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INFLUENCE OF AUTOCHTHONOUS MICROBIOTA ON THE SICILIAN WINE PRODUCTION

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DOTTORANDO
Dott. Ciro Sannino

COORDINATORE DEL DOTTORATO
Prof.ssa Maria Antonietta Germanà

TUTOR
Prof. Giancarlo Moschetti

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DOTTORATO

General introduction: the role of microorgnisms in the winemaking process

In accordance to the Italian law (DPR n. 162, Gazzetta Ufficiale n. 73, 23/3/65), the wine is enologically defined as the product carried out by the alcoholic fermentation (partial or complete) of the grapes and/or grapes just crushed and/or grape must. The final content of ethanol of wine should be higher than 60% of the potential ethanol content calculated on the basis of the amount of reducing sugars transformed into ethanol during the fermentation process.

The concentration of microbial populations generally detected on grape surface is comprised between 10^3-10^5 colony forming unit (CFU)/g. Several species and/or strains per species of yeasts, lactic acid bacteria (LAB) as well as acetic acid bacteria (AAB) could be present on grape surface (Barata et al 2011; Francecsa et al 2011; Nisiotou et al 2011).

Up to day, although more than 200 different species of yeasts have been detected in wine environment, the species *Saccharomyces cerevisiae* has been characterized by the lowest frequency of isolation and this species often is at undetectable level (Davenport 1973, 1974; Fleet et al 2002; Barata et al 2011).

The presence of yeasts on grape depends on many factors such as the geographic location, the age of the vineyard (Parrish and Carroll 1985; Longo 1991; Martini et al 1980), the soil type (Farris et al 1990), the cultivar, the harvest technique, the state of maturation (Rosini et al 1982; Pretorius et al 1999) as well as the health state of the grapes (Prakitchaiwattana et al 2004).

On the other hand, the concentration of LAB on grapes is usually recognized at level lower than 10^3 UFC/g as well as their concentration into must just crushed is very low (Bae et al 2006; Fugelsang 1997; Lafon-Lafourcade et al 1983).

Only few species of LAB can grow in the must and in the wine (Konig e Frohlinch 2009) and *Oenococcus oeni*, the main LAB species involved during malolactic fermentation (Henick-Kling, 1993; Lonvaud-Funel 1995), is rarely isolated on grape surfaces (Renouf et al 2007).

The AAB population hosted by grapes is normally detected at very low concentations (10^2 - 10^3 CFU/g) and *Gluconobacter oxydans* is the species most frequent isolated. In case of damaged grapes, the concentration of AAB could increase up to 10^5-10^6 CFU/g (Barbe et al 2001).

Yeasts and LAB are the microbial groups that mainly affect the quality of the final products by fermentations during the entire vinification process. Furthermore, the interactions between the different microbial groups are important in order to understand the dinamycs and the reasons that affect the development of spoilage microorganisms responsible of *off-flavours* in to the final products.

The type of yeast species and/or strains per species could significantly affect the vinification process in terms of rapidity and regularity of alcoholic fermentation thus affect the quality of wines (Zambonelli 1998). Furthermore, the metabolic activities of yeasts such as the production of specific volatile organic compounds and/or organic acids could greatly contribute to define the aroma and flavour of wines.

The alcoholic fermentation is the main technological step of the vinification process and it significantly affects the sensory characteristics of wines (Henschke 1997). In

this process yeasts utilize grape juice constituents, mainly reducing sugars, to produce ethanol and several secondary metabolites determining the organoleptic complexity of the wine (Cole and Noble1997; Lambrechts and Pretorius 2000). These activities greatly vary with the yeast diversity as well as the tipicality of wine flavour could be closely related to the species and/or strains dominating during the fermentations (Fleet and Heard 1993; Fleet 2001).

From this perspective, several studies (Fleet 1992; Lema et al 1996; Romano 1997; Heard 1999; Lambrechts and Pretorius 2000) have been carried out on diversity of metabolites produced by yeasts during winemaking and on their effects on quality of wines.

Several yeast species and strains with their interactive growth and biochemical activities are involved during grape juice fermentation. The wine yeasts could originate from the microflora of the grapes, from the microflora present in the cellar environment as well as carried by birds, insects and air that represent a considerable sources of wine microorganisms. Usually the first phase of spontaneous alcoholic is characterized by growth of the species belonging to the genera *Hanseniaspora*, *Candida* and *Metschnikowi*a that largely originate from the grapes. Other species of the genera *Pichia*, *Issatchenkia* and *Kluyveromyces* may also grow at this stage. The concentration of yeasts is generally around 10^5–10^7 CFU/ml at the beginning of the alcoholic fermentation, after that it increases up to 10^7–10^8 CFU/ml and it remains constant until the end of tumultuous phase of fermentation. When the reducing sugars of grape must are completely metabolized by microorganisms, thus the content of ethanol increases in the wine, the yeast concentrations significantly

decrease. Generally, the presence of the species *S. cerevisiae* reaches detectable levels at the middle phase of alcoholic fermentation. During the spontaneous alcoholic fermentation several yeast species successionally grow as well as the growth of several strains per each yeast species is well recognized (Fleet 2001). Specifically, the number of different strains that grow during fermentation process varies in relation to the grape variety, the health state of grapes, the oenological process and it is significantly affected by microorganisms contaminating the cellar environment. However, several work showed that five or more strains of *S. cerevisiae* could be usually found during the different phases of spontaneous alcoholic fermentation (Schulz and Gaffner 1993; Henick-Kling et al 1998; Sabate et al 1998; Fleet 2001).

Obviously, when the alcoholic fermentation is carried out by selected strains inoculated into grape musts, the number of *S. cerevisiae* strains detectable during the winemaking process greatly decreases and the inoculated strains could dominate the entire process. In this case, the low diversity of the strain belonging to *S. cerevisiae* during the wine process could reduce the complexity of wine flavour as well as the wine tipicality (Fleet and Heard 1993; Fugelsang 1997; Lambrecht and Pretorius 2000).

The LAB, over the yeast populations, represent one of the most important microbial group associated to the wine environment. These microorganisms occur naturally on grapes and their ability to grow in grape juice and wine is well documented (Davis et al 1985; Bartowsky et al 2004; Neeley et al 2005). The growth of LAB in wine is influenced by many factors such as temperature, alcohol concentration, pH, nutrient

availability and sulphur dioxide (SO_2) concentration (Fugelsang 1997). LAB have a defining role in wine production since their activities can be beneficial or detrimental for the quality of wine, depending on the species and/or strain and also on the stage of vinification process at which they develop (Lonvaud-Funel 1999; Renouf et al 2005).

In particular, during winemaking the main LAB activity is represented by the malolactic fermentation. This process usually starts at the end of ethanol fermentation and it is known as biological deacidification based on the decarboxylation of L(−) malic acid to L(+) lactic acid and the production of CO_2. Malic acid, together with tartaric acid, determines the total acidity of wine. These acids represent more than 90% of the totality of wine organic acids.

Furthermore, malic acid (e.g., characteristic acidity in apples) is more acidic in taste than lactic acid (e.g., acidity of dairy fermented drinks). After malic acid bioconversion, a smaller amount of the milder acid is formed and wine is additionally saturated with CO_2 (Versari et al 1999; Davis et al 1985; Henick-Kling 1995).

Excepted the biological deacidification, the LAB activity is clearly represented by the impact of malolactic fermentation on wine aroma and taste. In this sense, the LAB biosynthesis of several metabolites such as acids, alcohols and esters reduce the undesirable plant or herb aromas and increase the level of fruit and flower flavours (Versari et al 1999; Davis et al 1986; Henick-Kling 1995; Maicas et al 1999).

The malolactic fermentation involves several different LAB species that mainly belong to the genera *Oenococcus*, *Lactobacillus*, *Pediococcus* and *Leuconoctoc*. Up

to now, strains of the species *Oenococcus oeni* (previously named *Leuconostoc oenos*) have been reported as the most efficient and appropriate in order to carry out the malolactic fermentation process (Maicas 2001; Versari et al 1999; Lopez et al 2007; Costello et al 1983).

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The aims of the PhD research thesis

Today, it is possible to define a "microbiological quality" for wine productions. This quality is affected by several factors that includes the health of grapes, the secondary aromas product by yeasts during alcoholic fermentation, the wine complexity obtained on lees of yeast during ageing, the taste balance generated by lactic acid bacteria during the malolactic fermentation, etc.

The high quality and typicality of many wines is due to the presence of yeast and LAB strains in the territory (or in the winery) particularly suitable for the fermentation of musts of specific grape varieties. These microorganisms, named as "autochthonous" yeasts, are naturally selected by various factors such as the environment, the tradition, the agronomic practices and the winery processes. This assumption sustains the study on wine yeasts and LAB ecology of specific environments.

With this perspective, **the first aim** of the present research thesis was to study the yeast ecology of Grillo grapes, the main cultivated grape variety in the production area of the Marsala wine and to isolate and select several strains belonging to the species *S. cerevisiae* characterized by high oenological aptitudes in order to use them for large-scale wine production.

With regards to wine yeast diversity, the present work also focused on the ecology of yeasts associated to vineyard environment during a specific time period: from the period just after the grape harvesting until the grape berry fruiting. The final scope was to check the presence of wine yeasts into vineyards when the grapes were not

formed and not present on plants in order to advance our knowledge on vineyard yeast ecology. To study this issue, the isolation and identification of the microbial population of a Portuguese vineyard was carried out analyzing the soil and different parts of plants, with particular attention to the nods of trunk vine plants.

Wine fermentation has been traditionally performed as a spontaneous process conducted by yeasts naturally present on the surfaces of grape berries and in cellar environment. Several studies focused on the ecology of wine yeasts and the works conducted by De Rossi (1935) could be considered, in Italy, as pioneer studies. Different works showed that spontaneous alcoholic fermentation is performed by many species and strains per each species of yeasts, that could significantly improve the sensory profiles of wines by producing several secondary chemical compounds.

In addition, the inter- and intra-specific biodiversity that characterizes the yeast microflora of a spontaneous fermentation is closely related to several environmental factors such as the soil and the clime, thus pedo-climatic factors, that could differ year by year.

Despite several researches aimed to understand and to manage the spontaneous fermentation, this process is still characterized by several potential risks.

Thus, the types of yeast species and their quantity at the beginning of the fermentation, the growth kinetics, the development and the persistence of each population during the entire vinification process mainly affect the organoleptic characteristics of the final wine.

On the base of the considerations above reported, **the second aim** of the present research thesis was focused on the monitoring of Sicilian wine production under

natural regime. The experimental wines produced following the natural regime were carried out by a spontaneous fermentation and without the addiction of any enological adjuvants, excluding also the addition of sulfites. The study was focused on wines obtained using grapes of Grillo and Catarratto varieties, that are largely cultivated as autochthonous Sicilian cultivars. The grapes grew under organic regime and the vinification processes were realized at large-scale production of wines commercially sold as "I.G.T. Sicilian wines.

The third scope of the present thesis was focused on development of a innovative winemaking process of Nero d'Avola grape cultivar in order to reduce the risks associated to spontaneous fermentations. Specifically, the experimental vinification was carried out by spontaneous alcoholic fermentation and based on use of pied de cuve fortified by ethanol addition. The "pied de cuve" represents the inoculum of a partially fermented must with fermentative cell yeasts into a fresh must. The alcoholic fermentation of the pied de cuve could be carried out by yeast starter, previously inoculated in the must or by yeasts naturally present in the must, thus by spontaneous fermentation.

This method allows to have a lot of active yeast cells able to start rapidly the alcoholic fermentation, to assure the presence of several strains of *S. cerevisiae* species at high concentrations during the entire vinification process carried out by spontaneous alcoholic fermentation.

Chapter 1. Yeast ecology of vineyards within Marsala wine area (western Sicily) in two consecutive vintages and selection of autochthonous *Saccharomyces ceravisiae* strains

1.1 Introduction

Yeasts responsible for the alcoholic fermentation of grape juice into wine are basically distinct in two groups: non-*Saccharomyces* (NS) species, that generally grow during the first stages of fermentation, and *Saccharomyces* strains, which complete the fermentation. The growth of NS yeasts during fermentation is mainly affected by alcohol and nutrient concentrations (Pretorius 2000); when the ethanol increases, yeasts of the genus *Saccharomyces*, especially *Saccharomyces cerevisiae*, become dominant.

Since the 80's, starter cultures belonging to the species *S. cerevisiae* became commercially available in order to drive the alcoholic fermentation and obtain wines with wanted characteristics (Subden 1987). However, despite the benefits due to the selected yeasts, in terms of effectiveness and ethanol yield (Reed and Chen 1978), their employment in winemaking is quite controversial. One of the main reasons of objection for the routine use of commercial starter yeasts is due to their massive prevalence, after a few days of fermentation, over the native microflora, with the consequent risk of loss of wine peculiarities (Valer et al 2005). Furthermore, the recent growing interest for wines with definite "*terroir*" characteristics determined a re-discovery of wine fermentation by using indigenous yeasts occurring on grapes and/or in the winery environment (Francesca et al 2010; Le Juene et al 2006).

As a matter of fact, starter cultures selected from autochthonous *S. cerevisiae* are commonly employed in winemaking, not only because they ensure controlled fermentation, but also because they are fundamental to obtain wines with predictable quality and typicality. Although the inoculation of must with selected *S. cerevisiae* is expected to suppress the indigenous NS strains, several studies have revealed that NS yeasts can indeed persist during the various stages of wine production driven by pure cultures of *S. cerevisiae* (Martinez et al 1989; Mora et al 1990).

Regarding natural fermentations, *Saccharomyces* and NS yeasts do not coexist passively, but they interact. Under these conditions, some oenological traits of NS yeasts are not expressed, or may be modulated by *S. cerevisiae* cultures (Ciani et al 2006; Anfang et al 2009). During spontaneous fermentation the succession of the different yeasts, with an appreciable variability in their ratio, determines the formation of the sensorial complexity in wines. NS yeasts contribute to the aroma complexity of wine due to their secondary metabolites (Soden et al 2000). Some authors reported that these yeasts produce extracellular enzymes which provide typical aromatic notes that link the wines to the production region (Charoenchai et al 1997; Pretorius et al 1999).

During the first stages of spontaneous fermentations, the large biodiversity of yeasts derives from vineyards and cellars (Le Juene et al 2006; Ciani et al 2004). Besides the influence of climate conditions, age of vineyards and oenological practices (Santamaria et al 2005; Zott et al 2008), one defining factor affecting the microbial structure at the beginning of wine production may be represented by the environmental contamination of commercial starter *S. cerevisiae* strains. The massive

and continued use of these strains may determine their dissemination in a restricted area (Valero et al 2005). Although the commercial strains are spread not too far from the winery, this phenomenon could be relevant in areas characterized by a high number of cellars, since it may influence negatively the final wines.

The modern trend of wine market is going towards products with particular peculiarities. Among special wines, including fortified and non-fortified wines, Marsala produced in the homonymous area of western Sicily is historically known outside Italy since 1773 thanks to the English trader John Woodhouse. Marsala enjoys a Denominazione di Origine Controllata (DOC) status that is a recognition of quality (controlled designation of origin). This product requires a base wine for its production and the cultivar Grillo is one of the most cultivated grapevine in Sicily to be fermented to this purpose.

Keeping in mind that wine production still remains a very traditional process, especially in areas where a long history and typicality of products is felt as an affection to the territory, the objectives of this study were to: examine the qualitative structure and the quantitative development of indigenous yeasts during the fermentation of Grillo cultivar, which represents the base wine for Marsala DOC product; to characterize *S. cerevisiae* isolates at strain level; and to investigate on the oenological potential of *S. cerevisiae* strains.

1.2 Materials And Methods

1.2.1 Sample collection

Ten vineyards (Table 1) of the "Grillo" variety were sampled for grapes and berries within the Marsala wine production area (Sicily, Italy) during the harvesting of two consecutive vintages (2008 and 2009). All vineyards were at least 10 km far from the closest winery. The sampling was made in three 100 m^2-subareas (representing three replicates of the same vineyard) distant approximately 100-300 m from one another. In each vineyard, fifteen grapes and 3.0 kg of grape berries (five grapes and 1 kg of barriers from each sub-area) were randomly collected from undamaged grapes. All samples were then stored at 4 °C during transport.

Grape samples (G) were placed into sterile plastic bags containing a washing isotonic peptone solution (10 g/L Bacto Soytone, 2 mL/L Tween 80) and incubated at 30°C for 3 h to collect the microorganisms hosted on peel surface (Renouf et al 2005).

Berries were crushed by stomacher (BagMixer® 400, Interscience, Saint Nom, France) for 5 min at the highest speed to obtain must that was transferred into sterile flasks (5 L-volume) and maintained at 17 °C until total sugar consumption. The samples collected for analysis were: grape must just pressed (M1), must at 1/5 (M2), 3/5 (M3) and 5/5 (M4) of sugar consumption.

1.2.2 Microbiological analysis

Cell suspensions recovered from grapes and must samples were serially diluted in Ringer's solution (Sigma-Aldrich, Milan, Italy). Decimal dilutions were spread plated (0.1 mL) onto Wallerstein laboratory (WL) nutrient agar (Oxoid, Basingstoke, UK),

incubated at 28°C for 48-72 h, for the counting of total yeasts (TY) and onto modified ethanol sulfite agar (MESA), prepared as reported by Francesca et al 2010, incubated at 28 °C for 72 h, to detect presumptive *Saccharomyces* spp. (PS). Both media were supplemented with chloramphenicol (0.5 g/L) and byphenil (1 g/L) to inhibit the growth of bacteria and moulds, respectively. Analyses were carried out in duplicate.

Statistical analyses were conducted using STATISTICA software (StatSoft Inc., Tulsa, OK, USA). Microbial data were analysed using a generalised linear model (GLM) including the effects of vineyard (V = Guarrato, Lago Preola, Madonna Paradiso, Mazara del Vallo, Mothia, Musciuleo, Pietra Rinosa, Pispisia, Tre Fontane and Triglia Scaletta), year (Y = 2008, 2009) and sample type (S = G, M1 to M4) and all their interactions (V*Y*S); the Student "t" test was used for mean comparison. The *post-hoc* Tukey method was applied for pairwise comparison. Significance level was $P<0.05$.

1.2.3 Yeast isolation and identification

Yeasts were isolated from both growth media used for counts. Three colonies per morphology were collected from the differential medium WL, while 10 colonies were randomly picked up from MESA. All isolates were purified to homogeneity after several sub-culturing steps onto WL and at least two isolates (from each sample) sharing the same morphology were subjected to the genetic characterization.

The DNA extraction was performed using the InstaGene Matrix kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions.

In order to perform a first differentiation of yeasts, all selected isolates were analyzed by restriction fragment length polymorphism (RFLP) of the region spanning the internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene. The DNA fragments were amplified with the primer pair ITS1/ITS4 (Esteve-Zarzoso et al 1999) by means of T1 Thermocycler (Biometra, Göttingen, Germany) and subsequently the amplicons were digested with the endonucleases *Cfo*I, *Hae*III and *Hinf*I (MBI Fermentas, St. Leon-Rot, Germany) at 37 °C for 8 h. The isolates presumptively belonging to the genus *Hanseniaspora* were further digested with the restriction enzyme *Dde*I (MBI Fermentas) (Esteve-Zarzoso et al 1999). ITS amplicons as well as their restriction fragments were analysed twice on agarose gel using at first 1.5% (w/v) agarose and then 3 % (w/v) agarose in 1 × TBE (89 mmol/L Tris-borate, 2 mmol/L EDTA pH 8) buffer. Gels were stained with SYBR® safe DNA gel stain (Invitrogen, Milan, Italy), visualized by UV transilluminator and acquired by Gel Doc 1000 Video Gel Documentation System (BioRad, Richmond, USA). Standard DNA ladders were 1kb Plus DNA Ladder (Invitrogen) and GeneRuler 50 pb DNA Ladder (MBI Fermentas). Five isolates representative of each group were subjected to an additional enzymatic restriction targeting the 26 rRNA gene. After amplification with the primer pair NL1/LR6 the PCR products were digested with the endonucleases *Hinf*I, *Mse*I and *Apa*I (MBI Fermentas) (Baleiras-Couto et al 2005) and visualised as above described. One isolate per group was further processed by sequencing the D1/D2 region of the 26S rRNA gene and/or 5.8S-ITS rRNA region to confirm the preliminary identification obtained by RFLP analysis. D1/D2 region was amplified with primers NL1 and NL4 (O'Donnel 1993). PCR products were visualised as

above. DNA sequencing reactions were performed at Primmbiotech S.r.l. (Milan, Italy). The identities of the sequences were determined by BlastN search against the NCBI non-redundant sequence database located at http://www.ncbi.nlm.nih.gov.

1.2.4 Strain typing of *S. cerevisiae* isolates

Intraspecific characterization of the isolates belonging to *S. cerevisiae* species was carried out through two techniques: interdelta analysis with primers delta12 and delta 21 (Legras and Karst 2003) and microsatellite multiplex PCR based on the analysis of polymorphic microsatellite loci named SC8132X, YOR267C and SCPTSY7 (Vaudano and Garcia Moruno 2008). The PCR products were analyzed on agarose gel 2.0% (w/v) in 1 × TBE buffer and visualized as above reported.

1.2.5 Technological characterization of *S. cerevisiae* strains

All strains belonging to the species *S. cerevisiae* were evaluated for their potential in winemaking. The ability to produce H_2S was tested using a qualitative method performed on Bismuth Sulfite Glucose Glycerin Yeast extract (BiGGY) agar (Oxoid) (Jiranek et al 1995). H_2S was estimated by colony blackening after 3 days of incubation at 28 °C. A five-level scale was used for colour evaluation: 0 = white, 1 = beige, 2 = light brown, 3 = brown, 4 = dark brown, 5 = black. The resistance to various levels of ethanol (from 12 to 16 % v/v) and potassium metabisulphite (KMBS) (from 50 to 300 mg/L) were determined onto MESA. *S. cerevisiae* GR1 (Francesca eta al 2010) and NF213, belonging to the culture collection of DEMETRA Department (University of Palermo, Italy), producing low amount of

H_2S and resistant to high levels of KMBS and ethanol were used as control strains. Copper tolerance was evaluated as the ability of a strain to grow in presence of different concentration (50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 µmol/L) of $CuSO_4$ (Fiore et al 2005). The strains characterized by high production levels of acetic acid were indicated by the halo produced around colonies onto calcium carbonate agar plates after 7-day incubation at 25 °C (Caridi et al 2002). *S. cerevisiae* GR1 was used as negative control, while *Hanseniaspora uvarum* TLM14 (DEMETRA culture collection) as positive control. The growth at low temperatures was determined in Yeast Extract Peptone Dextrose (YPD) broth at 13 and 17 °C for five days. Growth patterns were examined through visual inspection of samples through a light microscope (Carl Zeiss Ltd) (Pretorius 2000; Di Maio et al 2012). Foam production was examined according to Regodón et al. (1997). All analysis were carried out in triplicate.

1.2.6 Microfermentations

The strains showing the best technological performances (low production of H_2S and acetic acid, resistance to ethanol, KMBS and $CuSO_4$, ability to grow at low temperatures, growth in suspended form and low foam production) were evaluated for their ability to ferment a grape must. Broth cultures in the stationary phase were washed twice in Ringer's solution and inoculated in 1 L of pasteurized Grillo grape must (pH 3.3, 21.6 °Brix, 151.6 mg/L yeast available nitrogen) added with KMBS (100 mg/L) at a final concentration of about 10^6 CFU/mL. Microfermentations were carried out at 13 and 17 °C. In order to allow CO_2 removal, the flasks were plugged

with a Müller valve containing sulphuric acid (Ciani and Rosini 1987) and the weight loss was monitored until the daily decrease was lower than 0.01 g (end of fermentation process). According to Ciani and Maccarelli (1998), fermentation power (FP) was evaluated as the ethanol amount (% v/v) produced at the end of the process, fermentation rate (FR) was calculated as CO_2 daily produced and fermentation purity (FPu) was calculated as acetic acid (g/L) per ethanol (% v/v) produced at the end of microfermentation. A control microfermentations was inoculated with *S. cerevisiae* GR1. At the end of fermentation, the wines were analysed for residual sugar, acetic acid and glycerol content following the standard methods of the Organization of Vine and Wine.

The same strains used for fermentation were also evaluated for their enzymatic activities: β-glucosidase activity (Hernendez et al 2003) was tested in presence of arbutin, esculin, 4-methylumbelliferil β-D-glucopyranoside (MUG) and 4-nitrophenyl β-D-glucopyranoside (p-NPG); proteolytic activity was assayed as reported by Bilinsky et al. (1987). All analysis were carried out in triplicate.

1.3 Results

1.3.1 Microbiological analysis

The viable counts of TY and PS populations investigated in this study are reported in Table 1. TY counts on the grape surface were in the range 3.54 – 6.92 and 3.16 – 6.08 Log CFU/g in vintage 2008 and 2009, respectively. On average, higher levels of TY were observed on grapes collected in 2008 ($P<0.05$), that were above 6 Log CFU/g for Mothia, Musciuleo, Tre Fontane and Triglia Scaletta vineyards. Data recovered

from MESA showed that, except samples from Guarrato vineyard in the vintage 2008 and Tre Fontane vineyard in the vintage 2009, grapes did not host yeasts ascribable to PS group at detectable levels.

The yeast populations analysed at different steps during sugar consumption were also monitored. TY load of M1 samples were higher than that detected on the corresponding grapes ($P<0.05$). Regarding PS populations, the concentrations found for M1 samples from Guarrato 2008 and Tre Fontane 2009 were higher ($P<0.05$) than those found in G samples and detectable levels were registered in six other M1 samples. During fermentation, both TY and PS counts increased significantly ($P<0.05$); although often M3 samples showed higher levels than M4, not always the highest concentrations were displayed by M3 samples, since in some cases it was registered for M4 or M2 samples.

In general, the effect of vineyard, year and sample type was found to significantly ($P<0.001$) affect count data of PS, while for TY the concentration levels were affected by vineyard ($P<0.001$) and sample type ($P<0.001$), but not by year. The combination of the three independent variables (V*Y*S) significantly affected both PS and TY counts.

1.3.2 Isolation and identification of yeasts

A total of 1144 colonies from WL and 987 from MESA were isolated, purified to homogeneity and separated on the basis of appearance of colony morphology on WL. At least two cultures from each sample were morphologically selected obtaining 1021 isolates (614 from WL and 407 from MESA) which were subjected to molecular

identification. After restriction analysis of 5.8S-ITS region and 26S rRNA gene, the isolates were clustered in 14 groups (Table 2): three of these groups (X, XI and XIII) were directly identified by comparison of restriction bands with those available in literature (Esteve-Zarzoso et al 1999;Cordero et al 2011; Muccilli et al 2011). These patterns corresponded to *Lachancea thermotolerans*, *Metschnikowia pulcherrima* and *S. cerevisiae* species. Eleven groups could not be identified by RFLP analysis, then the identification at species level was concluded by sequencing of D1/D2 domain of the 26S rRNA gene which was successful for all groups obtained by enzymatic digestions.

1.3.3 Yeast species distribution

The distribution of yeast species among vineyards and vintages, as well as their concentration estimated for each sample, are reported in Table 3. *Hanseniaspora uvarum*, *M. pulcherrima* and *Aureobasidium pullulans* were the species most frequently encountered on grapes and musts soon after pressing. In general, the concentration levels detected on WL were higher than those found on MESA. *S. cerevisiae* was never detected on grapes and only once in M1 (Mothia, 2008). However, in the last case, the concentration of *S. cerevisiae* was relevant (*ca*. 10^6 CFU/mL). The samples M2 and M3 were dominated by *H. uvarum*, *S. cerevisiae* and *Candida zemplinina* in both years reaching levels ranging between 6 and 8 orders of magnitude. *Hanseniaspora opuntiae* was also isolated in several M2 and M3 samples at high concentrations but only in the vintage 2009. At the end of fermentation process, *S. cerevisiae*, *H. uvarum* and *Pichia kudriavzevii* were detected in several

M4 samples of the two consecutive vintages and *C. zemplinina* only in 2008. Interestingly, in this technological step, the yeast levels found on MESA were comparable or even superimposable with those estimated on WL. Although in the samples obtained from Musciuleo and Pietra Rinosa vineyards *S. cerevisiae* was never isolated in both vintages, it resulted dominant, alone (in the majority of the vineyards analysed) or in combination with other species such as *H. uvarum*, *H. opuntiae* and *L. thermotolerans*, reaching concentrations within 6 – 8 Log CFU/mL. When *S. cerevisiae* was not detected, the species dominating the fermentation process were *H. uvarum*, *P. kudriavzevii* or *C. zemplinina*.

