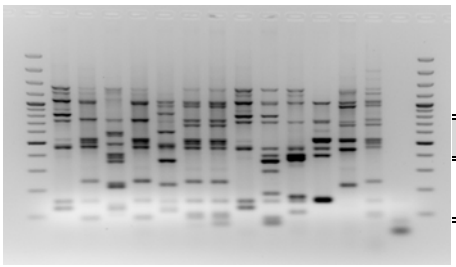
Sixteen indigenous *S. cerevisiae* strains were selected on the basis of typing and geographical origin. Two starters *S. cerevisiae* IOC 18-2007 strain and *S. bayanus* EC-1118 strain were used as controls. Two different media were used in the fermentation tests: "synthetic must" (Guerra et al.,1999) and sterile white must (Pignoletto). Yeast cultures were inoculated at a concentration of $1x10^6$ cell/ml in 100 ml flasks, stoppered by valves, statically incubated at 18°C (Guerra et al.,1999). At the end of fermentation aliquots of wine samples were centrifuged at 4500 *g* and the supernatants were analysed. Ethanol, glycerol and acetic acid were determined by enzymatic protocols. Sniffing tests of wines were realized with a taster panel (scores from 0 to 10). Statistical analysis of data was carried out through ANOVA (Statgraphics Centurion Plus 5.1).

3. Results and discussion

3.1.Biodiversity of yeast populations

The identification of yeast populations are reported in Table 1. *S. cerevisiae* was found in 3 samples of Oltrepò air, whereas it was not isolated in Franciacorta. In 24 must samples *S. cerevisiae* predominated in both areas but *Z. bailii* and *I. occidentalis* were commonly present. In 11 wines of Oltrepò *S. cerevisiae* was predominant, instead a great biodiversity was detected in 13 Franciacorta samples.

Figure 1 Example of electrophoretic run of *S.cerevisiae* Interdelta analysis



The analysis of interdelta profiles of *S. cerevisiae* isolated from musts and wines in Franciacorta showed the formation of six clusters with a percentage of similarity varying from 43% to 100%. On the other hand the Oltrepò strains isolated from air, must and wine grouped in eight
Different clusters with a percentage of similarity starting from 15% to 100%

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FRANCIACORTA		
Samples (n)	Isolates (n)	Yeast identification
Air (12)	8	*Aureobasidium pullulans* 50% *Cryptococcus laurentii* 50%
Must (13)	42	*Saccharomyces cerevisiae* 60% *Zygosaccharomyces bailii* 26% *Issatchenkia occidentalis* 14%
Wine (13)	56	*Saccharomyces cerevisiae* 72% *Pichia membranifaciens* 16% *Pichia fluxum* 6% *Zygosaccharomyces bailii* 4% *Issatchenkia orientalis* 1% *Torulaspora delbueckii* 1%
OLTREPO		
Samples (n)	Isolates (n)	Yeast identification
Air (11)	9	*Saccharomyces cerevisiae* 78% *Aureobasidium pullulans* 11% *Issatchenkia occidentalis* 11%
Must (11)	27	*Saccharomyces cerevisiae* 70% *Zygosaccharomyces bailii* 14% *Issatchenkia occidentalis* 4% *Hanseniaspora uvarum* 4% *Candida zemplinia* 4% *Candida diversa* 4%
Wine (11)	55	*Saccharomyces cerevisiae* 100%

Table 1. Yeast identification and incidence

3.2.Technological and quality characters of *S. cerevisiae* strains

The indigenous strains selected in this work showed an alcohol production comparable with the starters. Particularly in "synthetic must" ethanol ranged from 9,5 to 13,2 % v/v for Franciacorta strains, whereas from 13,0 to 13,8 % v/v for those isolated in Oltrepò. In Pignoletto must the alcohol production varied from 9,7 to 10,9 % v/v for Franciacorta strains, and from 9,8 to 12,0 % v/v for those isolated in Oltrepò. This finding could be caused by nutritional deficiencies, given the characteristics of Pignoletto must, which affected the metabolism of yeast. As regards quality characters all strains proved to produce glycerol in quantity comparable to starters, however at a lower level (0,08 to 0,25 g/L) . Acetic acid production was limited for all strains (0,17 to 0,37 g/l). Samples of wine obtained from fermentation assays were subjected to sniffing test. Pleasantness and intensity were evaluated: results obtained by ANOVA revealed that strains were significantly different ($p < 0,05$) and four of them showed the best performances.

5.References

Esteve-Zaezoso B, Belloch C, Uruburu F, Querol A (1999) Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. International Journal of Systematic Bacteriology **49:**329-337

Giudici P, Zambonelli C (1992) Criteri di selezione dei lieviti per enologia. Vignevini **9:** 29-34

Guerra E, Mannazzu I, Sordi G, Tangherlini M, Clementi F, Fatichenti F (1999) Characterization of indigenous *Saccharomyces cerevisiae* from the Italian region of Marche : hunting for new strains for local wine quality improvement .Annali di Microbiologia ed Enzimologia **49:**79-88.

Legras J, Karst F (2002) Optimisation of interdelta analysis for *Saccharomyces cerevisiae* strain characterisation; FEMS Microbiology Letters **221:** 249-255

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungi ribosomal RNA genes for phylogenetics. Innis MA, Gelfand DH, Sninsky 325-322

15th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, University of Napoli I – Federico II, Portici, 15-17 September, 2010

Biodiversity analysis of *Saccharomyces cerevisiae* strains isolated from Franciacorta and Oltrepò Pavese to improve sparkling wine production made by *champenois* method

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Introduction. The study of biodiversity within microbial populations involved in wine-making have recently become an object of growing interest due to the possibility of obtaining new strains with useful capabilities for the wine industry. The first step towards the development of new starters is the clonal selection of the wild yeasts isolated from natural environments associated with the wine-producing areas. Starters currently used in Lombardy for sparkling wines have been isolated from French territories on the basis of the quality characteristics of Champagne wine. Moreover a biodiversity analysis of yeasts populations in vinery districts of Lombardy has not still carried out. This PhD research project is aimed to the identification, typing and selection of *Saccharomyces cerevisiae* strains to evaluate the interspecific biodiversity and the potential use as new starters in the sparkling wine production made by *Champenois* method. Indigenous yeasts were isolated in Franciacorta (BS) and Oltrepo' Pavese (PV) and some *S. cerevisiae* strains were tested for their proper attitudes for winemaking

Materials and Methods

Identification and typing of yeasts

Samples of vineyard air, must before SO_2 addition and wine at the end of spontaneous or controlled fermentation have been collected in different Franciacorta and Oltrepò territories. The following techniques were used for the identification and typing of the isolates: Restriction Fragment Lenght Polymorphism (RFLP) of ITS, D1/D2 of 26S rDNA sequence analysis and Interdelta analysis (δ-PCR). Electrophoretical patterns have been analyzed through software Bionumerics (Applied Maths, Belgium). Dendrograms were constructed by the unweighted pair group method using arithmetic averages (UPGMA)

Fermentation tests of selected *S. cerevisiae* strains

Sixteen indigenous *S. cerevisiae* strains were selected on the basis of typing and geographical origin. Two starters *S. cerevisiae* IOC 18-2007 strain and *S. bayanus* EC-1118 strain were used as controls. Two different media were used in the fermentation tests: "synthetic must" and sterile white must (Pignoletto). Yeast cultures were inoculated at a concentration of $1x10^6$ cell/ml. At the end of fermentation, ethanol, glycerol and acetic acid were determined by enzymatic protocols. Sniffing tests of wines were realized with a taster panel (scores from 0 to 10). Statistical analysis of data was carried out through ANOVA (Statgraphics Centurion Plus 5.1)

RESULTS

FRANCIACORTA			OLTREPO'		
Samples (n)	Isolates (n)	Yeast identification	Samples (n)	Isolates (n)	Yeast identification
Air (12)	8	*Aureobasidium pullulans* 50%	Air (11)	9	***Saccharomyces cerevisiae* 78%**
		Cryptococcus laurentii 50%			*Aureobasidium pullulans* 11%
Must (13)	42	***Saccharomyces cerevisiae* 60%**			*Issatchenkia occidentalis* 11%
		Zygosaccharomyces bailii 26%	Must (11)	27	***Saccharomyces cerevisiae* 70%**
		Issatchenkia occidentalis 14%			*Zygosaccharomyces bailii* 14%
Wine (13)	56	***Saccharomyces cerevisiae* 72%**			*Issatchenkia occidentalis* 4%
		Pichia membranifaciens 16%			*Hanseniaspora uvarum* 4%
		Pichia fluxum 6%			*Candida zemplinia* 4%
		Zygosaccharomyces bailii 4%			*Candida diversa* 4%
		Issatchenkia orientalis 1%	Wine (11)	55	***Saccharomyces cerevisiae* 100%**
		Torulaspora delbrueckii 1%			

TABLE 1. Yeast identification and incidence

Technological and quality characters of *S. cerevisiae* strains

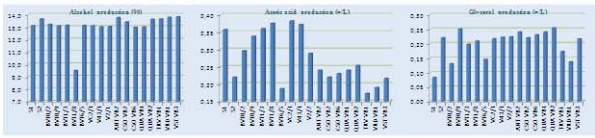

Figure 3 . Alcohol, glycerol and acetic acid production for Franciacorta and Oltrepò strains.

Samples of wine obtained from fermentation assays were subjected to sniffing test. Intensity (figure 4) and Pleasantness (figure 5) were evaluated: results obtained by ANOVA revealed that strains were significantly different ($p < 0,05$) and four of them showed the best performances

Biodiversity of yeast populations

The identification of yeast populations are reported in Table 1. *S. cerevisiae* was found in 3 samples of Oltrepò air, whereas it was not isolated in Franciacorta. In 24 must samples *S. cerevisiae* predominated in both areas but *Z. bailii* and *I. occidentalis* were commonly present. In 11 wines of Oltrepò *S. cerevisiae* was predominant, instead a great biodiversity was detected in 13 Franciacorta samples.

The analysis of interdelta profiles (example figure 1) of *S. cerevisiae* isolated from musts and wines in Franciacorta showed the formation of six clusters with a percentage of similarity varying from 43% to 100% (figure 2). On the other hand the Oltrepò strains isolated from air, must and wine were grouped in eight different clusters with a percentage of similarity starting from 15% to 100% by interdelta analysis

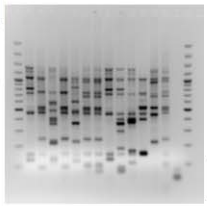

Figure 1. Image of a electrophoretic run of *S. cerevisiae* strains from Interdelta analysis

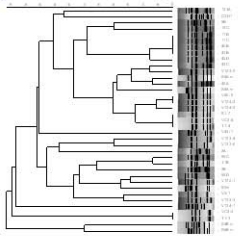

Figure 2. Example of dendrogram from Interdelta analysis of Franciacorta strains

Figure 4. Intensity of wine samples obtained from fermentation assays for franciacorta strains

Figure 5 . Pleasantness of wine samples obtained from fermentation assays for franciacorta strains

Work in progress

According to obtained results two strains for each production area have been chosen for re-fermentation wine tests in seven cellars of Franciacorta and Oltrepò. Assessment of strain dominance during the fermentation steps and its permanence in the territory during the vintage have been started for the second year. In order to find specific targets amplification for the traceability of the product, a protocol for the recovery of DNA from the yeast used in re-fermentation will be developed.