1.3.4 Typing of *S. cerevisiae* strains and geographic distribution

The 447 isolates belonging to the species *S. cerevisiae* were further genetically characterized. The interdelta analysis was able to separate the isolates in 51 groups, while microsatellite multiplex PCR recognized 44 different groups, showing a lower discriminatory power than the first technique. A dendrogram resulting from the cluster analysis of the 51 interdelta profiles is reported in Figure 1. Except a few strains found in the same vineyard in a given year (CS136 and CS179; CS338 and CS339) which clustered at high levels (>90%), no particular similarities were found among strains isolated within the same vineyard. Furthermore, no strain was found in different vineyards or vintages.

The distribution of *S. cerevisiae* strains among the vineyards analyzed (Table 4) was found to be non-homogeneous. When *S. cerevisiae* was found, the number of strains recognized was between 1 and 12; the sampling from Lago Preola, Madonna Paradiso

and Triglia Scaletta sites produced a very low number of strains, on the contrary Mothia, Pispisia and Mazara del Vallo were richer in *S. cerevisiae* biodiversity with 12, 11 and 10 different strains, respectively.

1.3.5 Technological screening of *S. cerevisiae* strains

The 51 *S. cerevisiae* strains were screened for their oenological characters (Table 5). Thirty-two strains were characterized by a low production of H_2S on Biggy agar plates (white - light brown colony) and resistance to high levels of ethanol (14-16% v/v). Moreover, 36 and 48 strains showed growth in presence of high concentrations of KMBS (150-300 mg/L) and $CuSO_4$ (400-500 mmol/L), respectively. Twenty-eight strains were found to produce low levels of acetic acid. The growth at low temperatures (13 and 17 °C) was positive for 22 strains, whereas all 51 developed in suspension. Only 5 strains were found to produce more than 2 mm of foam.

From the previous technological tests, 14 strains were selected and used as starters to ferment grape must at 13 and 17 °C in presence of 100 mg/L of KMBS. The results of the fermentation kinetics (Table 6) showed that, in terms of FP, FR and FPu, three strains (CS160, CS165 and CS182) showed better technological aptitudes than control strains.

After fermentation, enzymatic activities were determined as quality parameters (Table 6). The above three strains were characterised by optimal β-glucosidase activity, in particular onto agar plates containing esculin and MUG. However, no *S. cerevisiae* showed protease activity.

1.4 Discussion

Microbial dynamics are important during long-term fermentation processes, such as wine productions, since the availability of the grapes occurs once a year and an anomalous evolution of the microorganisms in the fermenting musts may determine low quality products and conspicuous economic losses for producers. Due to the renewed interest shown by consumers, several wines, including Marsala, are gaining importance.

In the recent years, the interest toward autochthonous yeasts to be used as starters in winemaking processes is increased and it is still on the increase. Some researchers found that yeasts and lactic acid bacteria harboured on grapes and acting during the spontaneous fermentations possess an important economic potential (Di Maio et al 2012; Francesca et al 2011). A wine produced with autochthonous yeast starters enjoys a status of tradition and typicality and is requested by expert wine consumers. Furthermore, the use of yeasts selected in a given geographical area represents a valuable technological alternative to the application of commercial starter cultures responsible for wine flavor standardization, as well as to the spontaneous fermentation that may lead to undesirable aroma developments.

The wine quality can be affected by the growth of different yeasts originating from the microbial communities hosted on grapes (Fleet 2008). In the present work, we pictured the structure of yeast communities present on grapes of Grillo cultivar, in must and during its steps of spontaneous fermentations, focusing on the technological selection of *S. cerevisiae* strains. Ten vineyards, representing the principal sites of Marsala wine production area, were sampled during two

consecutive years (2008 and 2009). Yeast counts reflected a non-homogeneous distribution among sampling sites and vintages, but, in general, the effect of vineyard, year and sample determined significant differences on the concentrations of TY and PS. The finding that the majority of yeasts occurring on grapes did not belong to the *Saccharomyces* genus is in agreement with previous reports (Sabatè et al 2002; Mercado et al 2007). On the other hand, the presence of PS populations increased during alcohol fermentation confirming that these stages of fermentation represent the right moments for the isolation of *Saccharomyces* strains.

The process of isolation resulted in the collection of 1144 yeasts. After restriction analysis of 5.8S-ITS rRNA region and 26S rRNA gene, 14 yeast groups were recognized. Only three of them were easily identified at species level, whereas for the other 11 groups, characterized by atypical restriction profiles of 5.8S-ITS, the sequencing of the D1/D2 domain of the 26S rRNA gene was necessary. Atypical polymorphism for this region is not surprising for yeasts, since many authors observed this behavior in several strains (Fernandez et al 2000; Kurtzman et al 2003; Tofalo et al 2009; Francesca et al 2012). At the end of the identification process, 14 species belonging to 10 genera (*Aureobasidium*, *Candida*, *Cryptococcus*, *Hanseniaspora*, *Issatchenkia*, *Lachanceae*, *Metschnikowia*, *Pichia*, *Saccharomyces* and *Wicheromyces*) were found.

The yeast communities present on the samples resulted complex. As previously stated by other authors (Sabatè et al 2002; Gonzales et al 2007), NS yeasts were dominant on grapes and in must soon after pressing, while only a few species (*H. uvarum*, *S. cerevisiae*, *C. zemplinina* and *P. kudriavzevii*) represented the prevailing flora during

the stages of fermentation. Although the frequency of the species is generally calculated on the total number of isolates collected from the different vineyards and in the entire period of observation, which may include consecutive vintages (Di Maio et al 2012; Mercado et al 2007; Li et al 2010; Romancino et al 2008), we found this approach arbitrary. The species proportion is unavoidably altered by the isolation process that is performed randomly. In this study we analyzed the yeast species distribution based on their effective concentrations (Table 3).

H. uvarum was the species mainly isolated during fermentation. In some cases it was found at levels of 10^7 - 10^8 CFU/mL in both vintages. Its high frequency of isolation at these stages, confirms a general behaviour observed for other grape varieties (Li et al 2010; Raspor et al 2006). This species is abundant in warm and hot regions and replaces its anamorphic form *Kloechera apiculata* (Boulton et al 1996). The distribution of *H. uvarum* in different geographic regions might be linked to the low altitude and high temperature (Jolly et al 2006), climatic factors that characterize the area of production of Marsala wine. Within *Hanseniaspora* genus, *Hanseniaspora guilliermondii* is the species reported to be mainly present in warm climates (Romancino et al 2006), but in our study it was isolated in a few samples, not above 10^7 CFU/mL, collected only during 2009 vintage. The species *Hanseniaspora opuntiae* was also isolated. Interestingly, this species was found when *H. uvarum* was absent and its presence was more frequent in the vintage 2009. *H. opuntiae* has been reported to be a member of the grape ecosystem (Nisiotou et al 2007) and to dominate the first stages of alcoholic fermentation (Bovo et al 2009), but no information is

available in literature on its presence at the late phases of the process. In this work *H. opuntiae* was detected at approximately 10^7 CFU/mL at 3/5 sugar consumption.

Another species isolated at high frequency on grapes and in must soon after pressing was *M. pulcherrima*. This result could be linked to the capability of this species to prevail by inhibiting the growth of different yeasts, including *S. cerevisiae* (Nguyen and Panon 1998). *A. pullulans* was also particularly present in these samples, but only in 2009 vintage. Generally, this species has been detected on unripe grape berries (Renouf et al 2005) and in grape musts (Francesca et al 2010; Sabatè et al 2003) and Verginer et al (2010) reported its influence in the flavour development of red wines. In the present study, strains of this species were isolated only from WL agar plates, even at 10^6 CFU/mL, showing their susceptibility to the selective conditions of MESA; hence, they do not represent potential wine contaminants. Among the yeast species isolated at low frequency, it is interestingly to note the presence of *Cr. flavescens* isolated on grapes at 10^4 CFU/g in a single vineyard and reported to be isolated on this matrix only once in China (Li et al 2010).

The spontaneous fermentations were then dominated by *H. uvarum, S. cerevisiae*, *C. zemplinina* and *P. kudriavzevii*. Despite the selective conditions of fermentation, NS populations reached levels of concentration comparable to the PS load until the end of fermentation. Several researchers have focused on the positive influence of NS yeasts emphasizing their potential application as starters in wine productions (Anfang et al 2009; Ciani and Maccarelli 1998; Loureiro and Malfeito-Ferreira 2003). Furthermore, the use of *Hanseniaspora* spp. in combination with *S. cerevisiae* has been reported to contribute positively to the complexity and aroma of wine (Ciani et al 2006; Moreira

et al 2008). This may be due to the capability of these yeasts, e.g. *H. uvarum* strains, to secrete several enzymes, such as *β*-glucosidase and proteases, that could contribute to the expression of varietal aroma of grapevine (Zott et al 2008; Jolly et al 2006). *C. zemplinina* was also isolated in several samples and at high concentrations (till 10^7 - 10^8 CFU/mL). These strains could represent an important source of starters to be employed for mixed fermentations with *S. cerevisiae*, since their interaction was demonstrated to increase the fermentation kinetics of grape must (Tofalo et al 2012). Moreover, some *C. zemplinina* strains are osmotolerants, producers of low concentration of acetic acid and high amounts of glycerol from sugars (Sipiczki et al 2011; Tofalo et al 2011) and may found application to reduce the ethanol content of wines produced by grape musts characterized by high sugar content, such as those produced in the Marsala area. Regarding *P. kudriavzevii*, it is usually detected on grapes (Li et al 2010) and in the early stages of alcoholic fermentation (Di Maro et al 2007), thus its finding at the latest stages of fermentation needs further investigation.

S. cerevisiae strains selected from indigenous populations of a given area might drive the alcoholic fermentation better than commercial starters (Lopes et al 2002). Due to their oenological importance, all *S. cerevisiae* cultures isolated in this work were investigated at strain level and subsequently characterized for their technological features. The combination of interdelta analysis and multiplex PCR determined the differentiation of the 447 isolates collected in 51 strains. The cluster analysis showed that none common pattern was found among strains isolated from different vineyards or vintages. Many authors claimed that autochthonous yeasts are linked to a specific area (Lopes et al 2002; Schuller et al 2005) and stable in consecutive years (Schuller

et al 2005). For many others, the occurrence of strains in the vineyards is only temporary, because several factors such as the climatic conditions, the grape treatment and sanity (Prakitchaiwattana et al 2004) and the degree of grape maturation (Rosini et al 1982) influence the structure of yeast communities on grapes. Based on their technological properties, especially on their ethanol resistance, a key factor for the production of wines with high alcohol content, 14 *S. cerevisiae* strains (isolated from five of the ten vineyards, mainly from Pispisia site during 2008 vintage) were selected and tested as starters in Grillo grape must showing interesting oenological features. Among these 14 *S. cerevisiae*, only two couples of strains (CS133-CS165 and CS338-CS339) found in the same vineyard in the same year shared a certain phylogenetical similarity, but no other strain was found in different vineyards or vintages. Three strains (CS160, CS165 and CS182) were characterized by a relevant FP, a capacity of paramount importance in this type of wine, since a high rate of sugar consumption is mandatory. Furthermore, they also showed better technological aptitudes than control strains.

In conclusion the yeast populations analyzed in ten vineyards located in the area of Marsala DOC wine, which have never been explored before, showed generally a stable structure, but some differences in species and concentration levels were found between the two consecutive years (2008 and 2009) object of study. Fourteen autochthonous *S. cerevisiae* strains displayed a technological potential to drive the fermentation of must into wine. Furthermore, another important result of this work is the presence of *H. uvarum*, *C. zemplinina* and, interestingly, *P. kudriavzevii* in place of or at comparable levels of *S. cerevisiae* in the stages of fermentation characterized

by high ethanol concentration. Thus, the technological investigation of these isolates is being prepared in order to design mixed strain starters for the preservation of the typicality of the wines.

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Table 1. Microbial loads[a] of samples collected from Grillo vineyards and microfermentations.

Samples[b]	Vineyards									
	Guarrato	Lago Preola	Madonna Paradiso	Mazara del Vallo	Mothia	Musciuleo	Pietra Rinosa	Pispisia	Tre Fontane	Triglia Scaletta
TY (2008)										
G	6.0±0.3	5.13±0.3	3.54±0.6	4.98±0.7	6.92±0.3	6.39±0.2	5.12±0.5	5.65±0.2	6.41±0.2	6.84±0.5
M1	6.25±0.3	5.60± 0.4	3.27±0.3	5.98±0.4	6.78±0.4	6.64±0.3	5.36±0.4	6.67±0.4	6.81±0.3	6.99±0.2
M2	7.38±0.4	6.87±0.8	7.15±0.2	7.08±0.2	8.28±0.3	5.99±0.5	5.77±0.4	8.24±0.4	7.17±0.0	7.46±0.2
M3	8.15±0.1	8.05±0.4	7.91±0.7	7.96±0.2	7.89±0.4	4.93±0.4	4.13±0.2	7.84±0.5	6.55±0.5	8.01±0.3
M4	8.09±0.4	4.79±0.4	4.42±0.4	8.09±0.5	7.98±0.6	2.93±0.1	1.39±0.5	7.54±0.6	4.16±0.1	7.21±0.5
PS (2008)										
G	2.47±0.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
M1	3.06±0.1	n.d.	n.d.	n.d.	3.92±0.1	n.d.	n.d.	n.d.	n.d.	n.d.
M2	6.20±0.1	3.56±0.2	3.12±0.2	5.88±0.7	7.14±0.2	5.08±0.1	3.29±0.4	6.5±0.8	5.23±0.3	5.71±0.6
M3	8.16±0.8	4.14±0.0	4.62±0.5	6.46±0.1	6.76±0.3	2.24±0.4	2.94±0.3	7.16±0.0	5.02±0.1	7.50±0.7
M4	7.36±0.5	3.81±0.2	3.44±0.3	7.48±0.3	7.02±0.7	1.0±0.0	n.d.	7.37±0.5	2.02±0.1	6.72±0.5
TY (2009)										
G	5.56±0.4	5.79±0.2	5.93±0.8	6.08±0.2	4.07±0.2	4.01±0.3	5.77±0.5	4.29±0.3	4.36±0.4	3.16±0.6
M1	5.25±0.8	6.30±0.3	6.09±0.6	6.6±0.3	5.0±0.3	5.54±0.4	5.25±0.4	5.03±0.5	5.29±0.4	3.98±0.5
M2	7.39±0.9	7.20±0.3	8.25±0.3	7.76±0.2	7.97±0.4	5.91±0.7	7.20±0.4	7.81±0.3	8.09±0.2	5.84±0.2
M3	7.59±0.4	7.27±0.5	8.78±0.7	7.38±0.4	7.83±0.6	4.26±0.5	7.09±0.2	7.55±0.2	7.85±0.6	6.77±0.4
M4	7.27±0.4	8.16±0.6	8.17±0.1	7.53±0.1	7.97±0.5	1.86±0.4	5.95±0.7	7.66±0.3	7.54±0.3	6.27±0.7
PS (2009)										
G	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.94±0.5	n.d.
M1	n.d.	2.13±0.7	1.84±0.4	n.d.	2.66±0.1	n.d.	n.d.	3.07±0.1	3.44±0.6	2.03±0.1
M2	5.47±0.3	5.47±0.1	7.76±0.6	2.87±0.3	5.64±0.5	3.85±0.9	6.30±0.4	5.22±	5.12±0.2	4.15±0.1
M3	7.4±0.0	7.21±0.5	8.77±0.4	5.10±0.1	6.60±0.8	3.12±0.2	5.85±0.3	7.54±0.7	7.22±0.3	5.92±0.6
M4	7.17±0.3	7.04±0.0	6.97±0.2	6.90±0.9	6.89±0.6	n.d.	5.62±0.9	7.07±0.1	6.97±0.1	6.16±0.1

[a] Log CFU/g for grape berries; Log CFU/mL for must samples.

Abbreviation: G, grape berries; M1, grape must just pressed; M2, grape must at 1/5 sugar consumption; M3, grape must at 3/5 sugar consumption; M4, grape must at 5/5 sugar consumption; TY, total yeasts on WL nutrient agar; PS, presumptive *Saccharomyces* on MESA.

n.d., not determined.

Table 2. Molecular identification of yeasts.

R.P.	Isolate code	5.8S-ITS PCR	Size of restriction fragments				26S PCR	Size of restriction fragments			Species (% identity)[a]	Accession No.
			*Cfo*I	*Hae*III	*Hinf*I	*Dde*I		*Hinf*I	*Mse*I	*Apa*I		
I	CS236	600	190+170+90	450+130	290+180+130	n.s.r.	1150	500+400+170	620+370+90+55	n.c.	*Aureobasidium pullulans* (99)	JX129904
II	CS15	500	205+175	450	240+125	n.s.r.	1100	370+270+220	n.c.	n.c.	*Candida apicola* (99)	JX129912
III	CS271	475	210+110	n.c.	235+235	n.s.r.	1100	340+210+75	750+130+90+65	n.c.	*Candida zemplinina* (100)	JX129898
IV	CS244	540	260+210	n.c.	300+180+60	n.s.r.	1100	410+200+105+85	400+380+250+65	n.c.	*Cryptococcus flavescens* (99)	JX129901
V	CS206	650	345+275	570+80	260+240+140	n.s.r.	1100	265+200+185+160+140	410+390+280	n.c.	*Cryptococcus magnus* (99)	JX129907
VI	CS231	750	335+115	n.c.	370+205+175+75	380+180+90+70+60	1190	n.c.	600+410+100+65	n.c.	*Hanseniaspora guilliermondii* (99)	JX129905
VII	CS203	750	335+115	n.c.	370+205+175+75	400+175+90+60	1100	400+170+100	n.c.	n.c.	*Hanseniaspora opuntiae* (100)	JX129909
VIII	CS234	750	335+115	n.c.	370+205+175+75	310+160+90+70+60	1100	400+170+100	500+400+100+65	n.c.	*Hanseniaspora uva rum* (99)	JX129914
IX	CS212	420	125+100+90+70	310+110	225	n.s.r.	1100	500+315+100+90+60	800+200+90	n.c.	*Issatchenkia terricola* (98)	JX129906
X	CS240	720	315+290	340+220+85	315	n.s.r.	1100	500+400+170	600+400+60	n.c.	*Lachanceae thermotolerans* (99)	JX129903
XI	CS51	400	200+90	300+100	200+180	n.s.r.	1100	n.c.	n.c.	n.c.	*Metschnikowia pulkerrima* (98)	JX129913
XII	CS280	500	215+190	400	230+160	n.s.r.	1150	500+400+125+100	1000+95	n.c.	*Pichia kudriavzevi* (98)	JX129897
XIII	CS325	880	380+360+140	340+255+175+140	375+130	n.s.r.	1100	500+210+190	1000+70	n.c.	*Saccharomyces cerevisiae* (99)	JX129896
XIV	CS27	650	650	700	310	n.s.r.	1130	500+250+190+170	1000+70	n.c.	*Wicheromyces anomalus* (98)	JX129911

All values for the 5.8S-ITS PCR, 26S PCR and restriction fragments are given in bp.
Abbreviations: R.P., restriction profile; n.c., not cut; n.s.r., not subjected to restriction.
[a] According to BlastN search of D1/D2 26S rRNA gene sequences in NCBI database.

Table 3a. Geographical and distribution[a] of yeast species during spontaneous fermentations (2008 vintage)

Species	Vineyards									
	Guarrato	Lago Preola	Madonna Paradiso	Mazara del Vallo	Mothia	Musciuleo	Pietra Rinosa	Pispisia	Tre Fontane	Triglia Scaletta
A. pullulans				G (4■)			G(5■)M1(5■)			
C. apicola									G(6■),M1(6■)	
C. zemplinina				M1(5■) M2(7■)			M2(5■)			
Cr. flavescens										
Cr. magnus										
H. guilliermondii										
H. opuntiae							M2(5■) M3(4■)			
H. uvarum	G(6■,2□) M1(6■,4□) M2(7■,□) M3(8■,7□)	M2(6■) M3(8■,4□) M4(8■,3□)	M2(7■,3□)M3(7■, 4□) M4(3□)	M2(7■,5□) M3(7■)	G(6■) M1(6■,3□) M2(8■,7□) M3(7■,6□)	M1(6■) M2(6■,5□) M3(4■,2□) M4(2■,1□)		M2(8■,6□) M3(7■)	M1(6■) M2(7■,5□)	G(6■) M1(6■) M2(7■,5□)
I. terricola										
L. thermotolerans	G(2□) M1(6■,3□) M2(7■)						M2(5■,3□) M3(4■)			
M. pulcherrima	G(6■) M1(6■)		G(3■) M1(3■) M2(7■)	M1(5■)				G(5■) M1(6■) M2(8■) M3(7■)	G(6■) M1(6■)	
P. kudriazdevi							M2(3□) M3(4■,2□) M4(1■)		M3(6■,5□) M4(4■,2□)	
S. cerevisiae	M2(6□) M3(8■,□) M4(8■,7□)			M3(7■,6□) M4(8■,7□)	M1(6■,3□) M3(6□) M4(7■,□)			M3(7■,□) M4(7■,□)		M2(7■,5□) M3(8■,7□) M4(7■,6□)
W. anomalus										

[a] The number reported between brackets refers to the highest concentration (Log cycle) of detection.
Symbols: ■, yeast count onto WL nutrient agar; □, yeast count onto MESA.
Abbreviations: *C.*, *Candida* spp.; *Cr.*, *Cryptococcus* spp.; *H.*, *Hanseniaspora* spp.; *I.*, *Issatchenkia* spp.; *L.*, *Lachancea* spp.; *M.*, *Metschnikowia* spp.; *P.*, *Pichia* spp.; *S.*, *Saccharomyces* spp.*; W.*, *Wickerhamomyces* spp.; G, grape berries; M1, grape must just pressed; M2, grape must at 1/5 sugar consumption; M3, grape must at 3/5 sugar consumption; M4, grape must at 5/5 sugar consumption.

Table 3b. Geographical and distributiona of yeast species during spontaneous fermentations (2009 vintage).

Species	Vineyards									
	Guarrato	Lago Preola	Madonna Paradiso	Mazara del Vallo	Mothia	Musciuleo	Pietra Rinosa	Pispisia	Tre Fontane	Triglia Scaletta
A. pullulans		G(5■) M1(6■)		G(6■) M1(6■)		G(4■)	G(5■) M1(5■)	G(4■) M1(5■)	G(4■) M1(5■)	
C. apicola										
C. zemplinina		M2(7■,5□) M3(7■,□) M4(8■,7□)			M1(5■,2□) M2(7■,5□)		M2(7■,6□) M3(5■,□)		M1(5■)	G(3■) M1(3■,2□) M2(5■,4□) M3(6■,5□) M4(6■,□)
Cr. flavescens					G(4■)					
Cr. magnus		G(5■) M1(6■)								
H. guilliermondii		M2(7■)	G(5■)						G(4■) M1(5■) M3(7■,6□) M4(7■,6□)	G(3■) M1(3■)
H. opuntiae	G(5■) M1(5■) M2(7■) M3(7■)	M1(6■)	M1(6■) M2(8■)	M2(7■) M3(7■)			G(5■) M1(5■) M2(7■)	M1(5■,3M) M2(7■) M3(7■)	M2(8■) M3(7■)	
H. uvarum	M3(7■,□) M4(7■,□)	G(5■) M1(2■,□) M2(7■,5□) M3(7■,□) M4(8■,7□)	M2(8■,7□) M3(8■)	M2(7■) M3(7■)	M1(5■) M2(7■,5□) M3(7■)	M2(3□) M3(4■,3□) M4(1■)			M1(5■,3□)	
I. terricola			G(5■) M1(6■)						G(5■) M2(5■)	
L. thermotolerans							M1(5■) M4(6■,4□)			
M. pulcherrima	M1(5■) M2(7■)	M1(5■) M2(6■)	M1(6■)	M1(6■)		M1(5■) M2(5■)		M2(7■) M3(7■)	M1(5■)	
P. kudriazdevi	M4(7■,□)	M1(6■) M2(5□) M3(7■)		M2(2□) M3(7■) M4(7■,6□)			M3(7■,5□) M4(5■,□)		G(1□) M3(7■)	
S. cerevisiae	M3(7■,□) M4(7■,□)	M3(7■,□) M4(8■,□)	M3(8■,□) M4(8■,□)	M3(7■,5□) M4(7■,6□)	M3(7■,6□) M4(7■,6□)			M2(7■,5□) M3(7■,□) M4(7■,□)	M1(3□) M2(5□) M3(7■,□) M4(7■,6□)	
W. anomalus										

[a] The number reported between brackets refers to the highest concentration (Log cycle) of detection.
Symbols: ■, yeast count onto WL nutrient agar; □, yeast count onto MESA.
Abbreviations: *C.*, *Candida* spp.; *Cr.*, *Cryptococcus* spp.; *H.*, *Hanseniaspora* spp.; *I.*, *Issatchenkia* spp.; *L.*, *Lachancea* spp.; *M.*, *Metschnikowia* spp.; *P.*, *Pichia* spp.; *S.*, *Saccharomyces* spp.*; W.*, *Wickerhamomyces* spp.; G, grape berries; M1, grape must just pressed; M2, grape must at 1/5 sugar consumption; M3, grape must at 3/5 sugar consumption; M4, grape must at 5/5 sugar consumption.

Table 4. Geographical and annual distribution of *S. cerevisiae* strains during spontaneous fermentations.

Vineyards	No. of *S.cerevisiae* isolates			No. of distinct patterns		
	2008	2009	Total	2008	2009	Total
Guarrato	28	43	71	2	3	5
Lago Preola	–	31	31	–	1	1
Madonna paradiso	–	33	33	–	2	2
Mazara del Vallo	26	38	64	4	6	10
Mothia	26	46	72	4	7	11
Musciuleo	–	–	–	–	–	–
Pietra Rinosa	–	–	–	–	–	–
Pispisia	34	47	81	5	7	12
Tre Fontane	–	48	48	–	7	7
Triglia Scaletta	47	–	47	3	–	3
Total	161	286	447	18	33	51

Table 5. Technological screening of *S. cerevisiae* strains.

Strain code	H_2S[a]	Ethanol[b]	KMBS[c]	$CuSO_4$[d]	$CaCO_3$[e]	13 °C[f]	17 °C[g]	Growth pattern[h]	Foam[i]
CS71	2	4	6	10	−	+	+	S	F0
CS72	4	2	5	9	−	−	−	S	F0
CS100	3	3	4	8	+	−	−	S	F0
CS127	1	2	5	8	−	−	−	S	F1
CS128	0	4	6	10	−	+	+	S	F0
CS129	3	3	4	8	+	+	+	S	F0
CS133	0	4	6	10	−	+	+	S	F0
CS136	1	2	3	8	+	−	−	S	F0
CS139	4	3	5	8	−	−	−	S	F0
CS148	1	4	5	10	−	+	+	S	F0
CS155	1	4	6	10	−	+	+	S	F0
CS160	2	4	6	10	−	+	+	S	F0
CS162	1	4	6	10	−	+	+	S	F0
CS165	0	4	6	10	−	+	+	S	F0
CS178	2	1	3	8	+	−	−	S	F1
CS179	4	3	3	9	−	−	−	S	F0
CS180	1	4	6	10	−	+	+	S	F0
CS182	2	4	6	10	−	+	+	S	F0
CS255	4	4	4	9	−	+	+	S	F0
CS267	3	3	4	8	+	−	−	S	F0
CS274	2	2	3	9	+	−	−	S	F0
CS275	4	3	4	10	+	+	+	S	F0
CS277	3	1	4	9	+	−	−	S	F1
CS278	4	4	3	8	−	+	+	S	F1
CS289A	4	3	4	7	−	−	−	S	F0
CS289B	4	3	3	8	+	−	−	S	F0
CS292	2	3	4	8	+	−	−	S	F0
CS295	3	1	3	9	−	−	−	S	F0
CS309	4	3	4	8	−	−	−	S	F0
CS310	4	4	4	7	+	−	−	S	F0
CS311	3	2	5	8	−	−	−	S	F0
CS313	3	3	5	9	+	−	−	S	F0
CS314	4	2	5	9	+	+	+	S	F0
CS315	3	1	4	7	−	−	−	S	F0
CS316	4	2	4	10	−	+	+	S	F0
CS317	2	2	3	9	+	−	−	S	F0
CS318	2	1	3	8	+	−	−	S	F0
CS319	3	1	3	9	+	−	−	S	F0
CS320	4	2	5	10	−	+	+	S	F0
CS321	4	3	4	9	+	+	+	S	F0
CS322	3	1	3	8	−	−	−	S	F0
CS323	2	2	3	9	+	−	−	S	F0
CS325	1	3	4	9	+	−	−	S	F0
CS326	1	3	3	8	+	−	−	S	F0
CS327	1	2	4	8	+	−	−	S	F0
CS328	1	2	3	8	+	−	−	S	F0
CS329	1	3	5	10	−	+	+	S	F0
CS331	1	4	6	10	−	+	+	S	F0
CS332	1	4	3	8	+	−	−	S	F1
CS338	1	4	5	10	−	+	+	S	F0
CS339	1	4	5	10	−	+	+	S	F0

[a] color of colony on Biggy agar plates: 0 = white; 1 = beige; 2 = light brown; 3 = brown; 4 = dark brown; 5 = black.