References

Esteve-Zaezoso B, Belloch C, Uruburu F, Querol A (1999) Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. International Journal of Systematic Bacteriology **49:**329-337

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White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungi ribosomal RNA genes for phylogenetics. Innis MA, Gelfand DH, Sninsky 325-322

Barrera-Cárdenas SM. (2011)
Genetic biodiversity of *Saccharomyces cerevisiae* strains from Lombardy wine districts and evaluation of indigenous yeast selected for the sparkling wine production made by *Champenois* method
16th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, Lodi, Sep 21-23
3rd Annual PhD report

Genetic biodiversity of *Saccharomyces cerevisiae* strains from Lombardy wine districts and evaluation of indigenous yeast selected for the sparkling wine production made by *Champenois* method

Shirley Mireya Barrera Càrdenas (shirley.barrera@unimi.it)
Department of Food Science Technology and Microbiology University of Milan, Italy
Tutor: Prof. Roberto Foschino

This PhD dealt with the study of genetic biodiversity of *Saccharomyces cerevisiae* strains isolated in *Franciacorta* (BS) and *Oltrepo' Pavese* (PV) to improve sparkling wine production made by *champenois* method. In particular, the strains were collected during 2009-2010 vintages, and from monitoring of spontaneous and controlled fermentations. Moreover, some *S. cerevisiae* indigenous strains were tested for their potential use as new starters through inoculated fermentations performed at pilot scale in seven cellars of wine areas.

Biodiversità genetica di ceppi di *Saccharomyces cerevisiae* in zone viticole della Lombardia e valutazione dei ceppi autoctoni selezionati per la produzione di vino spumante metodo classico

Questa tesi di dottorato ha riguardato lo studio delle biodiversità genetica dei ceppi di *Saccharomyces cerevisiae* isolati in Franciacorta (BS) e l'Oltrepò pavese (PV) per il miglioramento della produzione di vino spumante metodo classico. In particolare, i ceppi sono stati collezionati durante le vendemmie 2009-2010 e anche durante il monitoraggio di fermentazioni spontanee e controllate. Inoltre, alcuni ceppi autoctoni di *S. cerevisiae* sono stati saggiati per identificare il loro uso potenziale come nuovi starter attraverso fermentazioni controllate eseguite in spumantizzazioni sperimentali in sette cantine delle aree viticole.

Keywords: *Saccharomyces cerevisiae*, genetic biodiversity, indigenous strains, sparkling wine, *Champenois* method

1. Introduction

Selected yeast starters are nowadays widely used since they possess very good fermentative and oenological capabilities, contributing to both standardization of fermentation process and wine quality in order to ensure a reproducible product and to reduce the risk of wine spoilage. As the quality of wine is strictly related to microbial flora which develops during fermentation, there is a growing demand to differentiate, between the fermentative yeasts, autochthonous strains with typical oenological traits which could be considered representative of particular oenological districts. On the other hand, sparkling wine obtained through the classic method (*Champenois* method), represents a relevant cultural and outstanding economical fact in Italy. Actually in Lombardy about 8 million bottles are commercialized worldwide each year (65% of national production). However, starters currently used have been isolated from French territories on the basis of the quality characteristics of *Champagne* wine and biodiversity analysis of yeast indigenous populations in our region has not still carried out.
Accordingly, this PhD research project has been focused on the identification and typing of *Saccharomyces cerevisiae* strains isolated in *Franciacorta* and *Oltrepo' Pavese* areas to evaluate

the genetic biodiversity and the potential use as new starters in the sparkling wine production. In particular, this oral communication reports the main results of the following activities related to:

- **Genetic identification and typing of indigenous strains**

The first part of the PhD thesis regarded the evaluation of genetic biodiversity of *Saccharomyces cerevisiae* strains collected during two vintages. Identification, typing and selection of isolates were performed for those belonging to 2009 vintage and are currently developing the identification and typing for those collected during 2010 vintage;

- **Evolution of the yeast population during alcoholic fermentation**

The second part of the PhD thesis was focused on monitoring the yeast population during the controlled and spontaneous fermentations to determine their genetic diversity and the dominant specie in the process;

- **Controlled fermentations to test indigenous yeasts in *tirage* proves at pilot scale**

The third part of the PhD thesis aimed to investigate the potential use of indigenous *S. cerevisiae* strains as starters. For this, inoculation of base wines in defined conditions with 2 different strains from each area, at pilot scale in seven cellars were performed.

2. Materials and Methods

2.1. Sample collection of indigenous yeast

Vineyard air, must and wine were sampled during 2009-2010 vintages. In the case of vineyard air, the sampling was done using an Air Sampler *"MAS 100 Eco"* of VWR International and for the colonies isolation was used the YEPD medium (1% yeast extract, 2% peptone, 2% dextrose, 2% agar) modified in the following characteristics: 3.6 pH, addition of 200 mg/l SO_2 and 100 mg/l of Chloramphenicol. The plates were incubated in anaerobic conditions for five days. The must was sampled inoculating 100 ml in YEPD broth for selective enrichment. In the case of wine, the sampling was done transferring 100 ml of sample in sterilized flasks. WL (Merck, Germany) medium was used for the colonies isolations. The purified isolates were stored at -80° C in YEPD broth with 20% (v/v) glycerol.

2.2. Monitoring of yeast populations during alcoholic fermentation

Base wine was sampled during spontaneous and controlled fermentation from three cellars denominated *VZ*, *V* and *BV* during 2010 vintage. The sampling was done transferring 50 ml of wine in sterilized flasks every 48 h since must's extraction until the end of fermentation. Sampling was performed 5-7 times for each cellar and type of fermentation. After appropriate dilutions samples were plated on WL (Merck, Germany) and the same medium was used for the colonies isolation. The purified isolates were stored as described above.

2.3. Molecular identification and typing of isolates

Genetic analysis of the isolates was carried out by extracting genomic DNA according to Querol *et al*. (1992).

For the identification of isolates, the internal transcribed spacers between the 18S and 26S rDNA genes (ITS1-5.8S-ITS2) were amplified; then in order to confirm isolates belonging to *S. cerevisiae* species, ITS amplifications were subjected to restriction (RFLP-ITS) as described by Esteve-Zarzoso *et al*. (2000) modified by using 3U *Hin*6I (Fermentas, Lithuania). Yeast isolates that not showed characteristic banding from ITS-RFLP analysis for a specific species (Granchi *et al.*, 1999), were subjected to D1/D2 of 26S rDNA amplification. The amplicons were subjected to sequencing (Primm s.r.l. Milano) and the obtained sequences were compared through BLAST software with the species listed in databases. The typing was realized with all isolates identified as *S. cerevisiae* using Interdelta analysis (δ-PCR) according to Legras *et al*. (2003).). All

electrophoretical profiles were registered through Geldoc (1000 System, Bio-Rad Laboratories, California). Interdelta profiles were compared through software (Gel compare II, Bionumerics Applied Maths, Belgium). Dendrograms were constructed by the unweighted pair group method using arithmetic averages (UPGMA).

2.4. Controlled fermentations to test indigenous yeasts in *tirage* proves at pilot scale

The first step for the selection of new potential starters, was to test some strains according to technological and quality characters. For this, microfermentation assays in laboratory were realized. On the basis of the laboratory experiments, two strains denominated "strain 1" and "strain 2" were chosen in each production area for *tirage* proves in 2010 vintage. Wine tests were performed in four Franciacorta cellars and in three Oltrepò Pavese cellars. In each cellar, the experiment was realized as follow: a protocol for the *pied de cuve* production was carried out in order to multiply the yeast and to activate fermentation; immediately afterward, 50 liters of base wine were inoculated with the *pied de cuve* of each strain. Furthermore, a third test was carried out with the commonly starter culture used in the cellar at the same conditions. Fermentation was monitored and a different times determination of viable counts were realized. Samples were adequately diluted in sterile peptone water and spreads on plates of WL nutrient agar (Merck, Germany). The plates were incubated at 28 °C for three days. Simultaneously, cellular vitality was evaluated through the methylene blue method.
Past six months from *tirage* proves, samples of wine obtained from fermentation trials were subjected to sniffing test. Intensity and pleasantness were evaluated and results were process by ANOVA (Statgraphics Centurion Plus 5.1).

3. Results and Discussion

3.1. Biodiversity of yeast populations during 2009 vintage

The identification of yeast populations is reported in Table 1. *S. cerevisiae* was found in 3 samples of Oltrepò air, whereas it was not isolated in Franciacorta. In 24 must samples *S. cerevisiae* predominated in both areas but *Z. bailii* and *I. occidentalis* were commonly present. In 11 wines of Oltrepò *S. cerevisiae* was predominant, instead a great biodiversity was detected in 13 Franciacorta samples.

Table 1. *Yeast identification and incidence during 2009 vintage*

FRANCIACORTA		
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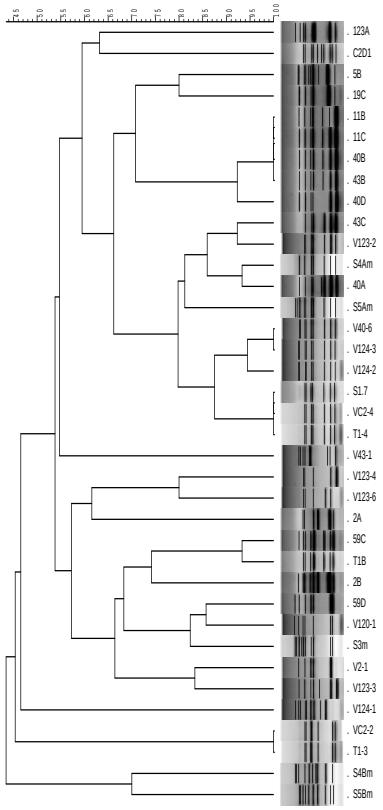


Figure 1. *Example of dendrogram of* S.

The analysis of interdelta profiles of *S. cerevisiae* isolated from musts and wines in Franciacorta showed the formation of six clusters. A percentage of similarity pattern varying from 43% to 100%. On the other hand the Oltrepò strains isolated from air, must and wine were grouped in eight different clusters. A percentage of similarity pattern starting from 15% to 100% by interdelta analysis.

The identification and typing of the 2010 vintage isolates are work in progress. Table 2 shows a comparison between numbers of isolates collected during 2009 and 2010 vintages in Franciacorta and Oltrepò Pavese areas. Differently to 2009 year, in the air samples of Franciacorta 2010, yeasts were not isolated. In this area, isolates 2010 from must represented 80% whereas isolates from wine represented 20% of the total. In Oltrepò Pavese area, the isolates from air represented 4% whereas the isolates from must represented 76% and the isolates from wine only 20% of the total. An effect vintage was observed since the isolates from must increased while in wine samples isolates decreased in both areas.