[b] 0, 0% (v/v); 1, 10% (v/v); 2, 12% (v/v); 3, 14% (v/v); 4, 16% (v/v) of ethanol contained in MESA plates at which strains showed growth.

[c] 50 mg l/l; 2, 100 mg/l; 3, 150 mg/l; 4, 200 mg/l; 5, 250 mg/l; 6, 300 mg/l of MBSK contained into MESA plates at which strains showed growth.

[d] 0, 0 μM; 1, 50 μM; 2, 100 μM; 3, 150 μM; 4, 200 μM; 5, 250 μM; 6, 300 μM; 7, 350 μM; 8, 400 μM; 9, 450 μM; 10, 500 μM of $CuSO_4$ contained into YPD agar plates at which strains showed growth.

[e] +, precipitation halo; − , non precipitation halo on $CaCO_3$ agar plates.

[f] +, growth; − , not growth at 13 °C in YPD broth.

[g] +, growth; − , not growth at 17 °C in YPD broth.

[h] S, suspended growth; F, flocculant growth in YPD broth.

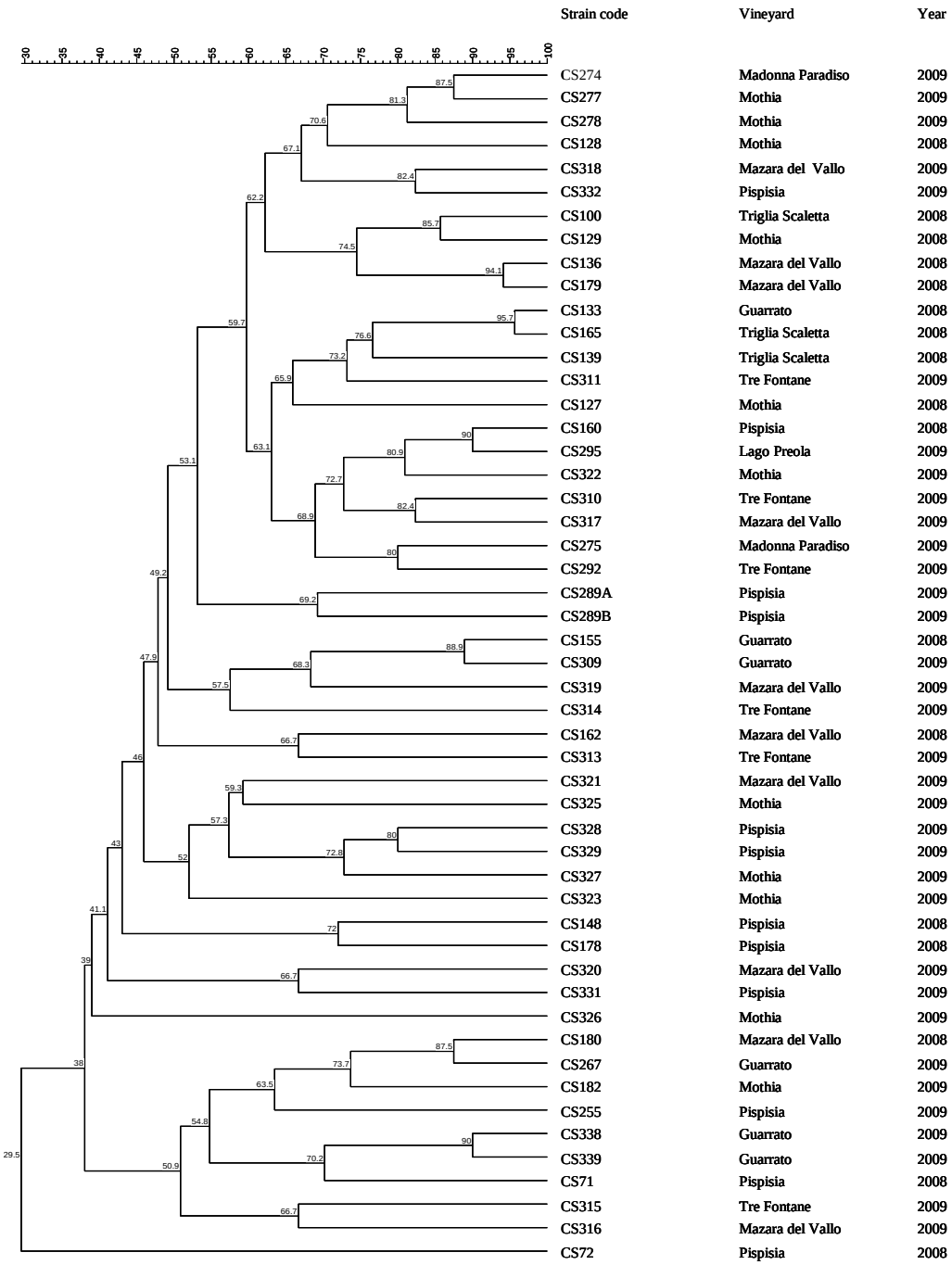
[i] F0, foaming lower than 2 mm; F1, foaming among 2 and 4 mm; F2, foaming greater than 4 mm.

Table 6. Kinetics of alcoholic microfermentations and enzymatic activities of preselected *S. cerevisiae* strains.

Strain code	Fermentation power[a]		Fermentation rate[b]		Volatile acidity[c]		Fermentation purity[d]		Residual sugar[e]		Glycerol content[f]		Glucosidase activity[g]				Protease activity[g]
	13 °C	17 °C	13 °C	17 °C	13 °C	17 °C	13 °C	17 °C	13 °C	17 °C	13 °C	17 °C	Esculin	Arbutin	MUG	p-NPG	
CS71	11.32±0.08	11.44±0.05	1.39±0.06	2.51±0.04	0.57±0.13	0.61±0.08	0.05±0.02	0.05±0.01	2.46±0.08	1.81.±0.08	7.40±0.08	7.43±0.08	+	−	+	+	−
CS128	11.27±0.21	12.73±0.08	1.79±0.12	3.20±0.31	0.34±0.05	0.35±0.21	0.03±0.01	0.03±0.02	2.30±0.02	1.30±0.01	7.56±0.02	7.61±0.01	++	−	+++	+	−
CS133	11.35±0.04	12.71±0.13	1.43±0.05	3.29±0.06	0.33±0.21	0.36±0.12	0.03±0.04	0.03±0.03	2.29±0.01	1.31±0.03	7.50±0.13	7.62±0.03	+++	−	+++	+	−
CS148	11.03±0.12	11.14±0.07	1.44±0.30	2.35±0.01	0.41±0.03	0.44±0.13	0.04±0.01	0.04±0.04	2.77±0.04	2.03±0.03	7.51±0.21	7.31±0.02	−	−	−	−	−
CS155	11.15±0.34	12.70±0.23	1.29±0.07	2.26±0.02	0.31±0.08	0.32±0.34	0.03±0.03	0.03±0.02	2.42±0.11	1.31±0.09	7.06±0.02	7.64±0.01	−	−	−	−	−
CS160	12.63±0.01	12.68±0.02	1.76±0.17	3.08±0.23	0.28±0.02	0.31±0.07	0.02±0.02	0.02±0.01	1.33±0.01	1.29±0.02	7.55±0.01	7.63±0.31	+++	−	++	+	−
CS162	10.12±0.11	11.84±0.11	1.28±0.07	2.64±0.04	0.51±0.05	0.55±0.03	0.05±0.02	0.05±0.01	3.24±0.03	1.62±0.12	6.97±0.02	7.01±0.12	−	−	−	−	−
CS165	12.67±0.14	12.50±0.01	2.09±0.02	2.64±0.06	0.27±0.11	0.30±0.21	0.02±0.02	0.02±0.03	1.27±0.01	1.42±0.03	7.61±0.04	7.59±0.21	++	−	++	+	−
CS180	12.49±0.03	12.59±0.31	1.08±0.04	2.20±0.17	0.36±0.01	0.45±0.21	0.03±0.03	0.04±0.04	1.41±0.03	1.43±0.06	7.03±0.01	7.56±0.05	+	−	+	+	−
CS182	12.41±0.23	12.84±0.03	1.39±0.03	3.25±0.24	0.41±0.06	0.47±0.31	0.03±0.01	0.04±0.02	1.52±0.09	1.26±0.04	7.39±0.11	7.63±0.01	++	−	+	+	−
CS329	11.29±0.12	11.81±0.06	1.07±0.04	2.31±0.04	0.57±0.25	0.65±0.28	0.05±0.02	0.06±0.02	2.09±0.02	1.61±0.21	7.52±0.31	7.52±0.03	+++	−	++	+	−
CS331	11.31±0.41	11.91±0.41	1.02±0.06	2.61±0.07	0.49±0.31	0.56±0.37	0.04±0.02	0.05±0.01	2.33±0.13	1.57±0.07	7.21±0.05	7.54±0.02	+++	−	++	+	−
CS338	11.25±0.01	11.45±0.09	1.07±0.09	2.25±0.21	0.59±0.07	0.58±0.18	0.05±0.03	0.05±0.01	1.98±0.05	1.78±0.02	7.20±0.01	7.33±0.11	+++	−	++	+	−
CS339	11.13±0.02	11.31±0.07	1.09±0.13	2.28±0.17	0.48±0.24	0.57±0.02	0.04±0.01	0.05±0.03	2.68±0.11	1.91±0.09	6.77±0.02	7.29±0.01	+	−	+	+	−
PC-1	11.87±0.11	12.01±0.04	1.24±0.19	2.77±0.11	0.41±0.20	0.48±0.11	0.03±0.01	0.04±0.08	1.88±0.12	1.67±0.10	7.19±0.09	7.33±0.14	+++	−	++	+	−
PC-2	12.03±0.23	12.34±0.06	1.27±0.02	2.63±0.04	0.44±0.12	0.47±0.01	0.04±0.21	0.04±0.03	1.61±0.02	1.49±0.03	7.41±0.03	7.53±0.12	++	−	+++	+	−

[a] Ethanol (% v/v) produced at the end of microfermentation.
[b] CO2 produced after 3 days of fermentation (CO_2/day).
[c] Acetic acid (g/l) produced at the end of microfermentation.
[d] Volatile acidity formed in relationship to ethanol produced at the end of microfermentation.
[e] Reducing sugars (g/l) at the end of microfermentation.
[f] Glycerol (g/l) produced at the end of microfermentation.
[g] Enzymatic activity: +++, high; ++,medium; +, low;− , not detected.

Figure 1. Dendrogram resulting from interdelta analysis of *S. cerevisiae* strains.



Chapter 2. Analysis of yeast ecology related to vineyards in Portugal

2.1 Introduction

In the wine production yeasts play an important role for the characteristics of the final product and the yeasts involved during winemaking belong to the ascomycetous group. The origin of wine yeasts is very difficult to determine (Mortimer 2000; Naumov 1996). Several studies demonstrated the presence of yeasts in the soil but only few of them is associated to the alcoholic fermentation (Botha 2006, 2011; Wuczkowski and Prillinger 2003; Slavikova and Vadkertiova 2003; Taovsik and Ovreas 2002; Bonkowski et al 2000; Spencer and Spencer 1997; Gray and Williams 1971; Do Carmo-Sousa 1969). A lot of studies showed that insects are carriers of fungi and bacteria (Stevic 1962; Lachance et al 1994) and, according to Mortimer and Polsinelli (1999), *Saccharomyce cerevisiae*, the most important fermenting yeast, needs a vector to contaminate the grapes and wine related-environments because it is not an air-borne contaminant. With this regard the aim of this work was to know how the yeasts, and in particular *S. cerevisiae*, could contaminate the grape.

2.2 Materials and Methods

2.2.1 Microbial isolation from vineyard soil

After the harvest of grapes in 2011 vintage, soil samples were collected from experimental vineyard at the Instituto Superior de Agronomia (Lisbon, Portugal,

latitude 38°42’31.57” N, longitude 9°11’14.01” W) and transported to the laboratory for microbial analysis.

The soil samples were collected from:

1. areas near to the cellar;
2. areas in the centre of vineyard;
3. areas far from the cellar.

Soil samples were taken with sterile utensil from several part of each row selected, they were mixed into stomacher bags and after that the samples were transported in the laboratory for microbiology analysis.

Ten grams of soil were distributed in 250 mL flasks with 90 mL of Ringer solution and incubated to 25 °C in agitation for 25 min.

After the incubation, the isolation was carried out by two different techniques:

- serial dilution of samples in saline solution to reduce the microbial load and direct plating on nutrient selective media;
- inoculation into enrichment media to support the development of the microbial species.

Yeast isolation was performed on two different media: (i) medium for total yeast composed of yeast extract 20 g/L, glucose 10 g/L, bactotryptone 10 g/L, agar 22 g/L, pH 5,0, after autoclaving add biphenyl (0,30 g/L) and chloramphenicol (10 mg/L) to inhibit the moulds and bacteria growing respectively. (ii) medium for isolation of *Saccharomyces cerevisiae* on yeast nitrogen base with 1% raffinose and 8% ethanol (Sampaio et al 2008).

2.2.2 Microbial isolation from *vitis vinifera* nods

The nods were taken in sterility and transported to the laboratory for the analysis.

The analysis were made as reported below:

- wash the sample under running tap-water for at least 10 minutes to remove adhering soil particles;
- bring the samples into the laminar air-flow and rinse it with 70% ethanol for 30s;
- place the samples into another beaker containing 1.0% sodium hypochloride (containing 0.1% Tween 80) for 10 minutes;
- to remove the disinfectant, rinse the samples with 70% ethanol for 30s and then five times with Phosphate buffer (PBS) 12.4 mM, ph 7.1;
- dry the samples with sterile paper towels (optional);
- crush the samples with the help of mortal-pastle;
- mix appropriate amount of PBS and spread on suitable media using serial dilution technique.

2.2.3 Microbial isolation from insects

Two different traps, characterized by two different solutions of volatile compounds, were placed in the vineyard and in the vinery in order to catch insects.

Solution A: acetic acid 4 g/L and ethyl acetate 1 g/L.

Solution B: phenylacetic acid 10 mg/L.

The insects trapped were incubated in tryptone soy broth for 48 h at 25 °C, after incubation were removed 0.5 uL of broth and spread on appropriate solid media and incubated at 25 °C for 48 h.

2.2.4 Phenotypic selection of yeast

The cell morphology of isolates were determinate with microscope after purification, and the colonies selected were stored in -80 °C in glycerol solution.

The isolates were subjected to Ascomycetous and fermentative yeast selection with urease test and fermentative test. Urease test was performed on Christensen's medium: bacteriological pepton 1.0 g/L, glucose 1.0 g/L, Nacl 5.0 g/L, KH_2PO_4 2,0 g/L, phenol red 0.012 g/L. Adjust pH 6.8 and dispense 4.5 mL in each tube and autoclave. When medium is cool add 0.5 mL of 20 % filter sterilized urea. Asomycetous yeast showed yellow colors in the tube.

With regards to the fermentation tubes, Durham column were arranged and the yeasts grew in the follow medium: glucose 20.0 g/L, yeast extract 5.0 g/L, KH_2PO_4 1.0 g/L, $(NH_4)_2PO_4$ 1.0 g/L. pH was adjusted at 5.0. Bubble formation into Durham tube confirmed the fermentation activity by yeasts.

2.2.5 Molecular identification

In order to perform a first differentiation of yeasts, all isolates were analyzed by restriction fragment length polymorphism (RFLP) of the region spanning the internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene. The DNA fragments were first amplified with the primer pair ITS1/ITS4 (Esteve-Zarzoso

et al 1999) by means of T1 Thermocycler (Biometra, Göttingen, Germany). The amplicons were then digested with the endonucleases CfoI, HaeIII and HinfI (MBI Fermentas, St. Leon-Rot, Germany) at 37 °C for 8 h.

ITS products, as well as restriction fragments were analysed on agarose gel twice using 1.5 and 3% (w/v) agarose in 1 × TBE (89 mM Tris-borate, 2 mM EDTA pH 8) buffer. Gels were stained with SYBR safe DNA gel stain (Invitrogen, Milan, Italy), visualized by UV transillumination and acquired by Gel Doc 1000 Video Gel Documentation System (BioRad, Richmond, USA). Standard DNA ladders were 1kb Plus and 50 pb (Invitrogen).

One isolate per group was further processed by sequencing of the D1/D2 region of the 26S rRNA gene and/or 5.8S-ITS rRNA region to confirm previous identification. D1/D2 region was amplified with primers NL1 and NL4 (O'Donnell 1993). The identities of the sequences were determined by BlastN search against the NCBI non-redundant sequence database located at http://www.ncbi.nlm.nih.gov.

DNA was extracted from fresh colony of yeast, and the following protocol was performed:

- two loopfulls of a culture grown were added in 2 mL tube with 1.8 mL of sterile H_2O and 200 uL of glass beads;
- centrifuge 3 min at 1300 rpm to pellet cells;
- store the pellet at -20 °C (at least 1h);
- add 250 uL of TE-Phenol, 250 uL chloroform, 500 uL of lysis buffer;

- disrupt cells for 20 min in a vortex;
- centrifuge 25 min at 13000 rpm;
- add 1 mL of EtOH (100%) in 1.5 mL tube + 400 uL of the supernatant;
- centrifuge 10 min at 13000 rpm;
- dissolve the pellet with 40 uL TE+RNase and put 15 min at 55 °C;
- diluite DNA 1:100 with sterile water for PCR analysis;
- store DNA at -20 °C.

2.3 Results and discussion

A total of 86 yeasts were isolated. In particular, 81 yeasts were isolated from the soil samples, 4 from nods and only 1 from insects (*Drosophila* spp.).

Only the 24 % of total number of isolates were preliminary identified as ascomycetes, confirmed by urease test. Moreover approximately 20% of total isolates showed fermentation in presence of glucose.

After restriction analysis of 5.8S-ITS region the isolates were clustered in 13 groups (Table 1). The restriction profile (RP) II was the mainly represented (29% of isolates), followed by RP I (22% of isolates) and RP XII (15.1% of isolates). Furthermore the RP I and II were found in all sampled parts of the vineyards. The yeast species associated to the main representative profiles were *Cryptococcus aerius*, *Rhodotorula glutinis* and *Lachanchea thermotolerans*. The species *Cryptococcus aerius* and *Rhodotorula glutinis* are commonly related to soil (Botha 2011; Gollner 2006). The specie *Lachanchea thermotolerans* was

also isolated from *Drosophila* spp. This yeast was formerly associated to *Drosophila* (Barata et al 2012,) but it was isolated also from leaf surface of plants (Slavikova et al 2007) and on grape berries (Settanni et al 2012). In the recent study this yeast was used with co-inoculum with *Saccharomyces cerevisiae* to enhance acidity and improve the overall quality of wine (Gobbi et al 2013).

The species *Rhodotorula glutinis* and *Aureobasidium pullulans* were isolated from plant nods. Several studies reported the presence of these yeasts in the soil and in parts of plants and/or on grape surphase (Botha 2011; C˘ade˘z et al 2010; Fleet 2003; Jolly et al 2003, 2006; Spencer and Spencer 1997; Raspor et al 2006; Renouf et al 2005), but up to now, no works showed the presence of this yeast species in nods of plants.

Despite the natural populations of *Saccharomyce*s are commonly associated with the different enological environments, including bark and soil in the proximity of the trees (Sniegowski et al 2002), in this work no strain belonging to this genus was isolated from any of the samples analysed (soil, nods or insects). The absence of these strains in soil could be due to its hostility. It is well known that different factors, e.g. soil character, climate, plant and soil communities may influence the microbial growth (Phaff and Starmer 1987), and this could be the case of *Saccharomyces*. However, this finding is not surprising, since Ascomycetous yeasts represent a low percentage of the total fungi present in soil (Wuczkowski and Prillinger 2004).

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Table 1. Molecular identification of yeasts

RP	Size of restriction fragments			Species (% identity)[a]
	*Cfo*I	*Hae*III	*Hinf*I	
I	298+215+98	620	216+120+96	*Rhodotorula glutinis* (99)
II	341+202+109	454	365+287	*Cryptococcus aerius* (100)
III	3431	432	219+190	*Kodamaea ohmeri* (99)
IV	185+173+92	447+149	283+178+127	*Aureobasium pullululans* (100)
V	254+208+77	553	201+180+106	*Cryptococcus terrestris* (100)
VI	299	432+150+90	330	*Cryptococcus membranefaciens* (99)
VII	348+308	510+84+52	375+282+231	*Cryptococcus magnus* (99)
VIII	322+299	420+110	277+157+113+81	*Rhodotorula nothofagi* (100)
IX	253+192+94	490+100	310+273	*Cryptococcus heveanensis* (99)
X	304	377+109	326+279	*Bullera dendrophila* (99)
XI	244+200+95	423+82+68	259+194+170	*Cryptococcus macerans* (100)
XII	319+285	313+212	348	*Lachanchea thermotolerans* (100)
XIII	207+84	282+99	195	*Metscnikowia fructicola* (98)

All values for the 5.8S-ITS PCR and restriction fragments are given in bp.
Abbreviations: R.P., restriction profile
[a] According to BlastN search of D1/D2 26S rRNA gene sequences in NCBI database.

Chapter 3. Microbiological and chemical monitoring of Cartarratto and Grillo wines produced under natural regime and at industrial level

3.1 Introduction

In the last years, the request for quality wines greatly increased and this phenomenon is still on the increase. Wine is probably one of the main fermented beverages for which the recognition of the "territoriality" is fundamental for its appreciation. The term "terroir", defined as an ecosystem in which the grapevine interacts with the environmental factors (soil and climate) affecting the quality and typicality of the wine produced in a particular location (Pereira et al 2006), refers to a concept basic during tasting. Several economic and social factors, such as international competition within the wine market and consumer demands for wines with innovative styles, are providing new challenges in winemaking (Bisson et al 2002; Pretorius and Hoj 2005). In this contest, numerous wine producers are convinced that the premium wine quality is made by "traditional" methods based on spontaneous fermentation (Cebollero et al 2000; Ranieri and Pretorius 2000) that could yield wines of unique and innovative characters that are particularly appreciated by specialized consumers. To this purpose, a new style of "natural" winemaking is gaining importance, since the resulting wines are obtained thanks to the action of spontaneous autochthonous agents and the use of chemical additives is not allowed (Guzzon et al 2011).

Chemical additives are used in foods to combat specific unwanted spoilage and pathogenic microorganisms that are defining for the shelf-life and safety of consumers, respectively. In case of wine, thanks to the ethanol content, low pH and

phenols, the use of chemical compounds does not represent a necessary condition to assure its stability and safety, although, in large-scale productions their employment avoid large economic losses. However, natural wines, produced without oenological additives, are generally carried out in small wineries where the control of grape healthy in vineyards and a right sanitization of the cellar (Guzzon et al 2011), as well as an optimal management of the process limit the risk of alterations.

The yeast species present on the grape surface are undoubtedly defining for the transformation of must into wine, but a relevant role may be played by the transformation environment (Guzzon et al 2011). Moreover, the composition of indigenous populations present in must may change during different vintages, since they are affected by the climatic conditions and/or agronomic practices (Fleet 2008). Furthermore, the cellar environment seem to be a source of microorganisms involved in the spontaneous fermentation of wines (Fleet 2008; Guzzon et al 2011). In this area, a recent work (Ocon et al 2013) has been focused on the role of the air of the cellars in the wine yeasts ecosystem. Ocon et al (2013) also showed that the air of different areas of the cellars were characterized by different concentrations and species of wine yeasts.

The complexity of wines obtained by spontaneous fermentation directly correlated with the nature of the process, initiated and completed by the combined action and/or succession of different species of yeasts (Lambrechts and Pretorius 2000). Several studies compared wines obtained by spontaneous fermentation with those produced using selected yeasts, showing substantial differences in chemical compound composition (Di Maio et al 2012), especially regarding the aromatic complexity and

fullness of palate structure detected at higher levels in spontaneous fermentation wines (Romano et al 2003), as well as in yeast species composition.

Another important factor is represented by the dynamics of succession of the different strains within a given species. Regarding *S. cerevisiae*, the action of different strains provide a better aroma complex and individuality to spontaneous fermentation wines than commercial yeasts (Fleet 2008). The results of spontaneous fermentation do not depend exclusively on yeasts, since lactic acid bacteria (LAB) also play a relevant role during winemaking (Renouf et al 2005).

The autochthonous microorganisms, in particular yeasts, strongly contribute to the expression of varietal characters (Jolly et al 2006; Zott et al 2008). On the other hand, commercial starter cultures, mainly belonging to the species *S. cerevisiae* drive the alcoholic fermentation and produce wines with wanted characteristics (Subden 1997), but their employment in winemaking is quite controversial, because of their massive prevalence, after a few days of fermentation, over the native microflora (Valero et al 2005). As a consequence, wine peculiarities, such as the complexity of aroma, may be lost. For this reason, besides autochthonous *S. cerevisiae*, non-*Saccharomyces* species are being object of oenological investigation (Soden et al 2000). With regards to selected strains, a wine strain collection obtained from a given area could be useful for local winemakers who want to produce wines with regional features and, at the same time, ensure the correct fermentation process. Several researches have been focused on the technological selection of yeasts in different wine areas throughout the world (Ocón et al 2010) with the aim to satisfy the increasing demand for wines with specific organoleptic profile. Settanni et al (2012)

also worked to select autochthonous *S. cerevisiae* strains with enological aptitude for Marsala wine production.

Infact, the "Marsala product" is the first Italian wine that enjoyed a controlled designation of origin (CDO) status and the Grillo as well as Catarratto cultivar are the grape varieties mostly cultivated in Sicily to produce commercial "IGT Sicilia" wines. Furhermore, the Grillo grapes are mainly used to produce the base wine for Marsala (Settanni et al 2012). The technology of production of this special wine (the Marsala) involves a base wine and the addition of cooked and/or concentrated and/or fortified grape musts and/or wine ethanol (D.P.R. 17 1996); after a long ageing in barrel, the mature Marsala wine must contains at least 17 % (v/v) of ethanol.

For these reasons, the objectives of the present study were to evaluate the microbiological, chemical and sensorial features of "Catarratto IGT Sicilia" and of Marsala base wine realised with Grillo cultivar during large-scale winemaking under the natural regime.

3.2 Materials And Methedos

3.2.1 Winemaking processes and sampling

The natural winemakings were performed in the "Azienda Agricola Barraco" (Marsala, TP, Italy) using the white grapes of Catarratto and Grillo cultivar during the vintage 2010. Both the cultivar were cultivated in the Marsala area wine production (western Sicily – southern Italy) and the vinification process of both cultivar were separately carried out.