Table 2. *Comparison of isolates number during 2009-2010 vintages*

	Franciacorta vintage		Oltrepò vintage	
Sample	**2009**	**2010**	**2009**	**2010**
Air	8	0	9	3
Must	42	92	26	57
Wine	56	23	55	15
Total isolates	**106**	**115**	**90**	**75**

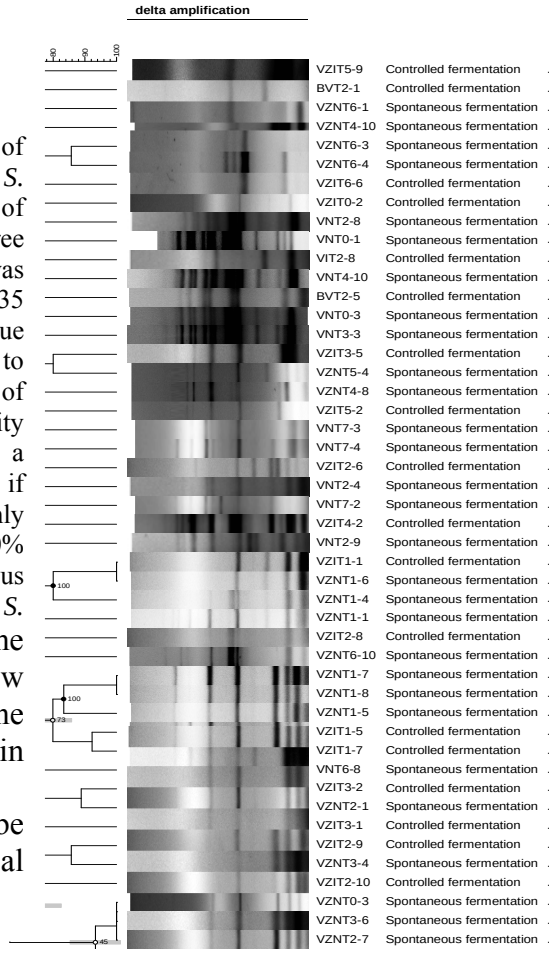
3.2. Biodiversity of yeast populations during alcoholic fermentation

The identification of yeast populations is reported in Table 3. Through the monitoring were collected 334 isolates of which ninety-eight percent were presumptively classified as *Saccharomyces cerevisiae* since analysis of RFLP-ITS of rDNA region generated a characteristic band of about 290-310 bp (results not shown). In *VZ* and *BVT* cellars, *S. cerevisiae* was predominant (100%) for both types of fermentations. In the case of *V* cellar, 3 isolates from controlled fermentation were belonging to the *Pichia membranifaciens* species (sequence identity 100%, coverage 100%) and 1 isolate from spontaneous fermentation was ascribed to *Hanseniaspora vinae* species (sequence identity 100%, coverage 99%). Although is common to found *Pichia membranifaciens* in the early stages of winemaking, this result could be a contamination index because the sample was collected from a controlled fermentation and in a later stage (sixth day of the fermentation). Instead, in the same cellar *Hanseniaspora vinae* isolated from the spontaneous fermentation in the early stages of fermentation, is coherent. Anyway as the fermentation progresses to higher concentrations of alcohol these species are substituted by more alcohol-tolerant strains of genus *Saccharomyces*. In fact *S. cerevisiae* was the predominant yeast with ninety-two percent (92%) for controlled fermentation and ninety-nine percent (99%) for spontaneous fermentation. The size of the populations at the beginning of controlled fermentation for all cellars was about 10^7 CFU ml^{-1}, whereas spontaneous fermentation was 10^6 CFU ml^{-1} . Viable cells decreased to 10^5 CFU ml^{-1} at the later stages of the process.

Table 3. *Identification of the yeast isolates from monitoring of three Franciacorta cellars*

Cellar	Type fermentation	Isolates (n)	Yeast identification
VZ	Controlled fermentation	70	*Saccharomyces cerevisiae 100%*
	Spontaneous fermentation	70	*Saccharomyces cerevisiae 100%*
V	Controlled fermentation	39	*Saccharomyces cerevisiae 92%* *Pichia membranifaciens 2%*
	Spontaneous fermentation	79	*Saccharomyces cerevisiae 99%* *Hanseniaspora vinae 1%*
BVT	Controlled fermentation	76	*Saccharomyces cerevisiae 100%*

Figure 2. *Dendrogram example of clustering of* S. cerevisiae *from Interdelta profiles*



Regarding the strain typing, the elaboration of Interdelta profiles involved 330 isolates of *S. cerevisiae* collected during the monitoring of controlled and spontaneous fermentation in three Franciacorta cellars. A repeatability of 97% was detected in our experimental conditions. 35 groups of isolates were ascribed to a unique strain. From data elaboration was possible to distinguish 258 strains (corresponding to 86% of the population investigated) with a similarity pattern varying from 58% to 97% showing a high level of biodiversity. In particular, even if in the *BVT* cellar wine-making was made only by a controlled fermentation, nevertheless 20% of the population was constituted by indigenous strains. The higher diversity among the *S. cerevisiae* isolates suggests that its genome may be more flexible and may allow more efficient adaptation to the continuously changing environment in the fermenting wine.

This remarkable biodiversity could be decisive in certain technological properties of wine .

3.3. Inoculated fermentations at pilot scale: test of indigenous yeasts for *tirage* proves

On the basis of the microfermentations results, three strains were used as starter cultures in inoculated fermentations at pilot scale in seven cellars producing sparkling wine: two indigenous strains and the starter used in each cellar. Figure 3 shows the trend of the strains in two of the cellar. As reported in Figure 3, the trend of cell concentrations of indigenous strains was very similar to that of the starter. However, at the end of fermentation, the interdelta profiles of the isolates were not corresponding to the inoculated strains suggesting that they were not implanted or dominant the fermentation.

Regarding to sniffing test results (Figure 4) of the sparkling wine, it was realized after the six months from *tirage* proves. In Oltrepò Pavese area, two strains: starter strain and "strain 2" belonging to cellar 1 were evaluated as significantly different. Instead in Franciacorta area, only the starter belonging to cellar 3 was resulted as significantly more pleasant.

Figure 3. *Cell concentrations from two cellar during the* tirage *proves*

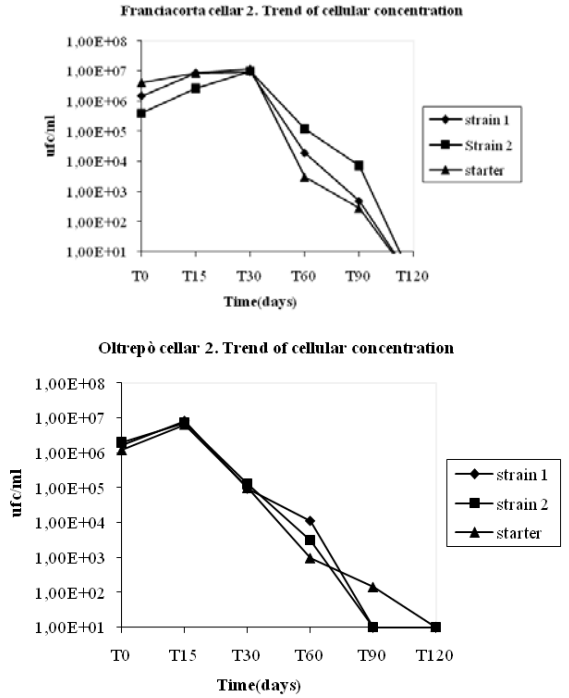
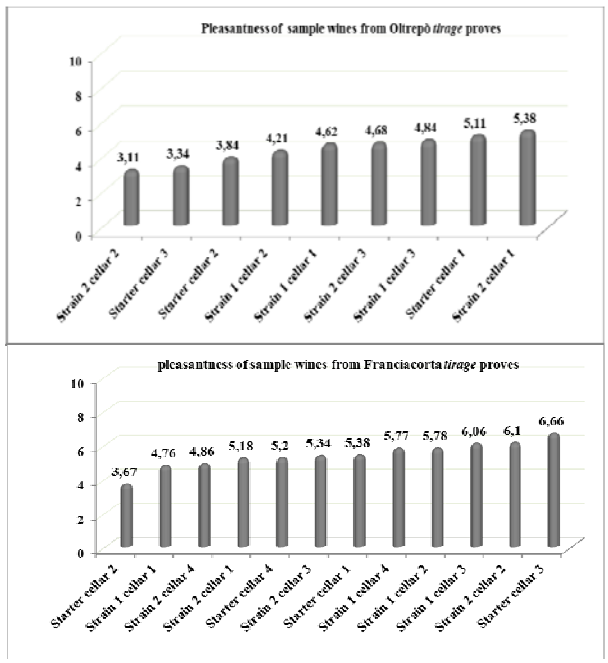


Figure 4. *Pleasantness results of sniffing test from sample wines past six months from the* tirage *proves*



6. Conclusions and Future Perspectives

The genetic investigation of *Saccharomyces cerevisiae* populations isolated in Franciacorta (BS) and Oltrepo' Pavese (PV) evinced that in this districts there was a significant biodiversity of this species. The results show that could be possible to take advantage of biodiversity for the development of new strains that can be considered representatives of these oenological areas. About 70% of the yeast populations present in Franciacorta and Oltrepò Pavese musts and wines corresponding to *S. cerevisiae* species, and some of them could have technological and quality characters for the sparkling wine production. As the quality of wine is strictly related to microbial flora, would remarkable to found indigenous strains that give wines with defined and improved characteristics. This is the first time that the biodiversity of yeast indigenous populations in our region is carried out. Even if the experimentation at pilot scale is work in progress, is expected that new starters can be used in the sparkling wine production made by *champenois* method.

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APPENDIX C

PhD thesis abstract

Sparkling wine obtained through the *Champenoise* method represents a relevant cultural and outstanding economical fact in Italy. There are two Lombardy sparkling wines belonging to *DOCG:* (*Denominazione di Origine Controllata e Garantita*) *Franciacorta DOCG* and *Oltrepò Pavese Metodo Classico* which high quality production is remarkable. Commercial starters belonging to *Saccharomyces* species currently used in Italy for sparkling wine production have been isolated from French territories on the basis of the quality characteristics of *Champagne* wine since they possess very good fermentative and oenological capabilities. Winemaking is a process on which interactions between *Saccharomyces* and non-*Saccharomyces* yeasts take place, influencing wine quality at both levels, sensory and chemical; so assessment of yeast biodiversity is relevant for understanding their evolution in winemaking and consequently increasing the control capability on such processes. Despite the Lombardy region is a very important region for sparkling wines production, a biodiversity analysis of yeast involved in the winemaking process in this region, has still not been carried out.
This PhD was focused on the study of yeasts population biodiversity involved in winemaking process in *Franciacorta* and *Oltrepò pavese* areas as well as the evaluation of technological aspects of indigenous *S. cerevisiae* to be used as potential new starters in the sparkling wine production made by *Champenois* method.
In particular, the main research activities and corresponding results were:

- *Genetic identification of yeast population involved in winemaking process in* Franciacorta *and* Oltrepò Pavese *areas*

Samples of vineyard air, must before SO_2 addition and base wine were collected during 2009, 2010 and 2011 vintages in *Franciacorta* and *Oltrepò pavese* areas. For genetic identification of yeast isolates, genomic DNA was extracted according to Querol et al.(1992). Analysis of *RFLP-ITS* (Fernandez-Espinar et al., 2000) and *D1/D2* of *26S rDNA* sequence (Kurtzman and Robnett, 1998) were performed. For the identification at strain level, interdelta analysis (δ-PCR) (Legras and Karst, 2003) was carried out for isolates identified as *S. cerevisiae* during the 2009 and 2010 vintages. The amplified fragments from δ-PCR were run in capillary electrophoresis (Tristezza et al., 2009). A data elaboration was performed calculating the genetic similarity according to Dice´s coefficient (Dice, 1945). Dendrograms were constructed by the unweighted pair group method using arithmetic averages (UPGMA). During this study it was possible to create a collection of 492 yeast isolates from 24 vineyards belonging to 19 wineries among *Oltrepò Pavese* and *Franciacorta* areas. A total of 13 genus and 25 yeast species were isolated and identified during vintages for 3 years. 186 *S. cerevisiae* isolates were obtained during 2009 and 2010 vintages and from fingerprinting, a great genetic biodiversity was obtained among the analyzed patterns which presented a genetic similarity in a range of 0.2% to 95% by which the 98% of the isolates were *S. cerevisiae* strain-specific.
In conclusion, through yeast biodiversity in *Franciacorta* and *Oltrepò Pavese* areas, it was possible to observe that there is a great opportunity to select autochthonous *S. cerevisiae* strains with typical oenological characters which could be representative of these oenological areas.