Forty quintals of grapes (in duplicate), per each cultivar, were manually harvested and subjected to stemmer-crushing. Musts were then placed in 50 hl stainless steel vats to let the fermentation take place by indigenous yeasts, naturally present on the grape surface and/or in the winery environment. Sulphites were not added.

The fermentations included, in the first 48 h after crushing, a maceration: the liquid phases were maintained in contact with the solid parts of grape (skin and seeds) at a constant temperature of 17 °C. After macerations, the entire bulk musts were pressed through an hydraulic press and the liquid phases were transferred in 25 hL stainless steel tanks. The fermentations continued at a controlled temperature of 20 °C for other five days according to the sugar consumption. Subsequently, the liquid mass were subjected to an ageing in steel tanks at a controlled temperature of 20 °C.

The samples for microbiological and chemical analyses were collected before crushing (five hundred grape berries), during fermentation, ageing and at bottling.

3.2.2 Microbiological analysis

Grape samples were placed into sterile plastic bags containing a washing isotonic peptone solution (10 g/L Bacto Soytone, 2 mL/L Tween 80) and incubated at 30 °C for 3 h to collect the microorganisms hosted on peel surface (Renouf et al 2005).

Cell suspensions recovered from grapes, must and wine samples were serially diluted in Ringer's solution. Decimal dilutions were spread plated (0.1 mL) onto Wallerstein laboratory (WL) nutrient agar, supplemented with chloramphenicol (0.5 g/L) and biphenyl (1 g/L) to inhibit the growth of bacteria and moulds, respectively, for the counting of total yeasts (TY). Cell suspensions were pour plated (1 ml) into de Man

Rogosa Sharpe (MRS) agar, glucose (5 g/L)-M17 (GM17) agar and medium for *Leuconostoc oenos* (MLO) agar (pH 4.8) (Caspritz and Radler 1983) for the counting of rod, coccus and acidophilic LAB, respectively. All media used for LAB growth were supplemented with cycloeximide (170 ppm) and biphenyl (1 g/L) to inhibit the growth of yeasts and moulds, respectively. All media were purchased by Oxoid (Basingstoke, UK) and chemical by Sigma-Aldrich (Milan, Italy). Incubation was at 28 ± 2 °C for 48-72 h for all microorganisms except acidophilic LAB incubated for 5 d. The incubation of LAB was under anaerobic conditions. Analyses were carried out in duplicate.

3.2.3 Yeast isolation and identification

Ten colonies for each morphology detected on each sample were purified onto WL agar, grouped on the basis of morphology and subjected to genetic characterization.

The DNA extraction was performed using the InstaGene Matrix kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions.

In order to perform a first differentiation of yeasts, all selected isolates were analyzed by restriction fragment length polymorphism (RFLP) of the region spanning the internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene. The DNA fragments were amplified with the primer pair ITS1/ITS4 (Esteve-Zarzoso et al 1999) by means of T1 Thermocycler (Biometra, Göttingen, Germany) and subsequently the amplicons were digested with the endonucleases *Cfo*I, *Hae*III and *Hinf*I (MBI Fermentas, St. Leon-Rot, Germany) at 37 °C for 8 h. The isolates presumptively belonging to the genus *Hanseniaspora* were further digested with the

restriction enzyme *Dde*I (MBI Fermentas) (Baleiras-Couto et al 2005). ITS amplicons as well as their restriction fragments were analysed twice on agarose gel using at first 1.5% (w/v) agarose and then 3 % (w/v) agarose in 1 × TBE (89 mmol/L Tris-borate, 2 mmol/L EDTA pH 8) buffer. Gels were stained with SYBR® safe DNA gel stain (Invitrogen, Milan, Italy), visualized by UV transilluminator and acquired by Gel Doc 1000 Video Gel Documentation System (BioRad, Richmond, USA). Standard DNA ladders were 1kb Plus DNA Ladder (Invitrogen) and GeneRuler 50 pb DNA Ladder (MBI Fermentas). Five isolates representative of each group were subjected to an additional enzymatic restriction targeting the 26 rRNA gene. After amplification with the primer pair NL1/LR6 the PCR products were digested with the endonucleases *Hinf*I, *Mse*I and *Apa*I (MBI Fermentas) (Baleiras-Couto et al 2005) and visualised as above described. One isolate per group was further processed by sequencing the D1/D2 region of the 26S rRNA gene to confirm the preliminary identification obtained by RFLP analysis. D1/D2 region was amplified with primers NL1 and NL4 (O'Donnel 1993). PCR products were visualised as above. DNA sequencing reactions were performed at Primmbiotech S.r.l. (Milan, Italy). The identities of the sequences were determined by BlastN search against the NCBI non-redundant sequence database located at http://www.ncbi.nlm.nih.gov.

3.2.4 Typing of *S. cerevisiae* isolates

The isolates identified as *S. cerevisiae* species were characterized at intra-specific level through two techniques: interdelta analysis with primers delta12 and delta21

(Legras and Karst 2003) and microsatellite multiplex PCR based on the analysis of polymorphic microsatellite loci named SC8132X, YOR267C and SCPTSY7 (Vaudano and Garcia-Moruno 2008). The PCR products were analyzed on agarose gel 2.0% (w/v) in 1 × TBE buffer and visualized as above reported.

Three commercial *S. cerevisiae* strains (Zymaflore VL2, Laffort; Premium Blanc-12V, Vason; Uvaferm CS2, Lallemand) commonly used in Catarratto and Grillo IGT Sicilia area were sampled and analysed as above reported as control strains in order to exclude their presence in winemaking.

3.2.5 Isolation and phenotypic grouping of LAB

After growth, colonies of various shapes (at least 10 with identical morphology) of Gram-positive (Gregersen KOH method) and catalase negative (determined by transferring fresh colonies from a Petri dish to a glass slide and adding H_2O_2 5%, v/v) bacteria (presumptive LAB) were randomly picked from count plates and transferred to the corresponding broth media. The isolates were purified by successive sub-culturing and stored in glycerol at −80 °C until further experimentations.

Rod and coccus-shaped LAB cultures were first grouped on the basis of cell disposition, growth at 15 and 45°C and CO_2 production from glucose. The last test was carried out in the optimal growth media (MRS for rod LAB and M17 for coccus LAB) containing all components except citrate, whose fermentation by certain LAB may determine gas formation. M17 contained glucose in place of lactose. The assay consisted of LAB inoculation into test tubes sealed with H_2O agar (2%, w/v). The strains negative to the assay were inoculated into test tubes containing the optimal

growth media prepared with a mixture of pentose carbohydrates (xylose, arabinose and ribose, 8 g/L each) in place of glucose. Coccus isolates were further sub-grouped on the basis of their growth at pH 9.6 and in presence of 6.5% NaCl.

3.2.6 Genotypic differentiation and identification of LAB

DNA extraction was performed as above reported for yeasts. Strain differentiation was performed by random amplification of polymorphic DNA-PCR (RAPD-PCR) analysis in a 25-µl reaction mix using single primers M13 (Stenlid et al 1994). Amplifications were performed by means of T1 Thermocycler (Biometra, Göttingen, Germany) applying the conditions reported by (Zapparoli et al 1998). RAPD profile were analyzed on agarose gel 1.5% (w/v) in 1 × TBE buffer and visualized as above. One representative culture for each cluster were identified by 16S rRNA gene sequencing as described by (Weisburg et al 1991).

3.2.7 Chemical analysis of conventional parameters

The composition of the wines was determined by means of a Winescan (FOSS) calibrated following EEC 2676 standard procedure (EEC 2676/90) for pH, total titratable acidity (TTA), volatile acidity, reducing sugars, ethanol, malic acid, lactic acid, citric acid, tartaric acid, promptly assimilable nitrogen (PAN), glycerol and dry extract. Total and free SO_2 were measured with the OIV method, while the end point was revealed by potentiometry as reported by Huerta Dıaz-Reganon M. D. (Ph.D. thesis, Alcala de Henares University, Madrid, 1996).

3.2.8 Phenolic components

Hydroxycinnamoyl tartaric acids (HCTA) were tested by HPLC (Di Stefano and Cravero 1992; Corona et al 2010). The standard employed was chlorogenic acid and the concentration of HCTA was expressed as chlorogenic acid equivalents. By processing these data (hypothetical identity of ε for chlorogenic and caftaric acids at 220 nm) and the data from the coefficients determined by injecting free hydroxycinnamic acids and chlorogenic acid, the concentration of caftaric, coutaric and fertaric acid was estimated. 2-S-glutathionyl caftaric acid was evaluated as caftaric acid equivalents. The analysis of fixed acids was performed by HPLC on an Agilent series 2100 instrument (Milan, Italy) equipped with a C18 column (EconosphereTM C18, 5 μm, 250 x 4.6 mm i.d., Lokeren, Belgium, part n° 70066), volume injected 20 μL, flow rate 0.6 mL/min., detection at 210 nm. Prior to injection, 0.5 mL of sample was stripped of phenolics by passing it through a 400 mg C18 Sep Pack cartridge (Sep Pak, Waters, Milan, Italy, part n° WAT036810), activated with 2 mL of methanol, followed by 3 mL of H_3PO_4 10^{-3} M and elution with H_3PO_4 10^{-3} M until a volume of 10 mL was reached.

3.2.9 Volatile organic compounds (VOCs)

Free volatiles were determined according to the method outlined by (Corona et al 2010). In brief, 25 mL of wine, charged with 1-Heptanol as internal standard (0.25 mL of 40 mg/L hydroalcoholic solution), diluted to 75 mL with distilled H_2O, were passed through a 1 g C_{18} cartridge (Isolute, SPE Columns, Uppsala, Sweden, part n° 221-0100-C) previously activated with 3 mL of methanol followed by 4 mL of

distilled H_2O. After washing with 30 mL of distilled H_2O, volatiles were recovered by elution with 12 mL dichloromethane, dehydrated and evaporated to 0.5 mL prior to injection into the gas chromatograph (PerkinElmer Autosystem XL, Milan, Italy) and GC-MS (Agilent 6890 Series GC system, Agilent 5973 Net Work Mass Selective Detector, Milan, Italy), both equipped with a DB-WAX column (Agilent Technologies, 30 m, 0.250 mm i.d., film thickness 0.25 μm, part n° 122-7032). Oven temperatures: 40 °C for 2 min (during splitless injection), from 40 to 60 °C, 40 °C min^{-1}, 60 °C for 2 min, from 60 to 190 °C, 2 °C min^{-1}, from 190 to 230, 5 °C min^{-1}, 230 °C for 15 min; injector 250 °C, Fid 250 °C, transfer line 230 °C, carrier helium 1 mL min^{-1}.; EM. 70 eV. The identification of volatiles was carried out by injection of commercial standards or others prepared in our laboratory (ethyl esters of 2-hydroxyglutaric acid) (Salgues et al 1986). Higher alcohols were determined on distilled wine through gas-chromatographic analyses with FID detector (GC PerkinElmer Autosystem XL) (Di Stefano 1980). The identification of the volatile compounds of higher alcohols, esters and acids was only tentative, not absolute. All solvents and reagents were purchased from WWR International (Milan, Italy). Chemical and physical determinations were performed in triplicate.

3.2.10 Sensory analysis

A descriptive method (Ente Nazionale Italiano di Unificazione, UNI 10957, 2003) was used to define the sensory profile of the experimental bottled wines in comparison to wines obtained by conventional winemaking and purchased from a market. A descriptive panel of ten judges was employed. The judges were trained in

some preliminary sessions, using different samples of IGT Sicilia Catarratto wines and commercial Marsala base wines, in order to develop a common vocabulary for the description of the sensory attributes of wine samples and to familiarize themselves with scales and procedures. Each attribute term was extensively described and explained to avoid any doubt about the relevant meaning. On the basis of the frequency of citation (>60%), several descriptors were selected to be inserted in the card, as follows: intensity of colour, odour intensity, odour complexity, off-odour, flowers, fresh fruits, mature fruits, citrus fruits, dry fruits, aromatic herbs, species (odour), sweet, acidity, bitter, salt (taste), hot and astringent (tactile in mouth) and off-flavour (taste). "Terroir" expression, was also evaluated.

The wine samples were randomly evaluated by assigning a score between 1 (absence of the sensation) and 9 (extremely intense) in individual booths under incandescent white lighting. The analysis was performed in triplicate. The resulting scores were averaged and compared. ANOVA test (STATISTICA software, StatSoft Inc., Tulsa, OK, USA) was applied to find significant differences among attributes of wines.

3.3 Results

3.3.1 Microbiological analysis

The results of microbial counts are reported in Table 1. TY recorded on Catarratto grapes was 6.28 Log CFU/g and increased of about one Log cycle after grape pressing, while on Grillo grapes was 4.70 Log CFU/g and remained constant after grape pressing. The concentration during alcoholic fermentation of TY increased in both vinifications reaching the highest concentration at the racking for Cratarratto

(7.93 Log CFU/mL) and at the second day of maceration for Grillo (7.79 log CFU/mL). The level of yeast count was stable at approximately 7 Log CFU/mL until the end of fermentation and it decreased during the ageing of wine reaching value around 4 Log CFU/mL in both winemakings. At bottling (34^{th} and 39^{th} day from the beginning of ageing for Catarratto and Grillo, respectively) TY concentration was not detectable.

LAB counts were estimated on three different media as reported in Table 1. Grape berries of Catarratto showed a LAB concentration on MLO (3.11 Log CFU/mL) higher than MRS (2.30 Log CFU/mL) and GM17 (2.29 Log CFU/mL); on Grillo, indeed, LAB concentration onto MRS (2.29 log CFU/mL) was almost superimposable to that displayed by GM17 (2.22 log CFU/mL), while no growth occurred onto MLO medium. After grape crushing, LAB population increased more than one Log cycle onto all media in both vinifications. After two days of alcoholic fermentation, LAB reached the highest values approximately 6 Log CFU/mL in all media for Catarratto and 6 log CFU/mL onto MRS and about 5 log CFU/mL onto GM17 and MLO for Grillo samples. A significant decrease in concentration was registered after racking in particular for acidophilic LAB (2.70 Log CFU/mL) during Cataratto vinification, whereas during Grillo winemaking the greatest reduction of LAB population was observed onto GM17.

During ageing a reduction of LAB population was observed onto all three media, till reaching values not detectable in correspondence of bottling, in both vinifications.

3.3.2 Isolation, identification and distribution of yeasts

A total of 1944 (867 and 1077 from Catarratto and Grillo samples, respectively) colonies from WL agar were isolated, purified to homogeneity on the same medium used for plate count and separated on the basis of colony morphology. At least ten cultures with different appearance from each sample were selected and 952 (423 and 529 from Catarratto and Grillo samples, respectively) isolates were subjected to molecular identification. After restriction analysis of 5.8S-ITS region and 26S rRNA gene, the isolates were clustered in fourteen groups (Table 2) reporting nine different species. Twelve of these groups were directly identified by comparison of the restriction bands with those available in literature (Esteve-Zarzoso et al 1999; Nisiotou and Nychas 2007; Tofalo et al 2009) and were allotted in the following species: *Aureobasidium pullulans* (group I), *Candida zemplinina* (group II), *Hanseniaspora guilliermondii* (group III), *Hanseniaspora guilliermondii* (group IV), *Hanseniaspora uvarum* (group V), *Metschnikowia pulcherrima* (group VIII), *Metschnikowia pulcherrima* (group IX), *Pichia guilliermondii* (group X), *Pichia guilliermondii* (group XI), *Rhodotorula mucillaginosa* (group XII), *S. cerevisiae* (group XIII) *S. cerevisiae* (group XIV). The groups VI and VII could not be identified by RFLP analysis and the identification at species level was concluded by sequencing of D1/D2 domain of the 26S rRNA gene which allotted the isolates into the species *Hanseniaspora uvarum* and *Issatchenkia terricola* respectively. This method was also applied to confirm previous species.

The distribution of yeast species and the highest concentrations estimated for each sample are reported in Table 2. The yeast diversity at species level for Catarratto was

superimposable to that recognized during Grillo vinification. In particular, for Caratarratto all nine species were easily detected on grape berries, while after pressing, only three species (*H. guilliermondii, M. pulcherrima* and *S. cerevisiae*) were present at dominating levels (the concentration estimated onto Petri dishes were the highest), furthermore *S. cerevisiae* resulted dominant alone during the entire alcoholic fermentation, even though the non-S*accharomyces* (NS) yeasts belonging to *A. pullulans, H. guilliermondii* and *H. uvarum* were isolated until the second day of this phase of vinification. During the wine ageing, the species *S. cerevisiae* was found, at concentrations lower than those registered in fermentation, no longer than the third day and after clarification, the only yeast species isolated was *P. guilliermondii*.

On the other hand, on Grillo grape berries only four sopecies were found (*H. uvarum, M. pulcherrima, P. guilliermondii, S. cerevisiae*). *Hanseniaspora* genus was detected from grape harvest to AF, while *M. pulcherrima* species was detected only on grape berries. *S. cerevisiae* and *P. guilliermondii* were isolated from all steps of winemaking and both resulted at high level during AF as well as ageing. In both vinifications no yeast was isolated at bottling.

3.3.3 Typing and distribution of *S. cerevisiae* strains

The 386 (179 and 207 from Catarratto and Grillo samples, respectively) isolates belonging to the species *S. cerevisiae* were further genetically characterized. The interdelta analysis was able to separate the isolates in 47 groups, while microsatellite multiplex PCR recognized 40 different groups (results not shown), showing a lower

discriminatory power than the first technique. The distribution of the different strains of *S. cerevisiae* during winemakings showed a high biodiversity in terms of strains during the different steps of wine productions (Fig. 1a, and Fig 1b). With regards to the Catarratto samples only four strains isolated from grapes (CTBRL 129) and must (CTBRL 63, CTBRL 87 and CTBRL 152) were identified during the alcoholic fermentation, while on Grillo grapes only two strais were found on must and during AF (GRBRL 12 and GRBRL 17). A high variety of strains (n= 16 and 14 from Catarratto and Grillo samples, respectively) at dominating levels was found during the alcoholic fermentations, in particular at day 3 and day 4 on Catarratto and at day 5 on Grillo. Subsequently, during the ageing, six (CTBRL155, CTBRL148, CTBRL156, CTBRL47, CTBRL56, CTBRL66) strains that were not identified in the previous steps were detected for Catarratto; whereas only one strain (GRBRL32) isolated from AF was detected for Grillo.

All strains presented genotypic profiles different from that showed by commercial strains used as control (data not shown).

3.3.4 Isolation, identification and distribution of LAB

On the basis of appearance, about ten colonies per morphology were isolated from each medium used for LAB counts (MRS, GM17 and MLO) at the highest dilutions of samples collected during both the vinifications. A total of 2174 (997 from Catarratto and 1177 from Grillo) bacterial cultures were picked up from agar plates and propagated in the broth media corresponding to those used for counts, applying the same incubation conditions. The cultures were purified as reported above and the

microscopic inspection allowed their separation in 1688 rods and 486 cocci. After Gram characterization and catalase testing, 1410 rods and 422 cocci were considered presumptive LAB cultures, as being Gram-positive and catalase-negative.

The combinations of the phenotypic characters considered for the analysis allowed the separation of the 1832 LAB cultures into 5 groups (Table 3), two for rods and three for cocci. CO_2 production from glucose was scored negative for the isolates of group E which were tested for growth in presence of pentose sugars, that evidenced their facultative heterofermentative metabolism.

About 30% of the isolates of each phenotypic group, 537 in total, was subjected to RAPD analysis using primer M13 (results not shown). The isolates analysed were divided into fifteen main clusters for the five phenotypic groups: four clusters for group A, two for group B, one for group C, two for group D and six for group E (results not shown). One strain for each RAPD profile was identified at species level by 16S rRNA gene sequencing. The BLAST search shared a percentage of identity with sequences available in the NCBI database of at least 97%. Five species belonging to *Enterococcus faecium*, *Lactobacillus hilgardii*, *Lactobacillus plantarum*, *Leuconostoc mesenteroides* and *Streptococcus macedonicus* were found in samples collected during both the vinifications.

The distribution of LAB species and their concentration estimated for each sample are shown in Table 4. During both vinification, grape berries showed the highest LAB diversity; *E. faecium* and *L. hilgardii* were no more detected during vinifications. On the contrary, *L. plantarum* was the species most encountered during the entire alcoholic fermentation (in both vinificationa) reaching the highest values of

concentration during the first days of fermentation. Also *Leuc. mesenteroides* was found at high dilutions of samples until the day 2 (Grillo) and until day 6 (Catarratto) of fermentation. During ageing and in both processes, *L. plantarum* was the only LAB species detected, but at lower concentrations than fermentation. *S. macedonicus* was found only at day 6 of ageing in Grillo vinification.

3.3.5 Chemical conventional parameters

The conventional parameters of samples collected during winemakings are reported in Table 5a and table 5b. In both processes, values of pH, TTA and tartaric acid were in the range of those commonly reported for commercial wines, instead total SO_2 and free SO_2 values were very low due to the absence of exogenous sulphides. Reducing sugars rapidly decreased during the first days of fermentations until bottling when these sugars were no more detected. On the contrary, ethanol and glycerol showed a rapid increase from the day 1 [0.53% (v/v) of ethanol; 0.77 g/L of glycerol, for Grillo] and day 2 [1.1% (v/v) of ethanol, 0.88 g/L of glycerol, for Catarratto] of fermentation to racking [5.8% (v/v) of ethanol and 4.98 g/L of glycerol for Catarratto and 4.27% (v/v) of ethanol and 4.97 g/L of glycerol for Grillo]. At the end of winemaking, ethanol and glycerol reached respectively 12.67% (v/v) and 7.19 g/L (Catarratto) and 14.72% (v/v) and 8.29 g/L (Grillo). PAN concentration varied greatly during the first two days of vinification until day 7 (Catarratto) and day 8 (Grillo) of AF at which it was not detectable. VA content was almost constant during winemaking and its maximum concentration (0.36 g/L) was estimated at bottling for Catarratto and at the day 5 of fermentation (0.5 g/L) for Grillo. Malic acid content

was almost stable during experimental process in both vinifications. Lactic acid concentration showed an irregular behaviour: the maximum level was registered at the day 2 of fermentation (1.65 g/L) for Catarratto and at racking (0.74 g/L) for Grillo.

3.3.6 Phenolic compounds

Figure 2a and 2b graphically reports the measurements of the HCTA for both processes. The *trans*-caffeil tartaric acid showed the highest increase during the entire period of sampling. Its maximum values (approximately of 60 mg/L for Catarratto and 95 mg/L for Grillo) were observed during the last three steps of fermentation and at the day 7 of AF for Catarratto and Grillo, respectively. After that *trans*-caffeil tartaric acid showed a rapid decrease until bottling (43.11 mg/L) in vinification of Catarratto; whereas its value was almost constant until the end of the process in Grillo winemaking. During the first day of AF, the 2-S-glutationil-*trans*-caffeil tartaric acid, known as Grape Reaction Product (GPR) (Salgues et al 1986), showed a rapid increase during the Catarratto vinification up to 40.99 mg/L (day-1 of fermentation), whereas the GPR showed a rapid decrease (up to 19.05 mg/L) from must to day 2 of AF in Grillo vinification.

3.3.7 VOCs and sensory evaluation

The concentration and composition of VOCs (Tables 6a and 6b) were almost similar in both vinifications. VOCs were composed of alcohols, esters, acetate esters and acids that were analysed at principal steps. Alcohols were principally represented by higher alcohols that showed an increasing trend until bottling. Among this group the highest concentrations were reached by isoamylic alcohol and isobutanol. Also levels of esters and acetate esters increased until bottling. In particular, diethyl succinate, ethyl octanoate, 4-OH-butyrate ethyl, hexanoate and ethyl decanoate showed the highest concentrations among esters, whereas ethyl acetate represented more than 97% of total acetate esters. Except for decanoic acid content that was almost constant, all acids reached high level of concentrations showing an increasing trend.

The results of sensory analysis of wine samples are represented in figures 3a and 3b. The majority of attributes examined were almost similar among wines and in both the vinifications. The significant ($p < 0.05$) differences among samples were found only for odour complexity, mature fruits, citrus fruit, aromatic herbs and "Terroir" expression. All wines did not show off-odours and off-flavours.

3.4 Discussion

Wine quality can be affected by the growth of different yeasts originating from the microbial communities hosted on grapes (Fleet 2008). The use of spontaneous fermentation represents a valuable technological alternative to the application of commercial starter cultures responsible for wine flavour standardization, as well as to the selected autochthonous cultures. The autochthonous yeasts could positively

contribute to wine quality and typicality but they are not able to represent completely the inter- and intra-specific biodiversity that characterize the spontaneous fermentations.

The present study was performed to investigate a natural winemaking of "Catarratto IGT Sicilia" and Grillo wine used as "Marsala base wine" for its microbiological and chemical composition. To this purpose, the vinifications based on spontaneous fermentation and carried out without any oenological additives were monitored at winery-scale. Samples were collected at each step of production, from grape harvest to wine bottling.

Microbiological results evidenced a substantial concentrations of yeasts during the entire process with values higher than 7 Log (CFU/mL) during the alcoholic fermentation of both experimentatyions. Even though the experimental vinification was carried out following the criteria of "natural wine", yeast evolution during the entire process was superimposable to that registered during conventional winemaking carried out with starter cultures and chemical additives (Fugelsand 1997). To our knowledge, no work has shown the maximum increase of LAB concentration during the tumultuous phase of alcoholic fermentation. Despite data reported in literature, also LAB concentrations reached the highest values at the beginning of AF in both vinifications. Generally, the LAB growth occurs at the end of AF when yeast activities greatly decrease and their cells undergo the lysis due to the wine stressing conditions such as high ethanol content, nutrient limitations and low pH (Vincenzini et al. 2005). LAB increase is commonly favoured by the absence of exogenous suplhites, but it is greatly inhibited by yeasts during AF also when this

process is carried out without suplhites addition (Granchi et al 2005). These bacteria have a defining role in wine production since their activities can be beneficial or detrimental for the quality of wine, depending on the species and/or strain and also on the stage of the vinification at which they develop (Fernandez-Espinar et al 2000). The growth of yeasts and LAB during spontaneous fermentation represents a complex phenomenon affected by several oenological factors. Since our study was not aimed to study the diversity of yeasts and LAB during one vintage and in one cellar, no correlations could be defined among our results and the specific technological conditions of the experimental vinifications.

The process of yeast isolation resulted in the collection of 1944 cultures. After restriction analysis of two distinct gene, fourteen yeast groups and nine species were recognized. The isolates belonging to the species *H. uvarum* (group VI) *I. terricola* (group VII) were characterized by atypical restriction profiles of 5.8S-ITS, a phenomenon that is not surprising for yeasts in this DNA region, since many authors observed this behaviour in several strains (Fernandez-Espinar et al 2000; Tofalo et al 2009; Francesca et al 2012). At the end of the identification process, nine species belonging to eight genera (*Aureobasidium*, *Candida*, *Hanseniaspora*, *Issatchenkia*, *Metschnikowia*, *Pichia*, *Rhodotorula* and *Saccharomyces*) were found. The yeast community present on the grapes was characterized by the highest interspecific biodiversity compared to the communities during the several phases of wine production. As previously stated by other authors (Sabatè et al 2002; Gonzales et al 2007), non-*Saccharomyces* (NS) yeasts were dominant on grapes and in must soon after pressing. Among NS yeasts, only *P. guilliermondii* was the main species

isolated during fermentation and ageing and at dominant level in both Catarratto and Grillo vinifications. This species is commonly found on grapes and during the first steps of AF (Ciolfi et al 2012; Romancino et al 2008) but at low concentrations. However, *P. guilliermondii* does not represent a species commonly used as starter or co-starter for wine fermentation, even though recent studies (Barata et al. 2006) reported this species to be responsible for the fermentation process and to affect the final quality. *S. cerevisiae* was also the main species isolated during fermentation and ageing and at dominant level. This species is commonly recognized as the main technological yeast due to its high vigour and power fermentation as well as its low production of acetic acid and off-flavour. Then, its dominance during alcoholic fermentation could represent a guarantee of quality of wines both from a microbiological and chemical point of view. In order to investigate whether the selection due to the winemaking process was defining also on the strain composition, *S. cerevisiae* community was analysed at intraspecies level. As expected from a spontaneous fermentation, different *S. cerevisiae* strains were detected at the various steps of production. The presence of a multi-strain *S. cerevisiae* population during AF could positively affect the organoleptic complexity of the final product due to different metabolic activities (Fleet 2003). The number of strains isolated at the highest level during AF of both vinifications was considerable and higher than those commonly reported in literature (Wang et al. 2013; Gonzalez et al. 2007).

In our study, the biodiversity of LAB population during the vinification process was also evaluated. LAB were phenotypically divided into five groups corresponding to five LAB species (*E. faecium, L. mesenteroides, L. hilgardii, L. plantarum* and *S.*

macedonicus). The highest LAB dversity was found on grape and in must just pressed. The species *E. faecium, L. mesenteroides, L. hilgardii, L. plantarum* are commonly associated with wine environments (Granchi et al 2005; Rodrıguez and Manca de Nadra 1995; Garcıa-Ruiz et al. 2009). Among these, *L. plantarum* was the species most frequently isolated. This species has been found to grow during manufacturing of other wines due to its ethanol tolerance and acidophilic characteristics (Rojo-Bezares et al 2007). Although, further investigations carried out for several vintages and in different cellars are necessary to define the LAB ecology of natural wine, to our knowledge, this is the first work that shows *Lb. plantarum* at high concentration during the entire winemaking and concomitantly with the maximum increase of yeasts during AF. The present study showed the presence of *S. macedonicus* into wine; this bacterium is typically associated to cheese environments (De Vuyst and Tsakalidou 2008).

The natural winemaking is strongly affected by microbial several spoilage issues (Vincenzini et al. 2005). For this reasons the vinification process was also monitored by chemical analysis and the results could be related to the metabolic activities of both yeast and LAB populations. The sugar consumption and the ethanol production showed a regular trend until bottling. Furthermore, the chemical analysis of samples revealed a consistent production of glycerol, at desirable level. Glycerol is produced through the glycero-pyruvic fermentation carried out by yeasts (Ough et al 1972). Generally, glycerol production is registered into grape musts characterized by high level of SO_2 and fermented by starters grown in aerobic condition (Fugelsang 1997) and during the first phase of the alcoholic fermentation. Final glycerol content into

wine is influenced by many factors depending on grape cultivar, fermentation temperature, sulphur dioxide addition as well as nitrogen and micronutrient concentrations (Belajova and Suhaj 2012). Furthermore, glycerol content in wine seems to be more strictly related to the yeast strains that dominate the fermentation process and than to the yeast concentration (Gardner et al 1993). Glycerol production is not limited to the phase during which a yeast increase is registered, since it represents a part of the total glycerol concentration produced during winemaking. In addition, the low oxygen availability during the ageing process could positively affect the glycerol production by yeasts (Gardner et al 1993; Hernandez-Cortes et al 2010).

However, when its concentration is higher than 5.2 g/L, glycerol plays a positive role in winemaking because provides the wine with fullness, sweetness and roundness sensations (Hinreimer et al 1955). Different yeast species producers of high amounts of glycerol from sugars (Sipiczki et al 2005; Tofalo et al 2011) may found application to reduce the ethanol content of wines produced by grape musts characterized by high sugar content, such as those produced in southern Italy.

Organic acids with low molecular weight were also monitored because they are of paramount relevance for final organoleptic characteristics of wine. During winemaking, VA was detected at low concentrations, probably due to the presence of yeast strains producing low concentrations of acetic acid. VA at high concentrations (more than 1 g/L) in wine are responsible for the generation of the off-flavours (Vincenzini et al 2005) and make the product unmarketable. Wines carried out by spontaneous fermentations are frequently characterised by high VA concentration due

to the proliferation of spoilage yeasts (Wang et al 2013). However, the processing of healthy grapes as well as a right sanitisation of the cellar equipment are two optimal conditions to limit the risk of wine spoilage (Guzzon and Settanni 2009).

Tartaric acid did not greatly vary during the entire process in particular in Grillo vinification and its changes were according to those registered during conventional vinifications (Radin et al 1994). The concentration of lactic acid showed an irregular behaviour during winemaking. It could be due to the activities of both yeast and LAB. In particular, a significant increase of lactic acid, associated to the low level of VA, was registered. This could be due to the homolactic fermentation of sugars carried out by *L. plantarum*.

Some studies showed that the chemical conversion of lactic acid into malic acid takes place also at low bacterial concentration and under stressing condition (Capucho and san Romano 1994) as well as during different phases of winemaking (Alexandrea et al 2004). Furthermore, *L. plantarum* represents the first species characterized by production of malo-lactic enzymes (Schutz and Radler 1974) and, recently, it has been employed to carry out the malo-lactic fermentation as an alternative to the species *Oenococcus oeni* (Lopez et al 2008).

VOCs were also monitored during the vinification, they are greatly influenced by yeasts and LAB activities (Valenteo et al 2007). Esters are of paramount importance to define the sensorial complexity of wines such as diethyl succinate (baked apple smell) and fatty acid esters (honey and wax smell). Higher alcohols, such as 1-hexanol, also contribute positively to the definition of the final profile of wine (herbaceous smell) if its concentration does not exceed 400 mg L^{-1} (Beltran et al

2002). The monitoring of HCTA was also performed since, in general, their concentration increases after the maceration phase and they could represent a risk for wine quality when they are oxidased to brown pigments and to volatile phenols (off-odours) by polyphenol oxidase (Ribereau-Gayon et al 2003). GPR compound, one of the most abundant HCTA detected represents a precursor for antioxidant compounds. To exclude organoleptic alteration of wine carried out by natural wine making the final wines were subjected to the sensory evaluation. No off-odours and off-flavour were detected.

In conclusion, during the natural winemaking, the main microbial populations (yeasts and LAB) were able to express metabolic activities and no negative impacts on wine chemistry and wine microbiology were detected. All conventional chemical parameters of wine were in agreement with those reported for the commercial wine production regulation in Marsala area and no off-odours and off-flavours were detected. LAB concentration, in particular that of *L. plantarum*, was found at high concentrations during the tumultuous phase of alcoholic fermentation, and this report is the first on this observation. As long as the cellar management is constant, on the basis of the results showed, the natural process could represent a valid alternative to the conventional winemaking to preserve the typicality of wine.

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Table 1. Microbial loads[a] of samples collected during natural winemaking processes of Catarratto and Grillo cultivar

Steps of winemaking	WL		MRS		GM17		MLO	
	CT	GR	CT	GR	CT	GR	CT	GR
Grape berries	6.28±0.72	4.70±0.00	2.30±0.99	2.29±0.12	2.29±0.34	2.22±0.15	3.11±0.07	n.d.
Must	7.04±0.20	4.85±0.00	4.07±0.05	3.91±0.37	4.11±0.04	3.55±0.27	4.08±0.04	3.29±0.02
Fermentation:								
day 1 - maceration	6.98±0.03	7.34±0.02	4.08±0.11	5.87±0-12	4.10±0.41	4.41±.0.21	4.16±0.04	4.39±0.04
day 2 - maceration	7.20±0.30	7.79±0.21	6.19±0.21	6.15±0.04	6.07±0.20	5.11±0.02	6.06±0.20	5.16±0.05
day 3 - racking	7.93±0.03	7.53±0.16	6.12±0.09	3.97±0.14	6.01±0.55	3.63±0.46	2.70±0.55	2.42±0.60
day 4	7.69±0.02	–	2.53±0.33	–	1.20±0.33	–	1.73±0.33	–
day 5	–	7.46±0.40	–	4.24±0.28	–	3.18±0.14	–	2.77±0.10
day 6	7.56±0.01	–	3.43±0.13	–	2.16±0.02	–	1.30±0.02	–
day 7	7.71±0.13	7.75±0.06	3.10±0.56	5.15±0.02	1.85±0.30	4.14±0.01	3.10±0.30	4.10±0.04
day 8	–	7.47±0.46	–	4.30±0.02	–	2. 35±0.23	–	4.50±0.65
day 9	–	7.10±0.21	–	4.36±0.11	–	2.29±0.15	–	4.31±0.13
Ageing:								
day 3 - clarification	6.87±0.01	7.35±0.04	1.02±0.88	4.39±0.55	0.30±0.13	1.74±0.87	1.50±0.13	4.15±0.21
day 6 - clarification	–	6.79±0.15	–	2.30±0.14	–	1.65±0.95	–	2.15±0.88
day 9 - transfer	–	6.75±0.02	–	1.78±0.02	–	n.d.	–	1.45±0.52
day 13	–	4.40±0.05	–	0.50±0.71	–	n.d.	–	1.02±0.70
day 14 -transfer	4.01±0.12	–	1.05±0.21	–	1.34±0.50	–	2.23±0.50	–
day 19 -transfer	4.57±0.04	–	1.20±0.33	–	1.38±0.12	–	1.15±0.12	–
Bottling	n.d.	n.d.	n.d.		n.d.	n.d.	n.d.	n.d.

[a]Log CFU/g for grape berries; Log CFU/mL for must and wine samples.
n.d., not detected (value < detection limit of method); CT, Catarratto cultivar; GR, Grillo cultivar.
–, not sample.

Table 2. Molecular identification and distribution[a] of yeasts

R.P.	Isolate code	5.8S-ITS PCR[b]	Size of restriction fragment			26S PCR[c]	Size of restriction fragment		Specie (% identity)[d]	Accession Number	Distibution
			*Cfo*I	*Hae*III	*Hinf*I		*Hinf*I	*Mse*I			
I	CtbrL8	620	180+160+90	470+150	280+160+130	1100	480+390+180+50	600+380+100+50	*A. pullulans* (99)	JX423556	Gb(6);F1(6)
II	CtbrL76	480	220+110+60	480	240+240	1100	340+320+210+90+50	710+140+70	*C. zemplinina* (99)	JX423554	Gb(6)
III	CtrbrL78B	750	320+310+105	750	350+180+160 +60	1120	n.c.	n.c.	*H. guilliermondii*[b] (99)	JX423565	Gb(6);M(7);F1(6);F2(7)
IV	GrbrL9	750	320+100+50	750	350+200+160+70+50	1120	420+400+180	520+480	*H. guilliermondii*[b] (99)	JX423571	M(4);F1,F2(7);
V	CtrbrL65	750	320+310+105	750	350+200+180	1100	390+180	500+420+100+50	*H. uvarum*[b] (99)	JX423558	Gb(6);F1(6)
VI	GrbrL22	750	340+125	750	350+200+170+60+50	1100	420+400+180	520+480	*H. uvarum*[b] (99)	JX423570	Gb(4);F1,F2,F7(7)
VII	CtrbrL79	460	125+100+90+80	300+120	220+90	1100	340+250+220	820+210+100+50	*I. terricola* (99)	JX423555	Gb(6)
VIII	CtrbrL43	400	205+100+95	280+100	200+190	1100	340+250+220	550+250+140+50	*M. pulcherrima* (98)	JX423553	Gb(6);M(7)
IX	GrbrL13	400	205+100+95	280+100	200+190	1100	360+250+235+220	580+260+140	*M. pulcherrima* (100)	JX423573	Gb(4);
X	CtrbrL5	600	300+265+60	400+115+90	320+300	1100	490+230+170	680+370+50	*P. guilliermondii* (99)	JX423568	Gb(6),A14(4);A19(4)
XI	GrbrL59	600	310+260+160	400+125+80	310+290+50	1100	500+240+180+160	700+360	*P. guilliermondii* (99)	JX423569	Gb,M(4);F1,F2,F3,F5,F7,F8,F9, A3(7);A6,A9(6);A13(4)
XII	CtrbrL26B	640	320+220	420 + 220	355+210+75	1100	510+420+210	380+270+240+140+70	*R. mucillaginosa* (100)	JX423557	Gb(6)
XIII	CtrbrL56	880	360+340+130	320+240+180+140	360+110	1100	490+210+190	n.c.	*S. cerevisiae* (100)	JX423563	Gb(6);M(7);F1(6);F2(7);F3(7); F4(7);F6(7);F7(7);A3(6)
XIV	GrbrL20	880	365+340+140	310+235+170+135	365+125+50	1100	500+200+180	n.c.	*S. cerevisiae* (99)	JX423572	Gb(4);F1,F2,F3,F5,F7,F8,F9, A3(7);A6,A9(6);A13(4)

All values for the 5.8S-ITS PCR, 26S PCR and restriction fragments are given in bp.

Abbreviations: R.P., restriction profile; n.c., not cut; *A.*, *Aureobasidium* spp.; *C.*, *Candida* spp.; *H.*, *Hanseniaspora* spp.; *I.*, *Issatchenkia* spp.; *M.*, *Metschnikowia* spp.; *P.*, *Pichia* spp.; *R.*, *Rhodotorula* spp.; *S.*, *Saccharomyces* spp.; Gb, Grape berries; M, must; F1, first day of fermentation (maceration); F2, second day of fermentation (maceration); F3, third day of fermentation (racking); F4, fourth day of fermentation; F5, 5th day of fermentation; F6, sixth day of fermentation; F7, seventh day of fermentation; F8, 8th day of fermentation; F9, 9th day of fermentation; A3, third day of ageing (clarification); A6, 6th day of ageing (clarification); A9, 9th day of ageing (transfer); A13, 13th day of ageing; A14, fourteenth day of ageing (transfer); A19, nineteen day of ageing (transfer).

[a] The number reported between brackets refers to the highest concentration (Log cycle) of detection.

[b] The 5.8S-ITS gene was also digested with *Dde*I endonuclease confirming the restriction profile reported by Esteve-Zarzoso and colleagues (1999).

[c] Restriction enzymes *Apa*I did not produce any cut fragment.

[d] According to BlastN search of D1/D2 26S rRNA gene sequences in NCBI database.

Table 3. Phenotypic grouping of LAB

Characters	Clusters				
	A (n = 378	B (n=35)	C (n=9)	D (n=55)	E (n=1355)
Morphology	Coccus	Coccus	Coccus	Rod	Rod
Growth:					
15 °C	+	+	–	+	+
45 °C	–	+	+	–	–
pH 9.6	–	+	–	n.d.	n.d.
6.5% NaCl	+	+	–	n.d.	n.d.
CO_2 from glucose	+	–	–	+	–
Growth in presence of pentose carbohydrates	n.d.	n.d.	n.d.	n.d.	+

n.d. not determined

Table 4. Molecular identification and distribution[a] of LAB.

Species	Isolate code	Phenotypic group	Genotipic identification			Distribution of LAB on different media		
			RADP-PCR profile	16S rRNA sequencing		MRS	GM17	MLO
				% homology[b]	Acc. No.			
Enterococcus faecium	GRBRBL443	B	Group I	98	KC351905		Gb(2)	
Enterococcus faecium	GRBRBL444	B	Group II	98	KC351906		Gb(2)	
Leuconostoc mesenteroides	GRBRBL104	A	Group III	99	KC351900		Gb(2);M(3);F1(4);F2(5)	
Leuconostoc mesenteroides	GRBRBL105	A	Group IV	99	KC351903		Gb(2); M(3)	
Leuconostoc mesenteroides	CtbrBL226	A	Group V	99	JX426116			Gb(3)
Leuconostoc mesenteroides	CtbrBL480	A	Group VI	99	JX423551		Gb(2);M(4);F1(4) F2(6); F3(6); F4(1)	
Lactobacillus hilgardii	GRBRBL106	D	Group VII	98	KC351904	Gb(2)		
Lactobacillus hilgardii	CtbrBL372	D	Group VIII	99	JX423552			Gb(3)
Lactobacillus plantarum	GRBRBL101	E	Group IX	99	KC351898	Gb(2)	Gb(2);M(3)	
Lactobacillus plantarum	GRBRBL102	E	Group X	99	KC351899	Gb(2);M(4);F1(4);F2(6);F3(3);F5(4);F7,F8,F9,A3(4),A6(2);A9(1)		M(4);F1(4);F2(6);F3,F5(2);F7,F8,F9,A3(4);A6(2);A9,A13(1)
Lactobacillus plantarum	CtbrBL22	E	Group XI	99	X426117	Gb(2)		Gb(3)
Lactobacillus plantarum	CtbrBL103	E	Group XII	99	JX426118	Gb(2)		Gb(3)
Lactobacillus plantarum	CtbrBL25	E	Group XIII	99	JX426119	Gb(2);M(4);F1(4);F2(6)		Gb(3);M(4);F1(4);F2(6)
Lactobacillus plantarum	CtbrBL487	E	Group XIV	99	JX423550	Gb(2);F3(6);F4(2);F6(3);F7(3) A3(1);A14(1);A19(1)	F6(2); F7(1);A3(1);A14(1);A19(1)	Gb(3);F3(2);F4(1);F6(1);F7(3) A3(1);A14(2);A19(1)
Streptococcus macedonicus	GRBRBL433	C	Group XV	100	KC351907		A6(1)	

[a] The number reported between brackets refers to the highest concentration (Log cycle) of detection.

[b] According to BlastN search of 16S rRNA gene sequences in NCBI database.

Abbreviations: Gb, Grape berries; M, must; F1, first day of fermentation (maceration); F2, second day of fermentation (maceration); F3, third day of fermentation (racking); F4, fourth day of fermentation; F5, 5th day of fermentation; F6, sixth day of fermentation; F7, seventh day of fermentation; F8, 8th day of fermentation; F9, 9th day of fermentation; A3, third day of ageing (clarification); A6, 6th day of ageing (clarification); A9, 9th day of ageing (transfer); A13, 13th day of ageing; A14, fourteenth day of ageing (transfer); A19, nineteen day of ageing (transfer).

Table 5a. Chemical analysis of conventional parameters of Catarratto vinification

Parameters	Grape berries	Must	Fermentation						Ageing			Bottling
			day 1 (maceration)	day 2 (maceration)	day 3 (racking)	day 4	day 6	day 7	day 3 (clarification)	day 14 (transfer 1)	day 19 (transfer 2)	
pH	3.20±0.1	3.33±0.01	3.42±0.01	3.33±0.01	3.35±0.01	3.35±0.01	3.31±0.01	3.34±0.01	3.39±0.01	3.42±0.01	3.47±0.01	3.48±0.01
Reducing sugars (% w/v)	194.50±0.08	214.15±0.07	208.76±0.03	188.71±0.07	110.50±0.06	84.67±0.06	25.81±0.37	14.90±0.24	1.68±0.07	1.46±0.06	1.21±0.02	n.d.
Alcohol (% w/v)	n.d.	0.33±0.03	0.49±0.02	1.10±0.01	5.80±0.01	7.37±0.01	10.98 ±0.12	11.67±0.02	12.66±0.01	12.65±0.1	12.62±0.01	12.67±0.01
Glycerol (% w/v)	n.d.	0.26±0.07	0.89±0.03	0.88±0.04	4.98±0.03	5.55±0.05	5.35±0.01	5.99±0.02	7.17±0.01	7.09±0.05	7.15±0.02	7.19±0.01
PAN (mg L^{-1})	94.57±1.27	115±1.08	135±0.88	34.02±1.10	32±0.78	26±0.95	3.20± 0.05	n.d.	n.d.	n.d.	n.d.	n.d.
TTA	4.80±0.18	5.51±0.07	5.98±0.09	6.08±0.04	5.64±0.08	5.98±0.01	6.74±0.03	6.86±0.01	6.58±0.01	6.34±0.01	5.70±0.01	5.03±0.01
VA	n.d.	0.07±0.01	0.19±0.01	0.11±0.02	0.25±0.01	0.28±0.20	0.21±0.01	0.20±0.01	0.22±0.01	0.25±0.02	0.24±0.01	0.36±0.02
Tartaric acid (g/L)	5.27±0.09	4.76±0.01	5.33±0.08	6.71±0.08	3.87±0.08	4.07±0.01	2.99±0.06	3.61±0.01	3.89±0.01	3.68±0.02	3.66±0.03	3.01±0.03
Citric acid (g/L)	0.63±0.08	0.65±0.01	0.10±0.01	0.22±0.02	0.33±0.2	0.28±0.02	0.41±0.02	0.29±0.02	0.10±0.02	0.15±0.01	0.12±0.01	0.10±0.01
Malic acid (g/L)	0.98±0.08	1.29±0.01	1.40±0.02	1.57±0.03	1.54±0.04	1.48±0.05	1.47±0.01	1.49±0.01	1.50±0.01	1.35±0.01	0.54±0.02	0.10±0.01
Lactic acid (g/L)	n.d.	n.d	0.10±0.03	1.65±0.08	0.39±0.02	0.28±0.01	0.41±0.02	0.52±0.02	0.62±0.04	0.69±0.03	1.25±0.02	1.63±0.02
Total SO_2 (mg/L)	n.d.	n.d.	n.d.	11.51±0.23	24.11±0.02	31.91±0.12	37.21±0.52	34.98±0.88	30.89±0.48	26.89±0.25	14.89±0.22	21.89±0.22
Free SO_2 (mg/L)	n.d.	n.d.	n.d.	2.10±0.65	7.00±0.58	11.00±0.65	9.00±0.11	13.09±0.91	9.89±0.18	4.09±0.58	2.89±0.80	5.89±0.80

Abbreviation: PAN, promptly assimilable nitrogen; TTA, total titratable acidity (tartaric acid g/L); VA, volatile acidity (acetic acid g/L).

Table 5b. Chemical analysis of conventional parameters of Grillo vinification

Parametrs	Grape berries	Must	Fermentation							Ageing				Bottling
			day 1 (maceration)	day 2 (maceration)	day 3 (racking)	day 5	day 7	day 8	day 9	day 3 (clarification)	day 6 (clarification)	day 9 (transfer)	day 13	
pH	3.3±0.1	3.33±0.01	3.41±0.01	3.38 ±0.01	3.32 ±0.01	3.33 ±0.01	3.30 ±0.01	3.31 ±0.01	3.33±0.14	3.39 ±0.01	3.43 ±0.02	3.42 ±0.01	3.48 ±0.01	3.47±0.25
Reducing sugars (g/L)	194.50±0.08	243.15±0.1	230±0.01	210.35 ±0.3	155.87± 0.4	70.36 ±0.06	40.81 ±0.2	24.41 ±0.1	13±0.01	3.18 ±0.07	2.27 ±0.02	2.09 ±0.02	1.81 ±0.02	n.d.
Alcohol (% v/v)	n.d.	0.33±0.11	0.53±0.19	2.29 ±0.09	4.27 ±0.01	10.04 ±0.01	12.82 ±0.1	14.32 ±0.1	14.49±0.06	14.63 ±0.01	14.7 ±0.01	14.75 ±0.1	14.73 ±0.1	14.72±0.11
Glycerol (g/L)	n.d.	0.68±0.09	0.77±0.09	1.17 ±0.02	4.97 ±0.04	5.78 ±0.03	5.89 ±0.09	5.86 ±0.01	6.01±0.08	8.45 ±0.01	8.64 ±0.04	8.78 ±0.01	8.38 ±0.01	8.29±0.01
PAN (mg/L)	98.83±0.21	98.83±0.1	108.83±0.2	87.68±0.04	80.68±0.14	37.5±0.17	26±0.10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
TTA	5.14±0.18	5.14±0.07	5.27±0.01	5.32 ±0.09	5.06 ±0.04	5.16 ±0.22	5.33 ±0.01	5.93 ±0.02	5.84±0.16	5.69 ±0.02	5.45 ±0.02	5.36 ±0.02	5.15 ±0.01	5.11±0.06
VA	n.d.	0.12±0.01	0.13±0.01	0.14 ±0.01	0.37 ±0.01	0.5 ±0.014	0.27 ±0.02	0.22 ±0.01	0.25±0.09	0.27 ±0.01	0.26 ±0.02	0.31 ±0.02	0.36 ±0.01	0.34±0.16
Tartaric acid (g/L)	6.29±0.08	6.3±0.01	6.56 ±0.02	5.84 ±0.09	5.26 ±0.07	5.26 ±0.01	4.77 ±0.01	4.99 ±0.06	5.01±0.54	5.02 ±0.01	4.86 ±0.03	4.80 ±0.04	4.18 ±0.02	4.15±0.14
Citric acid (g/L)	0.20±0.08	0.20±0.01	0.22 ±0.01	0.28 ±0.01	0.22 ±0.01	0.18 ±0.01	0.16 ±0.01	0.18 ±0.01	0.17±0.18	0.15 ±0.01	0.11 ±0.01	0.10 ±0.01	0.12 ±0.01	0.11±0.18
Malic acid (g/L)	0.10±0.06	0.39±0.01	0.44 ±0.09	0.30 ±0.01	0.04 ±0.04	0.03 ±0.07	0.03 ±0.05	0.04 ±0.01	0.04±0.14	0.04 ±0.01	0.03± 0.01	0.04 ±0.01	0.04 ±0.08	0.04±0.64
Lactic acid (g/L)	n.d.	n.d.	0.09 ±0.01	0.1 ±0.01	0.74 ±0.04	0.67 ±0.03	0.36 ±0.01	0.25 ±0.01	0.27±0.24	0.51 ±0.06	0.47 ±0.02	0.49 ±0.01	0.52 ±0.02	0.51±0.68
Total SO_2 (mg/L)	n.d.	n.d.	n.d.	11.55±0.21	23.96±0.12	31.35±0.11	37.36±0.41	33.92±0.28	32.87±0.15	30.76±0.45	27.01±0.22	14.34±0.21	22.03±0.12	13.87±0.11
Free SO_2 (mg/L)	n.d.	n.d.	n.d.	2.12±0.56	7.04±0.54	11.03±0.43	9.06±0.231	12.98±0.72	11.51±0.25	9.74±0.11	3.99±0.57	2.87±0.79	5.89±0.70	5.87±0.24

Abbreviation: PAN, promptly assimilable nitrogen; TTA, total titratable acidity (tartaric acid g/L); VA, volatile acidity (acetic acid g/L)

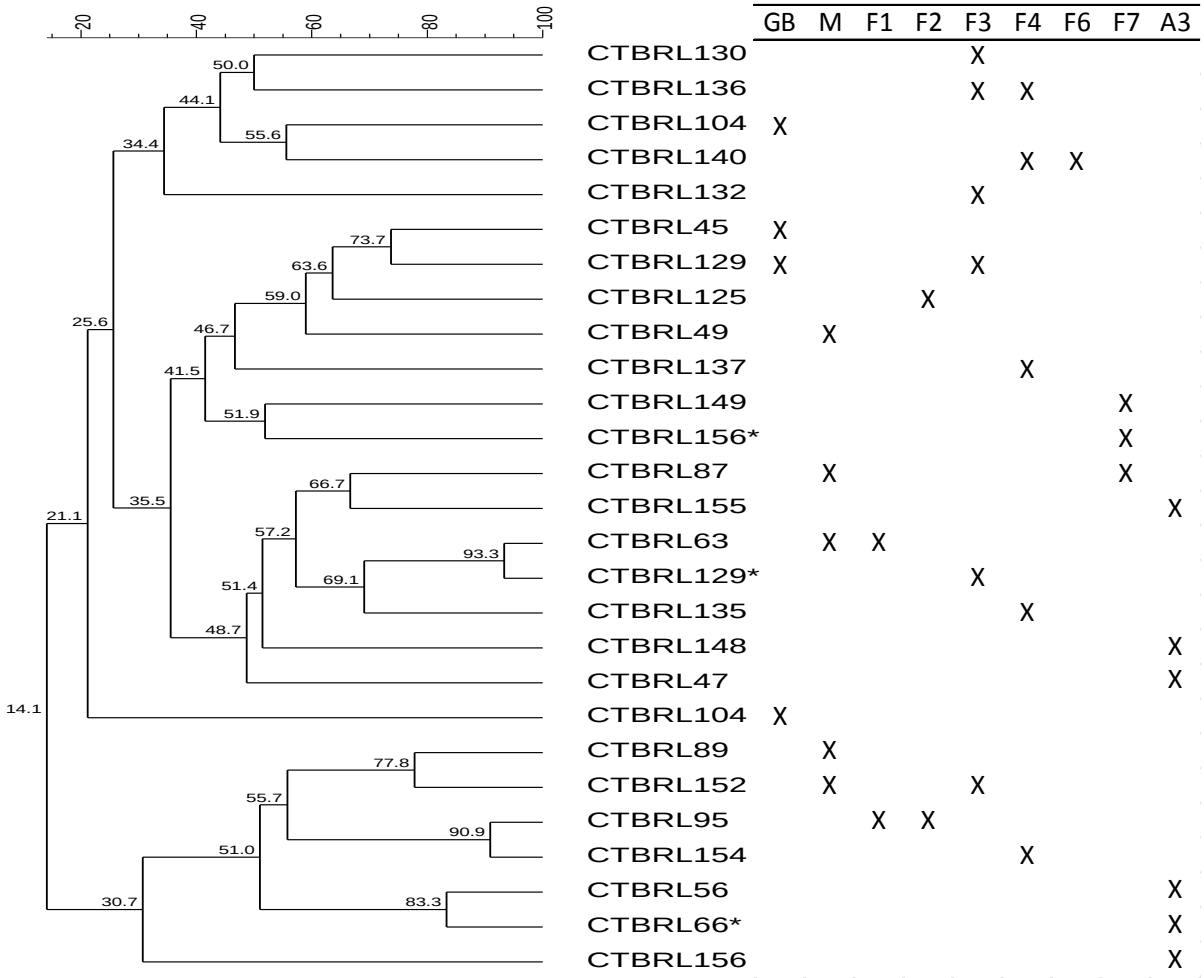
Table 6a. Volatile organic compounds of Catarratto vinification