- *Yeast population evolution during controlled and spontaneous fermentations*

A monitoring of yeast populations evolution during controlled and spontaneous alcoholic fermentations was carried out in three *Franciacorta* wineries during 2009 vintages. Genetic identification of the isolates was performed as described above except for capillary electrophoresis. The amplified fragments from δ-PCR were directly elaborated through software Gel compare II (Bionumerics Applied Maths, Belgium) and Dendrograms were constructed by the unweighted pair group method using arithmetic averages (UPGMA). A total of 323 isolates were collected from both controlled and spontaneous fermentation of which ninety-eight percent (98%) were

presumptively classified as *S. cerevisiae* according to the results of *RFLP-ITS* of *rDNA* analysis. Through δ-PCR a total of 319 genetic profiles were obtained and analyzed. From the elaboration of the results, 164 different strains among controlled and spontaneous alcoholic fermentation was distinguished (corresponding to 51% of the population investigated). The highest *S. cerevisiae* biodiversity was found in a winery from controlled fermentation with 53 different genetic patterns corresponding to 82% of the yeast population.
In conclusion, this study represented a first approach to population dynamics of oenological yeasts in an important viticulture region as *Franciacorta*. The obtained results have an important significance for the local industry, especially since the controlled fermentations are not always lead by commercial starter strains only, but even by a high percentage of indigenous strains that took place in the process.

- *Selection and evaluation of indigenous* Saccharomyces cerevisiae *strains for Sparkling wine production*

From the two previous phases, sixteen indigenous *S. cerevisiae* strains were selected and their technological and qualitative characterizations were performed. By the microfermentations in laboratory, ethanol, glycerol and acetic acid production were tested by enzymatic protocols. Sniffing tests of wines were realized with a tasters panel and statistical analysis of data was carried out through ANOVA (Statgraphics Centurion Plus 5.1). On the basis of microfermentation and sniffing results, two strains of each *Oltrepò Pavese* and *Franciacorta* area were chosen to be used as indigenous starters in *tirage* proves at pilot scale in different *Franciacorta* and *Oltrepò pavese* wineries. The test was monitored at different time intervals thorough viable counts determination, cellular vitality evaluation and yeast genetic identification in order to determine the indigenous strain dominance and its permanence during fermentation. In addition, samples of wine were subjected to tasting six months after *tirage* proves to evaluate the intensity and pleasantness. Statistical analysis of data was carried out through ANOVA (Statgraphics Centurion Plus 5.1).
In conclusion, four indigenous starters representative of each area demonstrated a high potential to implant and carry out the fermentation successfully. In addition, this potential was supported through tasting-wine proves on which wines produced by these indigenous starters were sensorially accepted at 6 months of aging. This result represents an opportunity of developing indigenous starters formulation to improve the *Franciacorta DOCG* and *Oltrepò Pavese Metodo Classico DOCG* production. From these results, future prospects are promising.

- *Development of a protocol for recovery of yeast DNA from sparkling wines*

Simultaneously to the previous research, a study to set up a protocol for extraction and amplification of yeast DNA from sparkling wine was carried out, aimed to a development of a molecular test that allows the verification of the sparkling wine authenticity.. Sparkling wine bottles from both *Oltrepò Pavese* and *Franciacorta* territories obtained during *tirage* test in wineries were used. Wine samples were filtered at different time intervals and four different DNA extraction protocols were tested.
In conclusion, through this study a protocol based on the combination of multiple methods *Magnetic Beads+Organic Solvents+Real-time PCR* was developed. This molecular approach could be used to improve the traceability for the *DOCG* sparkling wines in Lombardy, allowing the use of *S. cerevisiae* as "trace element" during the process of sparkling wine authentication and as a helpful criterion to investigate *DOCG* status. Future developments of the protocol will be focused on applying the same DNA extraction protocol to commercial bottles ready for the consumer market.

PhD thesis Italian abstract

Il vino spumante ottenuto con il metodo *Champenoise* rappresenta in Italia un aspetto culturale rilevante e, di fatto, anche un importante aspetto economico. Attualmente in Lombardia vi sono due tipi di vino spumante a denominazione di origine controllata e garantita: Franciacorta DOCG e Oltrepò Pavese Metodo Classico, la cui qualità produttiva è di notevole importanza. Gli starter commerciali ad oggi usati in Italia per la produzione di vino spumante sono stati isolati da territori francesi sulla base delle caratteristiche di qualità del vino *Champagne*, poiché possiedono capacità fermentative ed enologiche di ottimo livello. Nel processo di vinificazione delle basi (*Cuvée*) svolgono un ruolo importante le interazioni tra lieviti *Saccharomyces* e non-*Saccharomyces*, influenzando la qualità del vino sia a livello sensoriale che a livello chimico. La valutazione della biodiversità dei lieviti nella produzione di vino risulta conseguentemente necessaria per capirne l'evoluzione della popolazione e per incrementare la capacità di controllo nel processo. Nonostante la Lombardia sia una regione molto importante per la produzione di vino spumante, un'analisi della biodiversità dei lieviti coinvolti non è stata ancora portata a termine.
Questo Dottorato di ricerca è stato indirizzato sia allo studio della biodiversità della popolazione dei lieviti utilizzati in Franciacorta e Oltrepò Pavese, che alla valutazione degli aspetti tecnologici dell'utilizzo di *S. cerevisiae* indigeni come potenziali starter nella produzione di vino spumante con metodo *Champenoise*.
In particolare, le principali attività di ricerca si sono indirizzate verso:

- *Identificazione genetica della popolazione dei lieviti coinvolti nella produzione di vino in Franciacorta e Oltrepò Pavese*

Campioni di aria di vigneti, mosto prima dell'aggiunta di SO_2 e vino di base sono stati raccolti durante le vendemmie del 2009, 2010 e 2011 da entrambi le zone. In particolare, 24 vigneti appartenenti a 19 cantine fra Oltrepo' Pavese e Franciacorta sono stati campionati. Durante questo studio è stato possibile raccogliere una collezione di 492 lieviti la quale è stata identificata a livello di specie attraverso diverse tecniche molecolari. Inoltre, gli isolati risultati appartenenti alla specie *S. cerevisiae* raccolti durante le vendemmie di 2009 e 2010 sono stati identificazione anche a livello di ceppo. In termini di biodiversità, la collezione racchiude 13 generi e 25 specie di lieviti diverse, identificati lungo i tre anni consecutivi. Per quanto riguarda la specie *S. cerevisiae,* durante le vendemmie 2009 e 2010, sono stati raccolti 186 isolati, fra i quali, è stata riscontrata una grande biodiversità genetica.
In conclusione, la valutazione della biodiversità della popolazione dei lieviti è stata importante in quanto si è potuto verificare la diffusione e ricchezza, anche da un punto di vista ecologico presente in questi territoriSi evidenzia l'opportunità di selezionare ceppi di *S. cerevisiae* autoctoni delle zone di Franciacorta e d Oltrepò Pavese che potrebbero presentare dei caratteri enologici tipici di ciascun'area.

- *Evoluzione della popolazione dei lieviti durante la fermentazione spontanea e controllata*

In tre cantine di Franciacorta è stato portato a termine un monitoraggio dell'evoluzione della popolazione dei lieviti durante la fermentazione spontanea e quella controllata nella vendemmia 2009. Sono stati raccolti un totale di 323 isolati dei quali il 99% è risultato appartenente alla specie *S. cerevisiae.* Dunque, 319 isolati sono stati inoltre caratterizzati a livello di ceppo. Questa analisi ha permesso la distinzione di 164 ceppi diversi tra fermentazione spontanea e controllata, corrispondenti al 51% della popolazione analizzata. La più grande biodiversità genetica è stata riscontrata durante la fermentazione spontanea in una cantina: alla analisi delle sequenze interdelta sono stati riscontrati 53 patterns genetici differenti corrispondenti all'82% della popolazione.
In conclusione, questa sperimentazione rappresenta un approccio iniziale allo studio delle dinamiche dei lieviti enologici in un'importante regione vitivinicola come la Franciacorta. I risultati ottenuti hanno un significato importante per l'industria locale, soprattutto poiché è stato verificato

che la fermentazione controllata non è sempre guidata soltanto dagli starter commerciali, ma anche da un'alta percentuale di ceppi indigeni che prendono parte all'intero processo.

- *Selezione di ceppi indigeni di* S. cerevisiae *per la produzione di vino spumante*

Dalle due fasi precedenti, sono stati scelti e saggiati 16 ceppi indigeni di *S. cerevisiae* per una caratterizzazione tecnologica e qualitativa. tra questi due ceppi sono stati selezionati sia per l'Oltrepò Pavese che per la Franciacorta per essere utilizzati come starter durante prove di *Tirage* a scala ridotta in diverse cantine delle due zone. Attraverso diverse prove di controllo è stata valutata la dominanza dei ceppi indigeni e la loro permanenza durante la fermentazione. Inoltre, dei campioni di vino sono stati sottoposti ad una prova di degustazione per la valutazione di intensità e gradevolezza del prodotto dopo sei mesi dalla prova di *Tirage*.
In conclusione, quattro starter indigeni rappresentativi di ciascuna area hanno dimostrato un alto potenziale per iniziare e portare a termine la rifermentazione con successo. Inoltre questa capacità è stata confermata dalle prove di degustazione, nelle quali i vini prodotti con questi ceppi sono stati giudicati accettabili dopo 6 mesi di invecchiamento. Questo risultato dimostra la possibilità di sviluppo di starter indigeni per migliorare la DOCG Franciacorta e Oltrepò Pavese Metodo Classico. Dai risultati ottenuti, le prospettive future sono molto promettenti.

- *Sviluppo di un protocollo per recuperare dal vino spumante il DNA del lievito*

Contemporaneamente alla ricerca precedente, è stato messo a punto un protocollo per l'estrazione e l'amplificazione del DNA del lievito dal vino spumante, allo scopo di sviluppare un test molecolare che permetta la verifica dell'autenticità del vino spumante come possibile elemento di ausilio per il riconoscimento DOCG. Sono state utilizzate bottiglie di vino spumante provenienti sia dalla Franciacorta sia dall'Oltrepo Pavese dopo le prove di tiraggio. I campioni di vino sono stati filtrati in tempi differenti e sono stati testati quattro protocolli differenti di estrazione di DNA.
In conclusione, grazie a questo studio è stato sviluppato un protocollo di estrazione di DNA basato su un metodo multiplo che ha previsto l'utilizzo di biglie magnetiche, solventi organici e PCR- *real time*. Questo approccio molecolare può essere utilizzato per migliorare la tracciabilità dei vini spumante DOCG in Lombardia, permettendo di utilizzare il lievito *S. cerevisiae* come elemento tracciante durante il processo di autenticazione del vino. Gli sviluppi futuri del protocollo saranno indirizzati all'applicazione di esso a bottiglie di spumanti pronte ad uso commerciale.