Compounds	Fermentation			Bottling
	day-3 (racking)	day-4	day-7	
Higher alcohols (mg/L)	107.65	240.54	243.42	297.22
Isoamylic alcohol	47.81±1.21	152.31±0.94	155.37±1.23	179.67±1.11
Isobutanol	28.58±0.61	43.70±1.09	38.41±1.31	62.88±3.33
n-propanol	18.00±0.88	23.38±0.60	18.38±0.98	25.93±1.34
Phenyl 2-ethanol	13.26±0.38	21.15±0.60	31.26±0.89	28.74±0.81
Other alcohols (µg/L)	819.01	1256.19	1181.09	1370.91
1-Hexanol	773.77±54.71	1155.16±81.68	1083.09±76.59	1246.71±88.16
3-Methyl pentan-1-olo	n.d.	21.20±1.05	33.89±1.68	54.48±2.70
cis-3-Hexenol	19.82±1.26	32.54±2.07	29.10±1.85	32.76±2.08
trans-3-Hexenol	25.42±1.62	47.29±3.01	35.01±2.23	36.96±2.35
Esters (µg/L)	3589.15	8703.08	12097.11	24070.83
Diethyl malate	n.d.	72.13±2.55	103.60±3.66	62.02±2.19
Diethyl succinate	32.30±1.83	2671.43±91.12	2463.93±82.81	15506.04±877.15
Ethyl 4-OH-butyrate	154.25±7.64	495.94±24.55	2279.18±112.81	1642.22±81.29
Ethyl 9-decanoate	n.d.	311.96±13.24	339.82±14.42	292.56±12.41
Ethyl decanoate	582.37±24.71	1039.67±44.11	1359.48±57.68	1526.01±64.74
Ethyl hexanoate	1048.28±44.47	1475.06±62.58	1751.93±74.33	1097.83±46.58
Ethyl lactate	50.57±2.86	295.49±16.72	322.18±18.23	782.58±44.27
Ethyl octanoate	1721.38±121.72	2341.40±165.56	3476.99±245.86	3161.57±223.56
Acetate Esters (µg/L)	13246.81	84346.42	114903.07	145431.60
Ethyl acetate	9150.11±379.21	79580.21±933.01	110150.09±776.12	141650.02±811.03
Hexyl acetate	130.00±7.35	130.03±7.36	102.61±5.80	85.22±4.82
Isoamyl acetate	1401.36±49.55	2082.45±73.63	2104.09±74.39	2251.80±79.61
Phenyl-2-ethanol acetate	2565.34±181.40	2553.73±180.58	2546.28±180.05	1444.56±102.15
Acids (µg/L)	12316.04	12844.36	18775.80	17724.96
Butyric acid	n.d.	32.16±1.14	28.49±1.01	35.39±1.25
Decanoic acid	3981.91±197.09	2383.43±117.97	4270.78±211.39	3506.01±173.54
Hexanoic acid	2724.18±96.31	4118.60±145.61	4826.92±170.66	5205.97±184.06
Octanoic acid	5609.95±238.01	6310.17±267.72	9649.61±309.40	8977.59±380.89

Table 6b. Volatile organic compounds

Compounds	Fermentation			Bottling
	day3 (racking)	day 7	day 9	
Higher alcohols (mg/L)	231.97	380.57	463.34	564.09
n-propanol	17.59±0.78	29.99±1.23	30.79±1.34	39.58±1.76
Isobutanol	33.70±2.02	45.69±2.57	67.18±3.32	88.87±4.32
Isoamyl alcohol	138.17±3.45	230.07±4.54	299.41±4.32	383.86±5.01
Phenyl 2-ethanol	42.51±5.02	74.82±6.45	65.96±3.32	51.78±8.31
Other alcohols (µg/L)	2075.24	2470.39	2255.35	2309.35
1-Hexanol	1930.36±122.85	2252.87±143.37	2058.67±131.01	2111.70±134.39
trans-3-Hexenol	31.79±2.02	42.08±2.68	35.96±2.29	29.13±1.85
cis-3-Hexenol	87.15±6.16	87.63±6.20	70.86±5.01	62.26±4.40
3-Methyl pentan-1-olo	25.94±1.47	87.81±4.97	89.86±5.08	106.26±6.01
Esters (µg/L)	2999.7	4434.04	5888.56	5944.13
Etyl 3-OH-butanoate	22.31±0.95	83.60±3.55	119.95±5.09	109.69±4.65
Ethyl 4-OH-butyrate	652.54±18.46	1560.70±44.14	2098.05±59.34	2000.40±56.58
Diethyl malate	18.27±0.65	47.67±1.69	71.98±2.54	102.10±3.61
Isoamyl 4-OH Butyrate	15.29±0.65	92.59±3.93	148.53±6.30	134.59±5.71
Ethyl lactate	145.98±10.32	357.34±25.27	404.59±28.61	511.90±36.20
Diethyl succinate	325.68±23.03	689.88±48.78	888.33±62.81	1511.84±106.90
2-Ethyl hexanoic acid	7.49±0.32	22.24±0.94	23.62±1.00	53.59±2.27
Ethyl hexanoate	583.28±33.00	620.79±35.12	589.71±33.36	386.79±21.88
Ethyl octanoate	859.96±48.65	591.25±33.45	890.94±50.40	724.81±41.00
Ethyl decanoate	346.46±19.60	358.77±20.30	644.69±36.47	395.68±22.38
Ethyl 9-decanoate	22.44±1.59	9.21±0.85	8.17±0.58	12.74±0.90
Acetate esters (µg/L)	43886.79	62494.85	80567.85	130121.5
Ethyl acetate	38920.01±456.87	57540.12±651.02	76170.24±792.12	126300.11±856.29
Isoamyl acetate	1227.32±43.39	1536.58±54.33	1512.54±53.48	1800.09±63.64
Hexyl acetate	76.13±3.77	51.41±2.54	34.04±1.69	32.03±1.59
Phenyl-2-ethanol acetate	3663.33±129.52	3366.74±119.03	2851.03±100.80	1989.31±70.33
Acids (µg/L)	4970.84	12161.33	9480.24	14057.92
Butyric acid	9.22±0.39	21.41±0.91	18.07±0.77	13.95±0.59
Isovalerianic acid	172.49±7.32	379.07±16.08	352.98±14.98	230.99±9.80
Hexanoic acid	1737.96±61.45	2338.77±82.69	2120.06±74.96	1967.80±69.57
Octanoic acid	1872.11±79.43	5076.39±215.37	2885.35±122.42	3514.85±149.12
Decanoic acid	455.53±16.11	2472.05±87.40	1796.24±63.51	1532.53±54.18
Monoethyl succinic acid	723.53±25.58	1873.64±66.24	2307.54±81.58	6797.80±240.34

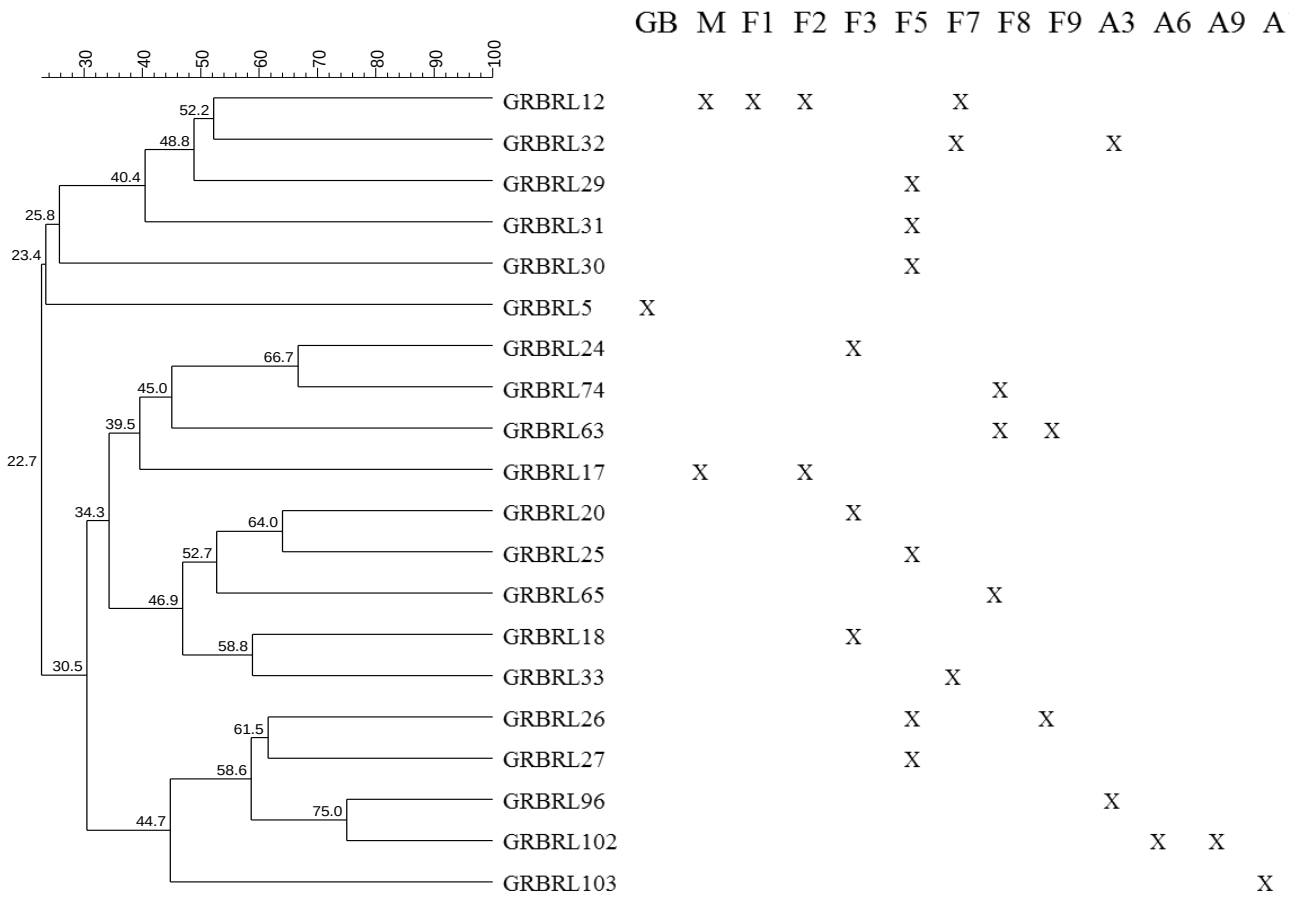
Figure 1a. Dendogram of interdelta profiles of *S. cerevisiae* strains and their distribution during Catarratto vinification



	GB	M	F1	F2	F3	F4	F6	F7	A3
CTBRL130					X				
CTBRL136					X	X			
CTBRL104	X								
CTBRL140						X	X		
CTBRL132					X				
CTBRL45	X								
CTBRL129	X				X				
CTBRL125				X					
CTBRL49		X							
CTBRL137						X			
CTBRL149								X	
CTBRL156*								X	
CTBRL87		X						X	
CTBRL155									X
CTBRL63		X	X						
CTBRL129*					X				
CTBRL135						X			
CTBRL148									X
CTBRL47									X
CTBRL104	X								
CTBRL89		X							
CTBRL152		X			X				
CTBRL95			X	X					
CTBRL154						X			
CTBRL56									X
CTBRL66*									X
CTBRL156									X

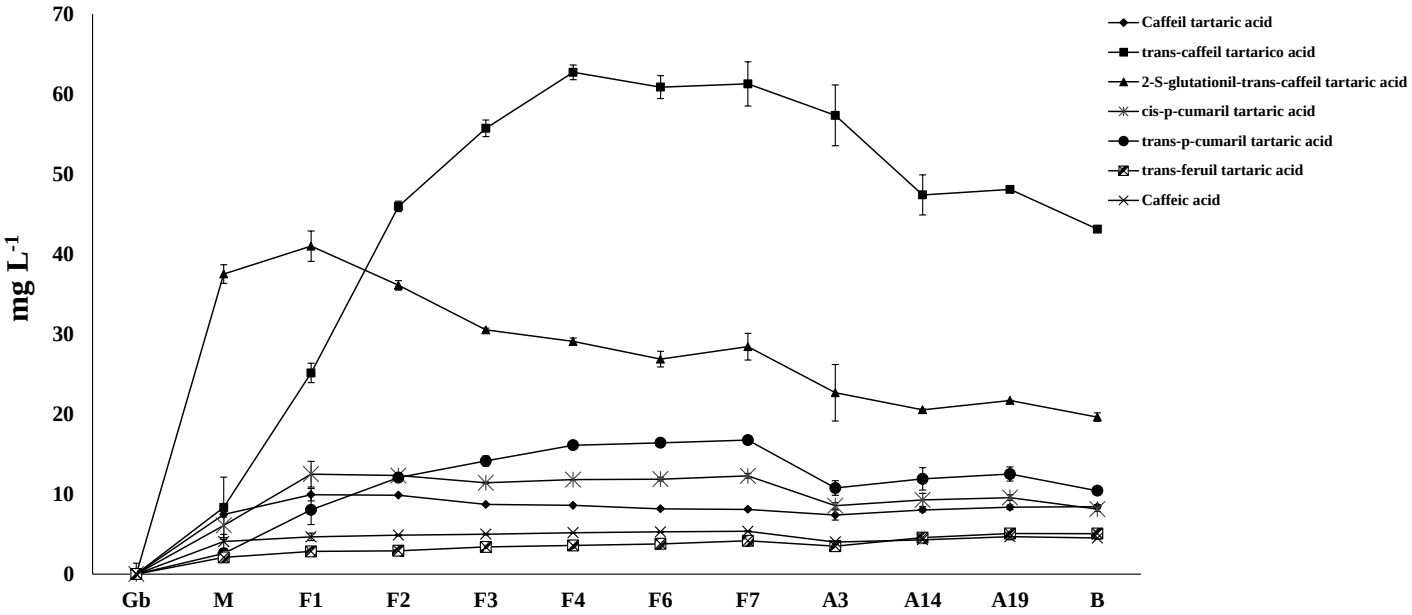
Abbreviations: Gb, Grape berries; M, must; F1, first day of fermentation (maceration); F2, second day of fermentation (maceration); F3, third day of fermentation (racking); F4, fourth day of fermentation; F6, sixth day of fermentation; F7, seventh day of fermentation; A3, third day of ageing (clarification).

Fig. 1b. Dendogram of interdelta profiles of *S. cerevisiae* strains and their distribution during Grillo vinification



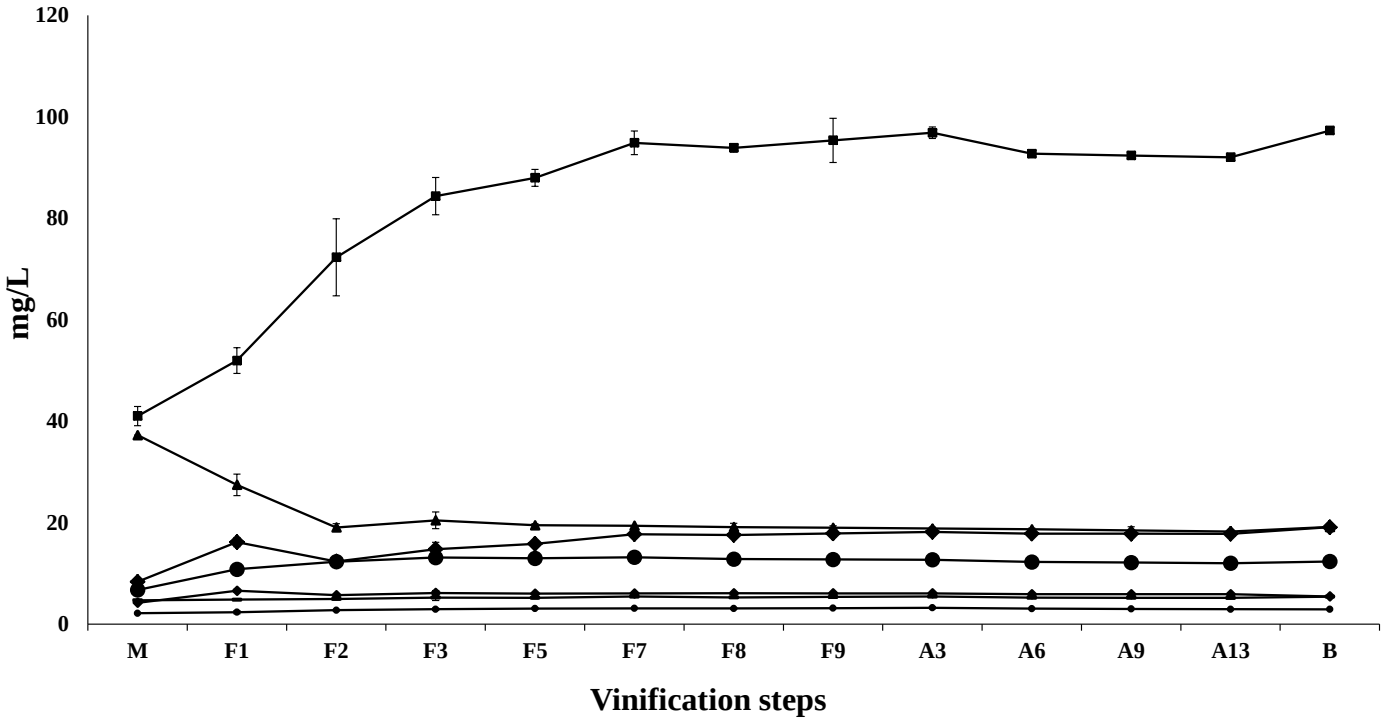
Abbreviations:
M, must;
F1, 1th day of fermentation (maceration);
F2, 2nd day of fermentation (maceration);
F3, 3rd day of fermentation (racking);
F5, 5th day of fermentation;
F7, 7th day of fermentation;
F8, 8th day of fermentation;
F9, 9th day of fermentation;
A3, 3rd day of ageing (clarification 1);
A6, 6th day of ageing (clarification 2);
A9, 9th day of ageing (transfer);
A13, 13th day of ageing.

Figure 2a. Distribution of hydroxy cinnamic acids during winemaking of Catarratto cultivar



Abbreviations: Gb, Grape berries; M, must; F1, first day of fermentation (maceration); F2, second day of fermentation (maceration); F3, third day of fermentation (racking); F4, fourth day of fermentation; F6, sixth day of fermentation; F7, seventh day of fermentation; A3, third day of ageing (clarification); A14, fourteenth day of ageing (transfer 1); A19, nineteen day of ageing (transfer 2); B, bottling.

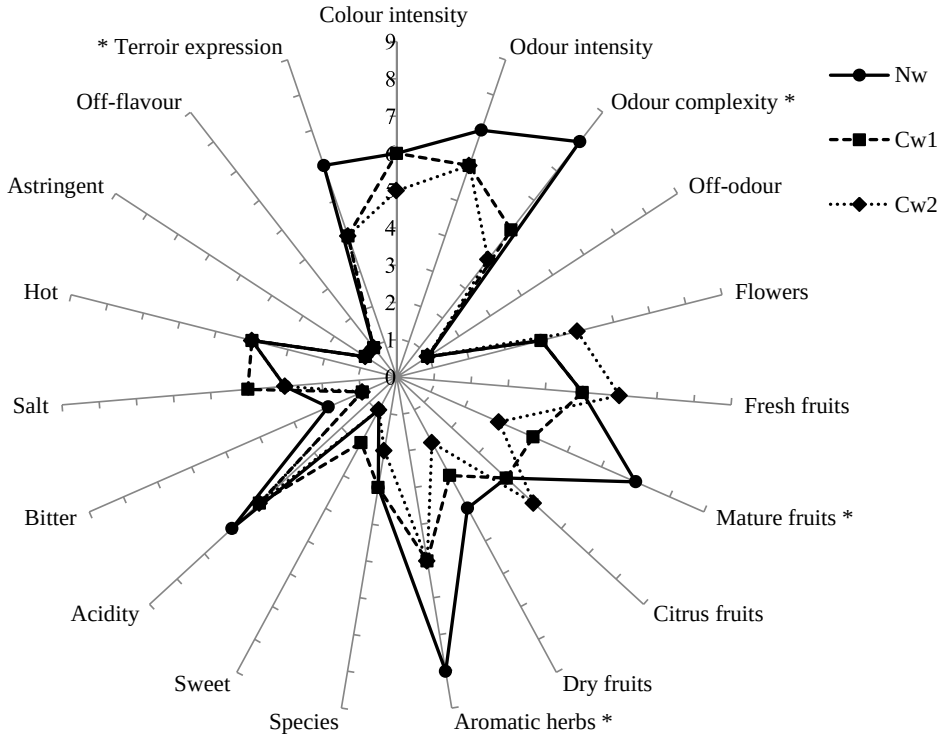
Fig. 2b. Distribution of hydroxycinnamoyl tartaric acids during winemaking of Grillo cultivar



Abbreviations: M, must; F1, 1th day of fermentation (maceration); F2, 2nd day of fermentation (maceration); F3, 3rd day of fermentation (racking); F5, 5th day of fermentation; F7, 7th day of fermentation; F8, 8th day of fermentation; F9, 9th day of fermentation; A3, 3rd day of ageing (clarification 1); A6, 6th day of ageing (clarification 2); A9, 9th day of ageing (transfer); A13, 13th day of ageing; B, base wine

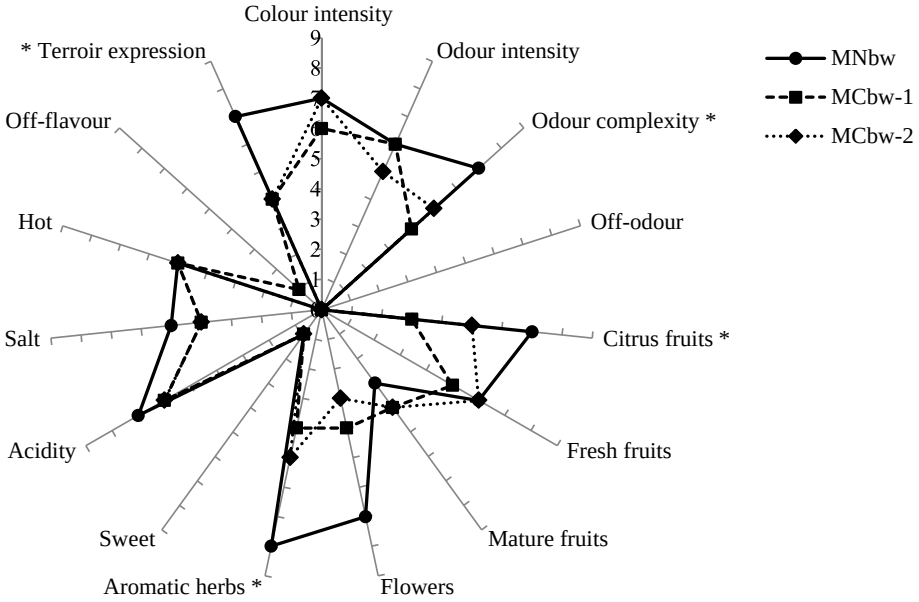
Symbols: small diamond, caffeil tartaric acid; square, *trans*-caffeil tartaric acid; triagle, 2-S-glutationil-*trans*-caffeil tartaric acid; big circle, *cis*-p-cumaril tartaric acid; big diamond, *trans*-p-cumaril tartaric acid; small circle, *trans*-feruil tartaric acid; horizontal bar, caffeic acid

Figure 3a. Sensory profiles of Catarratto wines.



Nw, Catarratto natural wine; Cw1 and Cw2, conventional wines.

Fig. 3b. Sensory profiles of Grillo wines (Marsala base wines)



MNbw, natural Grillo wine; MCbw-1, conventional Grillo wine 1; MCbw-2,conventional Grillo wine 2

Chapter 4. Innovative protocol for fermentation of natural wine and their microbial and chemical-physical monitoring

4.1 Introduction

The cultivar of Nero d'Avola is one of the most cultivated grapevine in Sicily (12000 Ha). The production of Nero d'Avola wines usually follows the technological phases of conventional vinification process that is based on the inoculum of yeast starter, on the maceration phase and ageing. In accordance to the increasing demand of natural wines by specialized consumers, the number of cellars producing Nero d'Avola wines based on spontaneous alcoholic fermentations is rapidly increasing.

As already reported by other authors, the winemaking is an heterogeneous microbiological process that involves the succession of different non-*Saccharomyces* and *S. cerevisiae* species. These yeasts exercise an important role for defining the color and flavour of the final product (Fleet 2003). Several studies focused on yeast population dynamics during wine fermentation (Le Jeune et al 2006; Li et al 2011; Lopandic et al 2008; Ocon et al 2010; Zott et al 2008) and considered the use of the selected indigenous yeasts for alcoholic fermentation to improve the complexity of wines (Renouf et al 2006).

Some wineries use a traditional yeast starter preparation method called "pied de cuve" to induce grape must fermentations. The pied de cuve method apply yeasts from a positively running fermentation to start a new must fermentation. Thus, the "pied de cuve" represents the inoculum of a partially fermented must with fermentative cell yeasts into a fresh must. The alcoholic fermentation of the pied de

cuve could be carried out by yeast starter, previously inoculated in the must, or by yeasts naturally present in the must, thus by spontaneous fermentation. When the fermented pied de cuve reaches the ethanol content of about 4-6 % (v/v), thus the must is enriched of viable fermentative yeast cells, the fermented pied de cuve is inoculated into a fresh must with a final pied de cuve-to-fresh must ratio of 1:10. In this way, the addition of the fermented pied de cuve into fresh must allows the inoculum of cell yeasts that rapidly drive the fermentation process. Obviously, the use of spontaneously fermented pied de cuve includes the same risks characterizing the spontaneous wine fermentations such the growth at high concentrations of spoilage microorganisms and/or the stuck of the alcoholic fermentations and/or the formation of off-flavours.

The aim of the present work was to show an innovative vinification process in order to assure a correct fermentation process based on the use of a spontaneous fermented pied de cuve. With this regards, we performed an experimental vinification by using the "fortified fermented pied de cuve" (FFPC) that is a pied de cuve first added with wine, thus fortified with ethanol contained in the wine, and after that the same pied de cuve is subjected to spontaneous alcoholic fermentation. The FFPC was inoculated into fresh must in order to carry out the alcoholic fermentation. The cultivar Nero d'Avola was used as model system.

4.2 Materials And Methods

4.2.1 Experimental winemaking and sample collection

The experimental winemaking was based on three technological phases: (i) the addition of ethanol, using Nero d'Avola wine, into fresh must that represented the fortified pied de cuve; (ii) the spontaneous alcoholic fermentation of the fortified pied de cuve; (iii) the inoculum of FFPC into fresh must and the monitoring of the entire winemaking processes by microbiological, chemical and sensory analysis.

The grapes of the "Nero d'Avola" cultivar were used as model raw material for the experimental vinifications that took place at the cellar of the "Centro di Ricerca per l'Innovazione della Filiera vitivinicola Ernesto del Giudice" located in Marsala (Trapani, Sicily, Italy) (37°78'+12°49').

The grapes were manually harvested in the vineyards located in Marsala province (37°45'+12°45'), stemmer-crushed and the resulting must (600 kg) was placed into a steel vat and added with potassium metabisulphite (KMBS) (5 g/q). Subsequently, the must was divided in three experimental trials (trials A, B, C) and each of them was further divided in three replicates for a total of 9 experimental steel vats containing 60 kg of must each. The must of the trial A was added with Nero d'Avola wine (vintage 2009) [pH 3.40; ethanol 13.1% (v/v), total acidity 5.85 g/L (tartaric acid); total SO_2 87 mg/L; free SO_2 20 mg/L] in order to generate an ethanol concentration of 1.5 % (v/v) into the must. The wine was added into must within a period of 3 h. The musts of the trial B were added with the same wine up to the ethanol content of 3% (v/v). Both trial A and B were spontaneously fermented. The musts of the trial C, representing the control trial, was not added with wine but

inoculated (10 g/q) with a commercial strain of *S. cerevisiae* that is generally used for production of commercial Nero d'Avola wines produced in the Marsala CDO area. The fermentations of FFPC took place at 21 °C and when the ethanol content of the FFPC reached value of 5-6% (v/v), all experimental trials were subjected to chemical (measurements of pH and of reducing sugars, total acidity, glycerol and ethanol contents) and sensory analysis as reported by Sannino et al. (2013).

On the basis of the results obtained by chemical and sensory analysis, one replicate for each experimental was selected and the bulk content (liquid and solid phases) was inoculated into steel vats with a capacity of 1.0 hL where the fermentation (8 days at 26 °C) took place. All vats were filled until a final fermented pied de cuve-to-fresh must ratio of 1:10 was reached. Diammonium phosphate and diammonium sulphate salts (1:1) (16 g/hL) were also added as activators of the fermentation process. During the tumultuous phase of alcoholic fermentation, but only after raising the cap, the content of each vat was mixed (three times per day) in order to facilitate the contact between the solid and liquid phases of the must. The scope of this action was to facilitate the contact between the liquid phase of the must with oxygen.

At the end of alcoholic fermentation the entire bulk content of each vats was pressed by hydraulic press and the resulting liquid phase (60 L) was transferred into stainless steel vats (capacity of 1 hL) where the spontaneous malo-lactic fermentation (MLF) (20 days at 23 °C) took place. At the end of MLF, in order to avoid the contact between the wine and the oxygen, all vats were sealed by nitrogen gas and stored for 7 months at 16 °C. At the end of ageing, the wines were filtered (5.0 µm pore size filter) and bottled. The final wines were stored at 16 °C and 80% of relative humidity

for 2 months. The entire vinification process, i.e., from must just crushed until wine bottling was performed in triplicate.

The samples were collected both during the pied de cuve preparation (from must just crushed to the end of fermentation) and the vinification process (from must just crushed to wine bottling).

4.2.2 Microbiological analysis

The samples collected during the experimental vinifications were serially diluted in Ringer's solution (Sigma-Aldrich, Milan, Italy). Decimal dilutions were spread-plated (0.1 mL) onto Wallerstein Laboratory (WL) nutrient agar (Oxoid, Basingstoke, UK) and incubated at 28 °C for 48–72 h to determine total yeast (TY) counts. To count the lactic acid bacteria (LAB), the sample dilutions were pour-plated onto Man, Rogosa, and Sharpe (MRS) agar (Oxoid) and on M17 agar (Oxoid) incubated at 28 °C for 48–72 h, for rod and coccus shape LAB, respectively. The sample dilutions were also pour-plated onto medium for Leuconostoc oenos (MLO) agar (Caspritz and Radler 1983) and incubated at 28 °C for 5 d. The latter medium was used for the enumeration of acidophilic LAB. The acetic acid bacteria (AAB) population was enumerated onto Kneifel agar medium (OIV 2010) and incubated at 25 °C for 10 d. The dilutions of samples collected after the MLF were also spread-plated onto *Dekkera*/*Brettanomyces* differential medium (Rodrigues et al 2001) and incubated at 25 °C for 14 d to detect presumptive *Dekkera*/*Brettanomyces* spp. The *Dekkera*/*Brettanomyces* population was also counted by filtering (0.45-µmpore size

filter, Sartorius, AubagneCedex, France) the samples using the same media and incubation conditions reported above. All analyses were carried out in duplicate.

4.2.3 Yeast isolation and identification

Yeasts were isolated only from WL differential medium. At least five colonies per morphology were randomly collected from the agar plates, purified to homogeneity after several sub-culturing steps onto WL, and at least three isolates (from each sample) sharing the same morphology were subjected to genetic characterization.

DNA extraction was performed using the InstaGene Matrix kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. In order to perform a first differentiation of yeasts, all selected isolates were subjected to restriction fragment length polymorphism (RFLP) of the region spanning the internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene as reported by (Esteve-Zarzoso et al 1999). Five isolates representative of each group were subjected to an additional enzymatic restriction targeting the 26 rRNA gene as reported by Settanni et al (2012). One isolate per group was further processed by sequencing the D1/D2 region of the 26S rRNA gene to confirm the preliminary identification obtained by RFLP analysis. D1/D2 region was amplified with primers NL1 and NL4 (O'Donnel 1993). Polymerase chain reaction (PCR) products were visualized as described by Settanni et al (2012). DNA sequencing reactions were performed at Primmbiotech S.r.l. (Milan, Italy). The identities of the sequences were determined by BlastN search against the NCBI non-redundant sequence database located at http://www.ncbi.nlm.nih.gov.

4.2.4 Strain typing of *Saccharomyces cerevisiae* isolates

The isolates belonging to the species *S. cerevisiae* were further characterized at intra-specific level by employing two techniques: interdelta analysis with primers delta12 and delta21 (Legras and Krast 2003) and microsatellite multiplex PCR based on the analysis of polymorphic microsatellite loci, i.e., SC8132X, YOR267C and SCPTSY7 (Vudano and Garcia-Moruno 2008). The PCR products were analyzed on 2% agarose gel (w/v) in 1x Tris/borate/EDTA buffer and visualised as reported by Settanni et al (2012).

4.2.5 Chemical analysis

The chemical composition of samples was determined by means of a Winescan (FOSS) calibrated following EEC 2676 standard procedure (European Economic Community 1990). Anthocyanin and total flavonoid contents were determined by spectrophotometry (Di Stefano 1980); volatile organic compounds (VOCs) (Giannotti and Di Stefano 1991) and higher alcohols (Di Stefano 1980) by gas-chromatograph (GC) and GC-mass spectrometry (MS).

4.3 Results

4.3.1 Microbiological analysis

The results of TY counts during the preparation of the FFPC are reported in the Table 1. The TY loads detected on grape berries and in the must just after crushing were 4.60 Log CFU/g and 5.44 Log CFU/mL, respectively. After the wine addition into must, both the trial A and B showed a significantly decrease of TY level; on the other hand, after the starter inoculum the TY concentration of the trial C increased than 1 Log. Subsequently, when the concentration of ethanol into FFPC was around 5-6% (v/v), TY concentration of all experimental trials increased up to 8.0 log CFU/mL. The LAB, AAB and *Dekkera*/*Brettanomyces* spp. populations were not detected during the entire period of FFPC preparation on all media tested.

The FFPC, at around 5-6% (v/v) of ethanol, was inoculated into a new fresh must in order to carry out the alcoholic fermentation in the winemaking process. The results of microbial counts detected during the vinification process, from grape harvest to wine bottling, are reported in the Table 1.

The TY concentration counted on grape berries was 4.78 Log CFU/g whereas that of must just crushed was 5.77 Log CFU/mL. After the addition of the FFPC, the TY levels of musts increased up to 7.20 Log CFU/mL (trial A) and 7.15 Log CFU/mL (trial B) that were higher than that (6.74 Log CFU/mL) detected in the trial C. During the alcoholic fermentation, the TY level increased in all trials reaching the highest values of 8.4 (trial A), 8.0 (trial B) and 8.1 (trial C) Log CFU/mL, respectively. After the racking all experimental trials showed a decrease of TY concentrations that were undetectable at the bottling phase.

During the experimental vinification, the LAB population reached detectable level only after the racking. Specifically, their concentration greatly increased by day 16 of vinification (the beginning of the MLF), on all media used in this study. Their highest concentration was reached at day 17 of ageing, both in trial A (6.51 Log CFU/mL) and trial B (6.64Log CFU/mL), and at day 23 of ageing in the trial C (6.49 Log CFU/mL). The LAB concentration decreased by day 36 of vinification on all media, and was estimated at undetectable level at the bottling phase.

Dekkera/*Brettanomyces* spp. and AAB populations were detected during the entire vinification process and in all experimental trials.

4.3.2 Isolation, identification and distribution of yeasts

A total of 2386 yeasts (512 from FFPC phase and 1874 from the vinification process) were isolated from count plates, purified to homogeneity and grouped on the basis of colony morphology on WL medium. A total of 20 colonies per each morphology were selected and subjected to molecular identification. After the restriction analysis of 5.8S-ITS region and 26S rRNA gene, the isolates were clustered into four groups (Table 2). Only the isolates belonging to the group I were directly identified as *Metschnikowia pulcherrima* by comparison of the restriction bands with those available in literature (Esteve-Zarzoso et al 1999). The identification of the isolates belonging to the groups II, III and IV was concluded by sequencing of D1/D2 domain of the 26S rRNA gene. This technique allotted the isolates in three species: *Pichia guilliermondii* (group II), *Hanseniaspora guilliermondii* (group III) and *S. cerevisiae* (group IV).

The distribution of yeast species and the corresponding concentrations estimated for each sample are reported in Table 2. During the FFPC preparation, non-*Saccharomyces* species were mainly detected before the addition of wine into must and only the species, *Metchnikowia pulkerrima*, *Hansenisapora guilliermondii* and *Saccharomyces cerevisiae* were found, whereas after this phase the species *S. cerevisiae* was the most frequently isolated and at the highest concentrations both in the trial A and B as well as in the trial C (control).

During the vinification phase, the species non-*Saccharomyces* were found at highest concentrations only on grape berries and in the must just crushed. The species *S. cerevisae* was detected only during the alcoholic fermentation and at the highest concentration during the entire fermentation process in all experimental trials. During the ageing phase, the species *S. cerevisiae* resulted at highest counts although *P. guilliermondii* and *M. pulcherrima* reaching the same concentration of *S. cerevisiae* (10^4 - 10^5 UFC/mL) at day 23 of ageing. At blotting phase, no yeast was found.

4.3.3 Typing and distribution of *S. cerevisiae* strains

Inter-delta analysis reported 49 different strains of *S. cerevisiae* (Fig.1), 22 of them were found during the FFPC preparation and 43 during the vinification process. Specifically, during the FFPC preparation the trial A showed 14 different strains, whereas 10 strains were detected from the pied de cuve of the trial B. Only two strains were found in the pied de cuve of trial C (control) and one of them showed the same molecular profile of *S. cerevisiae* strain used as starter. During the

vinification process, the trial A and the trial B showed 28 and 26 strains, respectively, whereas in the trial C 9 strains were found.

With regards to the strain distribution, during the FFPC preparation the strains 15 and 8 (the commercial starter) were most frequently isolated in the trial B and C, respectively. The trial A showed a high number of different strains at the same frequency of isolation.

During the vinification process, the strains 15, 22 and 8 were the most frequently isolated in the trial A, B and C, respectively. Furthermore, the strain 15 and 22 were mainly isolated at the beginning of alcoholic fermentation and at the end of ageing, respectively, both in the trial A and B. In the trial C, although the strain 8 (the commercial starter) dominated the alcoholic fermentation, the strain 33 was mainly isolated at the end of vinification.

4.3.4 Chemical conventional parameters and polyphenols compounds

The conventional parameters of the samples collected during the experimental process are reported in Table 3. During the alcoholic fermentation, the reducing sugar greatly decreased in all experimental trials, as well as a consistent increase of ethanol content was found. Glycerol concentration of the trial A (5.6 g/L) was higher than that found both in the trial B and C (4.6 g/L) at first day of fermentation, but the value of this compound was the same (7.7 g/L) in all trials at the end of alcoholic fermentation. The volatile acidity was found at very low content during the alcoholic fermentation in all experimental trials; whereas, at the end of ageing an increase up to about 0.43 (g/L of acetic acid) was found both in the trial A and B.

The pH and total acidity showed the same trend in all experimental trials. The malic acid content was the same (1.52 g/L) in all trials at the beginning of the alcoholic fermentation. On the other hand, the level of lactic acid content of the trial A and B (1.18 g/L) was higher than that found in the trial C (0.99 g/L).

The Table 3 also show the results of polyphenol analysis. The values of polyphenol content showed the same trend in all experimental trials. Both anthocyanins and flavonoids showed a consistent decrease from the beginning of the alcoholic fermentation until the bottling.

4.3.5 VOCs determination

The results of VOCs analyses carried out only on the samples collected during the vinification process are reported in the Table 4. Hexanoic acid, ottanoic acid and decanoic acid (fatty acids) reached the highest value at day 1 and day 2 of alcoholic fermentation in the trial A and at day 4 in the trial B. During vinification their concenration remained constant. The content of C6 alcohols (Hexanol, *trans*-3-hexenol, *cis*-3-hexenol) decreased during the experimentation showing almost the same values in all the experimental trials. Isoamylic acetate and 2-phenyl-etil-acetate were produced in all trials within the first two days of alcoholic fermentation. In particular both trial A and B showed values of isoamyl acetate (661.56 and 506.97 µg/L, respectively) and 2-phenyl-etil-acetate (62.13 and 64.90 µg/L, respectively) higher than the trial C (control) (560 and 98 µg/L, respectively). At the end of the ageing, the content of the isoamyl acetate significantly decreased in all experimental trials up to a values of about 280 µg/L. 2-phenyl-etil-acetate remained constant for

trial B and C (about 85 µg/L) instead in the trial A its content decreased until the bottling (43.73 µg/L). During the alcoholic fermentation, the methanol content was almost the same level in all experimental trials; only the trial B showed a methanol concentration (180 mg/L) higher than the other experimentations (about 162 mg/L) at the racking phase. During the ageing, the methanol content increased only in the trial C reaching the value of 187 mg/L.

4.3.6 PCA of chemichal compounds and VOCs

The figure 2 reported the analysis of principal chemical compounds during vinification. The figure shows that first and second component explained the 35.5% and 23.3% of total variance, respectively. The first component was positively related to the ethyl ester compounds (with exception of the ethyl 9-decanoate), to the fatty acid, to the superior alcohol and their acetate and to the hexanol compounds as well as to the total and volatile acidities. On the other hand, the first component was negatively correlated with sugar, pH, malic acid, total anthocyanins and flavonoids, with other C6 superior alcohols and with isovalerianic acid. The second component was negatively correlated with ethyl esters, organic acids, C6 alcohols, sugar, pH, volatile acidity and lactic acid.

4.4 Discussion

The aim of this work was to carry out and to validate, for a microbiological and physical and chemical point of view, an innovative vinification protocol based on use of FFPC and to produce wines with quality comparable or higher than that obtained with standard vinification and with use of commercial starter. For this purpose we used two different type of pied de cuve, and we inoculated this pied de cuve into a new grape must using the pied de cuve like incolum of viable microorganism cells. The vinification was monitored by polyphasic approach: analysis of yeast and LAB populations and chemical and physical analysis of all main technological steps.

The results of the yeast counts were similar to those of a conventional vinification based on the inoculum of selected commercial starter as well as the results of experimental trial (A and B) based on use of FFPC were superimposable to that obtained from the trial C, the control. LAB concentration was also similar to data reported in literature (Francesca et al 2010; Bae et al 2006; Yanagina et al 2008). Taking into account these results, it was important to carry out the identification at species level of all yeasts isolated during the vinification process. The RFLP analysis assembled the yeasts into seven group (Tab.2), but only one of them was directly identified at specie level, while for the other groups was necessary the sequencing of domain D1/D2 26S rRNA gene because their restriction profiles were not reported in literature. This data confirmed the results obtained by other authors in terms of atypical polymorphisms (Fernandez-Espinar et al 2000; Kurtzman and Robnett 2003; Solieri et al 2007; Tofalo et al 2009). This molecular technique is most used for a

rapid identification at specie level of wine yeasts, therefore the discover of new polymorphisms could be useful for a rapid identification of yeast by RFLP analysis.

Yeasts species distribution is reported in Table 2. The grape berries showed a very low yeast diversity and *S. cerevisiae* was the unique species isolated during the initial phases of AF. The presence of this species at dominant level during the fermentation showed that in a vinification carried out by FFPC the species *S. cerevisiae* could reach high concentration thus ensure the microbiological and chemical stability of winemaking. During the vinification other yeasts species were found such as *P. guilliermondii* and *M. pulcherrima*; these species usually are present at higher concentration in the first phases of spontaneous fermentation (Di Maro et al 2007; Gonzales et al 2007; Zott et al 2008; Csoma and Sipiczki 2008). In theory, during a vinification obtained with spontaneous alcoholic fermentation we would expect a yeast species diversity (expecially for non-*Saccharomyces* group) greatly higher than that detected in our experimental thesis. This result confirm the microbiological stability of the innovative vinification protocol showed in the present study.

Several *S. cerecisiae* strains were found during the experimental vinifications. Although the trial A and B were carried out by FFPC, the alcoholic fermentation of these trials were spontaneously performed. With this regards, the number of indigenous strains detected in the trial A and B were significantly higher than that commonly reported in literature for the conventional spontaneous alcoholic fermentation. Furthermore, the number of *S. cerevisae* strains both in the trial A and B was significantly higher than that of the trial C. Thus, the use of FFPC in order to start a spontaneous alcoholic fermentation could represent an innovative strategy of

vinification to allow the grow of a high number of *S. cerecisiae* strains that could reach the highest concentration during the entire vinification process. The presence of several *S. cerevisiae* strains during wineaking is reported to improve the complexity of sensory profile of final product.

With regards to the results of chemical analysis, few differences were found on the evolution of several compounds in the three trials during the AF. Alcohol and glycerol contents were mainly detected in the trial A, on the other hand the volatile acidity of trial A and B was higher than that detected in the control. The pH reduction was observed in all trials during the alcoholic fermentation, this result was probably due to a higher extraction of organic acids respect to the cations (Ca, K, Mg). During the MLF there was an increase of pH due to the LAB activity, while the increase during maturation of wine was due to the precipitation of potassium bitartrate. When MLF started malic acid content decreased and the lactic acid was produced.

During the first two days of alcoholic fermentation there was the extraction of polyphenols, at racking their content was similar in all experimental trials and the polyphenol decrease was not significant during the vinification between trials.

Several volatile compounds were produced by yeasts from alcoholic fermentation to blotting. The increase of the fatty acid content during the first steps of alcoholic fermentation produced in this phase ethyl esters specially in the trial A in the first two days and in trial B after day 4 of alcoholic fermentation. Subsequently, during the vinification process the concentration of fatty acids and ethyl ester was the same in the three trials. The same behavior was reported for superior alcohols produced by

yeasts. The trial C during the alcoholic fermentation showed a low content of alcohols and acetates, in particular of the isoamyl acetate and fatty acids with short chain. Fatty acid esters reached the lowest concentrations when wines showed 3.5% of ethanol, after this value their contents were similar among the trials.

The values regarding C6 alcohols were similar in the all trials, while the highest concentration of fix acid esters was detected in the trial B. The superior alcohol concentration was different among the experimental trials: 1-propanol and isoamyl alcohol showed the highest concentration in the trial C; whereas isobutyl alcohol in the trials A and C. This result report the different activity of the yeasts during the alcoholic fermentation regarding the production of keto acids as conseguence of the protein synthesis.

The analysis of principal chemical component showed a clear separation among the trials during vinification, this separation is shown by their position in opposite point along the axis of the first and second components. Volatile compounds and chemical and physical composition is indicated by dispersion (fig. 2).

In conclusion this study provides for the first time a complete overview on microbial population during the vinification based on use of FFPC. The addition of ethanol into pied de cuve, before the beginning of the alcoholic fermentation, could allow the selective growth of yeasts with tolerance to ethanol, thus to promote the growth of yeasts with potential oenological aptitudes and to favorite the development of a high diversity of *S. cerevisiae* strains during the entire vinification process. Taking into account that up to day our analysis were carried out in one cellar only for the first year of experimentation, further investigations on yeast and LAB ecology of wines

obtained with FFPC will be performed in other cellars. Moreover, samples collected during the second year of experimentation (already realized) will be analyzed in order to further validate the innovative vinification protocol showed in the present research PhD thesis.

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Table 1 . Microbial loads[a] of samples collected during the pied de cuve preparation and during winemaking process of Nero d'Avola cultivar

Steps of pied de cuve preparation	Trial A				Trial B				Trial C			
	WL	MRS	GM17	MLO	WL	MRS	GM17	MLO	WL	MRS	GM17	MLO
Grape berries	4.60±0.10	n.d.	n.d.	n.d.	4.60±0.10	n.d.	n.d.	n.d.	4.60±0.10	n.d.	n.d.	n.d.
Must	5.44±0.15	n.d.	n.d.	n.d.	5.44±0.15	n.d.	n.d.	n.d.	5.44±0.15	n.d.	n.d.	n.d.
Ethanol addition[b]/inoculum starter[c]	5.22±0.10	n.d.	n.d.	n.d.	5.02±0.02	n.d.	n.d.	n.d.	6.48±0.19	n.d.	n.d.	n.d.
day1- AF	7.93±0.12	n.d.	n.d.	n.d.	7.55±0.10	n.d.	n.d.	n.d.	7.03±0.15	n.d.	n.d.	n.d.
day2- AF	8.23±0.13	n.d.	n.d.	n.d.	8.90±0.30	n.d.	n.d.	n.d.	8.56±0.16	n.d.	n.d.	n.d.
Steps of winemaking process												
Alcoholic fermentation:												
Grape berries	4.78±0.00	n.d.	n.d.	n.d.	4.78±0.00	n.d.	n.d.	n.d.	4.78±0.00	n.d.	n.d.	n.d.
Must	5.77±0.00	n.d.	n.d.	n.d.	5.77±0.00	n.d.	n.d.	n.d.	5.77±0.00	n.d.	n.d.	n.d.
Pied de cuve inoculum	7.20±0.04	n.d.	n.d.	n.d.	7.16±0.16	n.d.	n.d.	n.d.	6.74±0.47	n.d.	n.d.	n.d.
day1- AF	7.56±0.42	n.d.	n.d.	n.d.	7.85±0.41	n.d.	n.d.	n.d.	7.61±0.62	n.d.	n.d.	n.d.
day2- AF	8.13±0.02	n.d.	n.d.	n.d.	7.91±0.14	n.d.	n.d.	n.d.	8.16±0.09	n.d.	n.d.	n.d.
day4- AF	8.47±0.55	n.d.	n.d.	n.d.	8.02±0.13	n.d.	n.d.	n.d.	8.16±0.00	n.d.	n.d.	n.d.
day7- raking	8.06±0.22	n.d.	n.d.	n.d.	8.01±0.06	n.d.	n.d.	n.d.	7.97±0.14	n.d.	n.d.	n.d.
Ageing:												
day3- transfer	7.07±0.15	2.17±0.13	2.32±1.48	2.10±1.58	7.03±0.07	1.87±0.66	1.08±0.04	2,82±0.69	7.18±0.26	2.31±0.23	1.18±0.12	1.52±0.04
day10- MLF	6.51±0.07	3.48±0.00	3.48±0.00	3.52±0.01	6.59±0.07	3.48±0.00	2.73±1.05	3,13±0.65	6.80±0.40	3.48±0.00	1.91±0.25	3.04±0.43
day13- MLF	6.24±0.25	4.44±0.28	4.42±0.04	4.69±0.19	5.64±0.01	4.51±0.60	4.24±0.05	4,60±0.84	5.88±0.71	4.76±0.16	3.99±0.06	4.46±0.17
day17- MLF	5.60±0.12	6.51±0.14	6.14±0.08	5.53±1.18	5.34±0.16	6.64±0.70	5.99±1.75	5,94±0.91	5.21±0.40	5.49±0.98	6.09±0.35	4.98±0.67
day23- MLF	4.65±0.09	5.20±0.89	5.22±0.01	5.37±0.64	4.49±0.06	6.31±0.12	5.15±0.71	6,30±1.05	4.30±0.77	5.56±0.56	5.34±0.52	6.49±1.33
day30- MLF	4.70±0.05	4.40±0.53	4.78±0.04	4.86±0.46	4.52±0.45	5.47±1.59	4.71±0.31	5,81±0.75	4.60±0.23	5.78±0.71	4.90±0.02	5.86±0.38
Bottling	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

[a] Log CFU/g for grape berries; Log CFU/mL for must and wine samples; [b]only for Trial A and Trial B; [c]only for Trial C.
n.d., not detected (value < detection limit of method).
AF, Alcoholic Fermentation; MLF, Malolactic Fermentation

Table 2. Molecular identification and distribution of yeasts

R.P.	5.8S-ITS PCR[a]	Size of restriction fragment				Specie (% identity)	Distribution		
							Pied de cuve preparation	Vinification	
								Alcoholic fermentation	Ageing
		*Cfo*I	*Hae*III	*Hinf*I	*Dde*I				
I	400	210+90+80	260+90	210+190	n.d	*Metschnikowia pulkerrima* (97)	GB[A,B,C (4)[b]]; M[A,B,C (5)]	GB[A,B,C (4)]; M[A,B,C (5)]	T[B (7)]; d13[C (5)]; d17[A,B (5)]; d23[B (4)]
II	620	310+260	390+130+90	320+300	n.d	*Pichia giulliermondii* (98)		GB[A,B,C (4)]; M[A,B,C (5)]	d17[A,B,C (5)]; d23[A,B,C (4)]
III	750	320+310+100	n.c	350+200+130+70	380+160+90+60	*Hanseniaspora guilliermondii* (99)	GB[A,B,C (4)]; M[A,B,C (5)]; EA[A,B (5)]	GB[A,B,C (4)]; M[A,B,C (5)]	d10[B,C (6)]
IV	880	380+360+140	320+240+170+140	380+120+50	n.d	*Saccharomyces cerevisiae* (98)	EA[B (5),C (6)];d1[A,B,C (7)]; d2[A,B,C (8)]	M [A,B,C (5)]; PDI[A,B (7), C (6)]; d1[A,B,C (7)]; d2[A,C (8), B (7)]; d4[A,B,C (8)]; R[A,B,(8), C (7)];	T[A,B,C (7)]; d10[A,B,C (6)]; d13[A (6), B,C (5); d17[A,B,C (5)]; d23, d30[A,B,C (4)]

All values for the 5.8S-ITS PCR, 26S PCR and restriction fragments are given in bp.

Abbreviations: R.P., restriction profile; n.c., not cut; n.d., nort detected; A, Trial A; B, Trial B; C, Trial C; GB, Grape berries; M, must; EA, Ethanol addition; AF, Alcoholic Fermentation; R, Racking; T, transfer; MLF, Malolactic Fermentation; d, days of steps.

a According to BlastN search of D1/D2 26S rRNA gene sequences in NCBI database.

b The number reported between brackets refers to the highest concentration (Log cycle) of detection.

Table 3a. Chemical and polyphenols compounds of Trial A

	Trial A													
Compounds	**Pied de cuve preparation**				**Vinification**									
					Alcoholic fermentation				**Ageing**					
	Must	**Wine adding**	**day1**	**day2**	**day1**	**day2**	**day4**	**day7**	**day10**	**day13**	**day17**	**day23**	**day30**	**Bottling**
pH	3.32±0.00	3.35±0.01	3.37±0.01	3.20±0.00	3.26±0.01	3.14±0.01	3.00±0.01	2.98±0.01	3.01±0.01	3.020±0.01	3.04±0.01	3.09±0.01	3.29±0.03	3.33±0.11
TTA	6.71±0.00	5.73±0.10	6.37±0.03	7.47±0.07	8.45±0.03	8.61±0.01	8.48±0.09	8.15±0.01	8.09±0.08	8.06±0.04	7.86±0.06	7.22±0.07	7.20±0.04	7.05±0.08
VA	0.10±0.00	0.19±0.00	0.15±0.00	0.25±0.01	0.25±0.02	0.14±0.00	0.12±0.02	0.16±0.01	0.17±0.00	0.16±0.00	0.19±0.01	0.38±0.04	0.43±0.01	0.46±0.08
Reducing sugar ($g\ L^{-1}$)	216.01±0.00	180.27±1.30	185.49±0.95	49.40±3.63	135.18±4.56	46.66±0.43	1.44±0.23	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ethanol (% w/v)	n.d	1.77±0.07	1.71±0.06	9.34±0.17	4.47±0.30	9.07±0.02	11.90±0.18	11.93±0.17	11.91±0.18	11.90±0.18	11.90±0.18	11.90±0.18	11.90±0.18	11.96±0.10
Glycerol (g/L)	n.d	2.85±0.09	2.58±0.07	5.46±0.06	5.58±0.51	6.24±0.07	7.61±0.04	7.78±0.03	7.79±0.04	7.80±0.11	7.81±0.02	7.83±0.21	7.84±0.10	7.85±0.01
Malic acid (g/L)	n.d.	n.d.	n.d.	n.d.	1.53±0.04	1.12±0.04	1.05±0.01	1.04±0.01	1.08±0.01	0.85±0.04	0.16±0.00	0.14±0.03	0.13±0.03	0.12±0.09
Lactic acid (g/L)	n.d.	n.d.	n.d.	n.d.	0.55±0.11	0.65±0.09	0.52±0.01	0.48±0.02	0.51±0.01	0.67±0.04	1.15±0.04	1.17±0.07	1.18±0.03	1.20±0.08
Glucose/fructose	n.d.	n.d.	n.d.	n.d.	126.99±4.97	47.44±0.11	0.59±0.02	0.79±0.02	0.58±0.01	0.66±0.03	0.58±0.01	0.61±0.04	0.69±0.08	n.d.
Dry extract	n.d.	n.d.	n.d.	n.d.	154.12±4.64	79.17±0.27	30.55±0.36	29.76±0.09	29.86±0.09	29.96±0.09	29.83±0.01	29.05±0.09	28.86±0.06	28.66±0.08
Flavonoids	n.d.	n.d.	n.d.	n.d.	1381.14±101.96	n.d.	1720.1±78.66	1606.8±81.57	1699.5±171.88	1691.26±61.18	1693.32±5.83	1477.02±26.22	1458.48±29.13	1347.24±163.14
Anthocyanins	n.d.	n.d.	n.d.	n.d.	479.44±60.60	n.d.	422.44±25.73	372.72±38.88	369.48±56.03	362.61±8.58	326.63±2.29	256.29±5.72	224.36±4.00	210.21±30.87

Abbreviation: TTA, total titratable acidity (tartaric acid g/L); VA, volatile acidity (acetic acid g/L)

Table 3b. Chemical and polyphenols compounds of Trial B

Trial B														
Compounds	**Pie de cuve preparation**				**Vinification**									
					Alcoholic fermentation				**Ageing**					
	Must	**Wine adding**	**day1**	**day2**	**day1**	**day2**	**day4**	**day7**	**day10**	**day13**	**day17**	**day23**	**day30**	**Bottling**
pH	3.32±0.00	3.34±0.01	3.39±0.01	3.22±0.01	3.27±0.00	3.14±0.01	3.00±0.01	2.98±0.02	3.00±0.01	3.02±0.02	3.04±0.04	3.11±0.05	3.30±0.02	3.31±0.04
TTA	6.71±0.00	5.86±0.03	6.52±0.19	7.15±0.03	8.23±0.06	8.64±0.01	8.63±0.06	8.23±0.04	8.14±0.06	8.08±0.10	7.82±0.30	7.34±0.17	7.26±0.06	7.28±0.03
VA	0.10±0.00	0.18±0.00	0.35±0.02	0.29±0.03	0.23±0.01	0.14±0.00	0.14±0.00	0.16±0.01	0.17±0.00	0.17±0.01	0.19±0.03	0.36±0.11	0.43±0.04	0.45±0.08
Reducing sugar (g/L)	216.01±0.00	158.97±0.20	162.93±5.97	50.94±1.76	149.61±5.46	48.155±2.11	1.140±0.03	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ethanol (% w/v)	n.d	3.11±0.09	3.39±0.01	9.18±0.08	3.73±0.31	9.02±0.10	12.05±0.00	12.07±0.01	12.06±0.13.	12.05±0.14	12.08±0.06	12.07±0.01	12.07±0.12	12.09±0.08
Glycerol (g/L)	n.d	4.67±0.08	4.37±0.27	5.14±0.08	4.71±0.30	6.02±0.03	7.45±0.01	7.67±0.05	7.68±0.07	7.69±0.11	7.70±0.01	7.71±0.13	7.72±0.21	7.72±0.01
Malic acid (g/L)	n.d.	n.d.	n.d.	n.d.	1.53±0.01	1.20±0.00	1.09±0.01	1.04±0.01	1.07±0.02	0.76±0.33	0.18±0.04	0.17±0.03	0.16±0.01	0.15±0.09
Lactic acid (g/L)	n.d.	n.d.	n.d.	n.d.	0.49±0.04	0.70±0.00	0.52±0.01	0.47±0.01	0.54±0.02	0.73±0.23	1.20±0.06	1.19±0.12	1.18±0.01	1.17±0.21
Glucose/fructose	n.d.	n.d.	n.d.	n.d.	141.8±5.22	48.9±2.18	0.5±0.10	0.8±0.05	0.5±0.01	0.6±0.01	0.5±0.04	0.6±0.16	0.6±0.02	n.d.
Dry extract	n.d.	n.d.	n.d.	n.d.	169.19±5.54	80.44±2.04	31.37±0.04	30.11±0.16	30.14±0.12	30.20±0.04	30.08±0.26	29.41±0.10	29.11±0.06	29.01±0.15
Flavonoids	n.d.	n.d.	n.d.	n.d.	1320.46±37.87	n.d.	1862.24±58.27	1804.56±87.40	1645.94±26.22	1625.34±8.74	1680.96±46.61	1528.52±215.58	1507.92±5.83	1357.54±276.76
Anthocyanins	n.d.	n.d.	n.d.	n.d.	409.91±22.87	n.d.	429.31±2.29	406.27±6.29	352.10±4.00	345.23±2.29	320.57±8.58	284.59±12.58	231.64±4.00	223.15±13.72

Abbreviation: TTA, total titratable acidity (tartaric acid g/L); VA, volatile acidity (acetic acid g/L)

Table 3c. Chemical and polyphenols compounds of Trial C

Trial C														
Compounds	**Pie de cuve preparation**				**Vinification**									
					Alcoholic fermentation				**Ageing**					
	Must	**Wine adding**	**day1**	**day2**	**day1**	**day2**	**day4**	**day7**	**day10**	**day13**	**day17**	**day23**	**day30**	**Bottling**
pH	3.32±0.00	3.28±0.02	3.18±0.04	3.35±0.04	3.26±0.02	3.14±0.01	3.00±0.02	2.97±0.01	2.99±0.01	3.00±0.01	3.07±0.03	3.09±0.03	3.27±0.04	3.29±0.01
TTA	6.71±0.00	5.56±0.11	6.79±0.16	7.86±0.09	7.94±0.01	8.45±0.04	8.53±0.08	8.19±0.02	8.13±0.01	8.07±0.02	7.79±0.03	7.52±0.15	7.52±0.30	7.51±0.24
VA	0.10±0.00	0.11±0.03	0.21±0.01	0.32±0.01	0.22±0.01	0.11±0.00	0.09±0.01	0.10±0.01	0.12±0.00	0.11±0.00	0.16±0.06	0.19±0.07	0.23±0.07	0.24±0.08
Reducing sugar (g/L)	216.01±0.00	188.18±16.10	169.05±9.40	68.20±6.61	149.55±3.51	62.16±1.68	0.83±0.02	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ethanol (% w/v)	n.d	0.34±0.12	2.30±0.40	8.28±0.23	3.54±0.02	8.23±0.11	11.94±0.10	11.95±0.11	11.95±0.22	11.96±0.01	11.96±0.15	11.97±0.10	11.97±0.05	11.98±0.10
Glycerol (g/L)	n.d	0.57±0.10	3.33±0.02	5.90±0.14	4.61±0.20	5.65±0.01	7.37±0.06	7.66±0.16	7.67±0.15	7.68±0.19	7.69±0.13	7.70±0.16	7.71±0.11	7.71±0.03
Malic acid (g/L)	n.d.	n.d.	n.d.	n.d.	1.52±0.01	1.04±0.00	0.98±0.02	0.90±0.04	0.94±0.06	0.79±0.04	0.37±0.33	0.35±0.21	0.33±0.30	0.32±0.01
Lactic acid (g/L)	n.d.	n.d.	n.d.	n.d.	0.49±0.00	0.74±0.01	0.60±0.04	0.52±0.07	0.59±0.01	0.72±0.04	0.96±0.25	0.98±0.23	0.99±0.20	0.99±0.18
Glucose/fructose	n.d.	n.d.	n.d.	n.d.	141.62±3.41	62.69±1.41	0.42±0.10	0.72±0.09	0.57±0.10	0.58±0.04	0.49±0.11	0.45±0.05	0.54±0.01	n.d.
Dry extract	n.d.	n.d.	n.d.	n.d.	168.8±3.6	93.6±1.8	30.4±0.2	29.5±0.0	29.9±0.0	29.8±0.1	30.2±0.4	29.7±0.9	29.2±0.5	29.1±0.6
Flavonoids	n.d.	n.d.	n.d.	n.d.	1346.30±26.22	n.d.	1767.48±40.79	1672.72±11.65	1571.78±26.22	1584.14±32.05	1532.64±145.66	1516.16±75.75	1413.16±34.96	1345.18±32.05
Anthocyanins	n.d.	n.d.	n.d.	n.d.	390.91±10.86	n.d.	426.89±26.30	401.02±3.43	339.97±4.00	349.27±8.00	320.17±12.58	301.57±25.15	272.06±39.45	217.89±8.58

Abbreviation: TTA, total titratable acidity (tartaric acid g/L); VA, volatile acidity (acetic acid g/L)

Table 4a. Volatile organic compounds of Trial A

Compounds	Trial A					
	Fermentation				Ageing	Bottling
	day-1	day-2	day-4	day-7	day-30	
Isoamyl acetate*	77.20±109.17	618.71±46.80	640.13±143.97	661.56±68.41	462.64±78.00	310.53±90.61
2-phenyl-etil-acetatate*	41.55±20.18	63.98±1.03	59.63±26.69	62.13±1.72	55.00±1.58	43.73±2.15
Ethyl hexanoate*	62.19±10.06	184.85±0.89	162.92±35.61	252.17±29.49	188.90±11.60	125.64±36.28
Ethyl octanoate*	119.16±3.71	154.93±6.11	190.69±59.05	241.38±36.21	191.13±15.90	167.58±36.28
Ethyl decanoate*	87.95±57.26	63.33±31.15	44.27±7.02	40.61±2.39	52.90±6.15	47.29±7.22
Ethyl 9-decanoate*	2.89±4.09	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
3-ethyl -2-fenil-OH-propinoate*	14.33±20.27	143.75±9.13	158.07±77.14	227.51±68.49	219.10±10.64	389.83±2.51
Ethyl lactate*	122.17±19.44	25.27±7.85	50.41±1.10	62.05±3.95	595.00±9.86	957.17±75.55
Diethyl succinate*	132.97±1.32	222.75±6.00	312.54±35.73	471.31±74.86	1261.14±63.09	5915.44±155.58
Diethyl malate*	10.60±14.99	11.19±4.18	18.30±2.13	13.89±0.84	44.03±3.53	210.80±10.54
Monoethyl succinic acid*	547.66±76.90	380.03±16.89	212.40±60.79	227.41±32.18	4968.96±703.45	7366.82±530.63
Hexanol*	1160.56±91.90	1119.84±154.63	1079.12±104.65	1065.95±17.00	1052.79±72.77	1180.40±124.69
trans*-3-hexenol	11.19±1.73	10.14±2.92	3.75±5.30	8.18±2.08	8.99±0.01	3.92±5.55
cis*-3-hexenol	107.58±11.69	98.80±55.72	90.02±18.04	90.17±4.66	82.39±41.69	74.61±27.29
Isovalerianic acid*	158.62±26.10	138.20±50.61	117.78±34.48	131.81±28.73	130.30±5.20	93.74±4.51
Hexanoic acid*	699.07±80.96	1002.56±6.99	967.85±87.23	933.15±88.26	1098.89±10.30	1215.93±10.98
Octanoic acid*	1050.22±111.58	1385.26±31.62	1238.55±76.31	1078.21±53.81	1455.08±167.22	1683.63±41.26
Decanoic acid*	437.35±119.44	364.26±1.75	308.64±35.30	364.24±149.97	359.20±78.65	542.07±15.01
9-decenoic acid*	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Methanol**	113.82±9.04	173.32±27.31	171.34±8.08	161.47±2.58	167.08±4.29	167.08±4.29
Ethyl acetate**	9.43±4.33	11.40±16.11	22.02±9.11	30.16±5.00	38.30±5.06	28.24±12.80
1-propanol**	16.21±1.32	31.16±2.82	40.59±0.46	40.45±3.79	40.31±1.06	35.32±9.64
Isobutilyc alcohol**	14.32±1.81	32.83±7.50	44.84±0.49	44.91±20.40	44.98±0.01	34.50±14.11
Isoamylic alcohol**	54.90±3.45	210.06± 14.89	281.15± 3.11	277.73± 22.29	274.32± 3.18	252.34± 25.10

*, ug/L; **, mg/L

Table 4b. Volatile organic compounds of Trial B

Trial B						
Compounds	**Fermentation**				**Ageing**	**Bottling**
	day-1	**day-2**	**day-4**	**day-7**	**day-30**	
Isoamyl acetate*	128.71±47.40	482.76±36.38	493.37±150.30	503.97±47.98	381.37±63.30	260.63±5.76
2-phenyl-etil-acetatate*	6.78±9.59	71.86±12.20	68.38±10.00	64.90±13.06	55.67±3.85	74.88±36.29
Ethyl hexanoate*	57.95±3.72	137.13±23.75	211.41±51.68	218.57±31.82	81.66±21.52	102.42±7.92
Ethyl octanoate*	109.87±38.55	163.93±8.14	250.36±67.86	203.12±0.72	128.02±20.65	153.23±3.32
Ethyl decanoate*	18.01±5.66	33.68±9.05	49.73±8.87	47.78±7.80	41.05±7.47	49.42±0.31
Ethyl 9-decanoate*	0.00±0.00	0.00±0.00	3.29±4.65	1.43±2.02	1.06±1.49	40.42±14.70
3-ethyl -2-fenil-OH-propinoate*	20.73±9.11	110.86±2.98	147.70±69.87	218.60±84.47	215.89±23.45	398.67±97.56
Ethyl lactate*	221.60±155.92	45.85±3.55	68.53±19.00	127.00±52.84	621.28±69.43	1514.23±409.59
Diethyl succinate*	295.13±0.77	404.80±62.69	335.45±134.19	583.88±102.66	1342.93±281.65	5602.25±361.82
Diethyl malate*	18.02±10.77	10.28±14.53	10.76±7.71	23.45±1.14	51.27±3.55	244.27±9.49
Monoethyl succinic acid*	875.72±271.91	247.34±77.62	151.26±66.90	372.07±59.65	5563.41±1057.41	8281.01±392.19
Hexanol*	1129.59±8.88	1093.96±28.48	1058.33±20.54	1120.25±10.04	1073.99±34.90	1062.93±35.19
trans*-3-Hexenol	12.39±0.36	8.88±1.66	8.86±0.20	7.96±0.88	8.07±2.46	6.81±0.95
cis*-3-Hexenol	102.20±4.81	76.34±1.11	74.50±14.84	91.00±6.40	94.83±14.82	98.67±12.23
Isovalerianic acid*	198.86±1.75	161.11±80.45	123.35±29.57	158.22±31.93	135.18±36.19	157.23±15.75
Hexanoic acid*	615.36±161.75	945.92±81.51	1233.30±391.48	929.41±57.28	1051.71±56.10	1125.40±122.38
Octanoic acid*	878.46±140.29	1330.38±196.05	1754.70±961.15	962.23±36.27	1281.78±122.14	1546.37±246.50
Decanoic acid*	256.05±35.22	382.57±84.64	552.02±436.33	295.05±50.24	281.67±35.70	236.11±256.98
9 decenoico acid*	0.00±0.00	0.00±0.00	24.81±35.09	6.58±9.30	6.73±0.49	6.88±9.72
Methanol**	183.60±4.92	180.65±32.38	177.71±39.90	179.91±18.36	182.11±4.76	182.11±13.05
Ethyl acetate**	9.65±1.74	24.47±3.34	27.66±11.00	30.85±6.07	20.37±18.36	5.67±1.61
1-Propanol**	22.46±0.26	34.13±4.98	40.85±1.34	44.02±13.05	43.55±2.01	36.70±0.65
Isobutilyc alcohol**	16.30±0.91	37.16±2.71	40.81±6.95	40.48±0.73	43.06±3.54	25.54±2.22
Isoamilyc alcohol**	68.67±0.17	229.67± 19.04	286.40± 16.37	283.45± 2.21	280.50± 7.65	237.27± 14.53

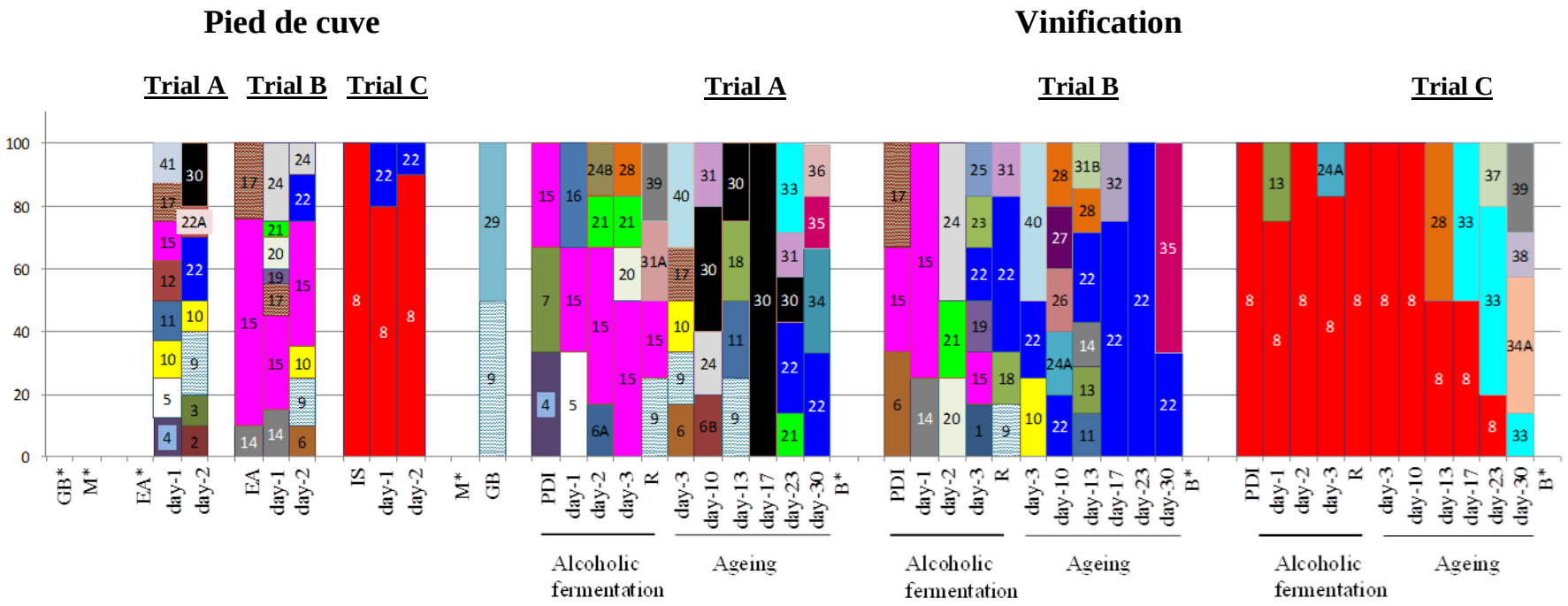
*, ug/L; **, mg/L

Table 4c. Volatile organic compounds of Trial C

Trial C						
Compounds	**Fermenation**				**Ageing**	**Bottling**
	day-1	**day-2**	**day-4**	**day-7**	**day-30**	
Isoamyl acetate*	124.37±8.60	473.26±211.08	516.65±170.34	560.04±47.94	489.90±4.56	290.13±106.35
2-phenyl-etil-acetatate*	13.84±0.00	43.76±12.98	67.00±29.07	98.03±57.48	60.04±0.79	98.10±1.29
Ethyl hexanoate*	28.42±5.58	148.69±12.29	240.64±16.08	214.78±21.06	119.82±0.53	125.97±15.74
Ethyl octanoate*	33.16±9.72	141.44±4.72	171.82±55.06	197.05±47.94	160.58±1.42	144.01±0.19
Ethyl decanoate*	16.28±8.48	53.86±41.89	38.85±10.41	59.03±15.22	35.69±15.29	32.25±11.11
Ethyl 9-decanoate*	0.00±0.00	0.00±0.00	1.53±2.17	0.00±0.00	0.00±0.00	0.00±0.00
3-ethyl -2-fenil-OH-propinoate*	13.46±4.18	135.44±43.18	197.81±36.96	256.77±229.40	273.71±2.27	345.03±26.40
Ethyl lactate*	11.51±5.60	44.05±13.30	54.80±7.24	81.58±2.31	403.46±26.38	1536.68±157.87
Diethyl succinate*	18.67±16.33	138.14±100.46	401.67±170.13	683.30±135.53	964.93±123.97	4344.77±906.55
Diethyl malate*	20.07±1.08	16.70±6.82	14.33±9.07	10.34±3.36	51.52±16.18	261.95±113.59
Monoethyl succinic acid*	37.23±21.28	221.61±117.42	212.26±45.41	239.05±4.57	5952.59±1374.12	7179.75±828.71
Hexanol*	1077.32±31.67	1131.28±143.14	1139.19±60.44	1090.31±17.23	1124.54±62.97	1165.67±135.84
trans*-3-hexenol	11.26±0.09	12.09±3.44	7.83±1.71	7.86±0.50	8.89±0.93	10.52±0.52
cis*-3-hexenol	115.33±5.44	103.91±7.23	90.66±9.63	92.14±3.03	94.12±1.67	114.87±8.18
Isovalerianic acid*	201.26±18.77	177.09±29.27	152.91±31.00	202.72±12.07	187.48±12.59	210.21±15.65
Hexanoic acid*	597.47±222.66	1136.90±69.02	922.91±127.35	1022.58±58.59	1160.26±28.41	1070.18±60.63
Octanoic acid *	831.99±360.56	731.28±1034.19	981.50±82.61	1086.57±77.46	1377.60±282.16	1330.43±4.56
Decanoic acid*	286.09±83.08	385.19±166.00	233.13±23.47	270.24±47.77	336.37±146.46	411.56±20.43
9-decenoic acid*	11.01±15.57	6.17±8.73	24.17±1.42	18.07±2.41	9.45±13.36	0.00±0.00
Methanol**	151.01±42.41	133.68±2.67	148.60±4.49	163.53±29.54	187.27±9.01	187.27±10.12
Ethyl acetate**	4.00±1.73	10.85±4.49	21.39±5.14	32.36±0.08	24.97±1.63	17.58±8.15
1-Propanol**	13.04±2.84	31.12±2.16	48.06±0.08	47.69±6.65	47.33±1.21	46.05±2.02
Isobutilyc alcohol**	14.65±3.375	37.25±1.54	40.18±13.71	47.73±3.23	46.10±2.93	42.04±6.55
Isoamilyc alcohol**	74.84±13.29	247.95±1.12	311.84±10.00	329.30±17.21	331.20±17.37	317.01±13.01

*, ug/L; **, mg/L

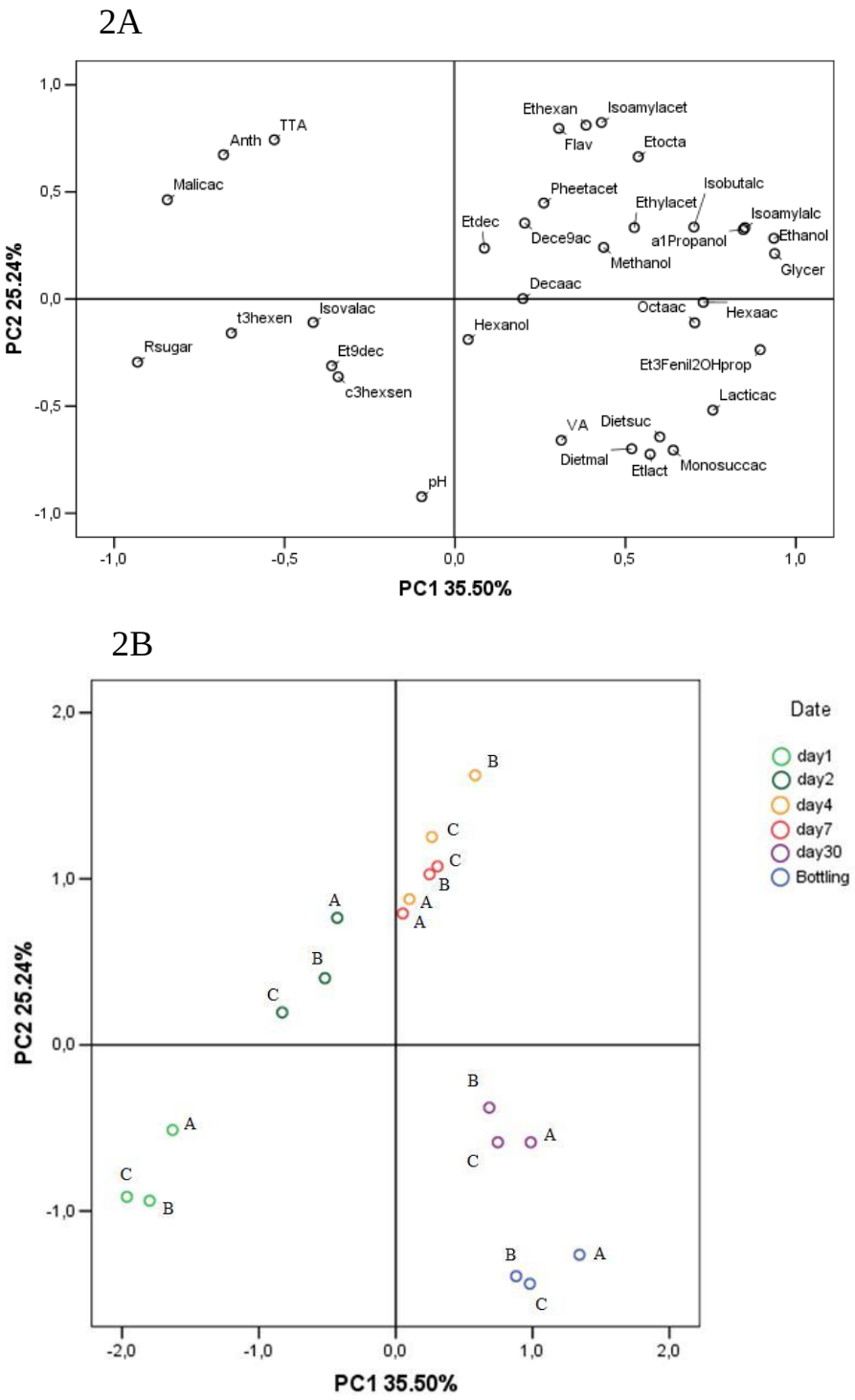
Fig 1. Distribution of *Saccharomyces cerevisiae* strains during pied de cuve preparation and vinification of Nero d'Avola



Symbols:

*, samples were not isolated *Saccharomyces cerevisae* strains; Gb, Grape berries; M, must; EA, Ethanol Addition; IS, Inoculum Starter; PDI, Inoculum of pie de cuve; R, raking; B, bottling

Figure 2. Analysis of principal chemichal compounds and VOCs



2A) Rappresentation of variables of first two components.

2B) Rappresentation of Trials A, B and C related the first two components

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