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Yeast population diversity on grapes during on-vine withering and their dynamics in natural and inoculated fermentations in the production of icewines

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Abstract

In this study, attention has been focused on microbial ecology, during an on-vine withering period of 120 days, of Mondeuse grapes, an autochthonous variety cultivated in the Western Alps in Europe, and on their fate during alcoholic fermentation. Laboratory fermentations have been followed in order to describe the yeast dynamics in both spontaneous and inoculated fermentations by means of culture-dependent and independent methods (PCR-DGGE). The ability of *Saccharomyces cerevisiae* to take over the fermentation has also been assessed by means of minisatellites analysis. The chemical composition has been evaluated on the final products. A biodiversity of the grapes has been detected during the withering period, despite the predominance of *Aureobasidium pullulans* and *Rhodotorula glutinis* thus empathising that withering conditions could affect yeast populations.

The spontaneous laboratory fermentation was characterized by a predominance of *Hanseniaspora uvarum*, *Metschnikowia fructicola* and *S. cerevisiae*. In the inoculated ones, counts of *S. cerevisiae*, belonging to the inoculated starter culture, became predominant after 7 days, even though the PCR-DGGE analysis showed the starter profile for the entire fermentation period.

Finally, when the *S. cerevisiae* isolates were characterized by the minisatellites analysis, it was determined that the inoculated culture was responsible for the alcoholic fermentation. As far as spontaneous fermentation is concerned, the autochthonous *S. cerevisiae* showed a good ethanol production yield, but left a high concentration of residual sugars.

Keywords: icewine, yeast ecology, grape withering, culture- dependent and independent methods

1. Introduction

Icewines, made from grapes that are left on the vine under cold weather conditions that allow the grapes to freeze, are typical dessert wines in which the freezing of grapes causes changes in their sugar concentration, pH, titratable acidity, ionic strength, viscosity, osmotic pressure and vapour pressure, as well as in their surface and interfacial tensions (Fennema, Powrie & Marth, 1973).

The environmental conditions necessary for production are only achieved naturally in a few countries, e.g. Canada, Germany and Austria and, in recent years, in China (Tian et al., 2009). In Italy, the climatic conditions suitable for the dehydration of grapes and the production of icewine are only found in mountain environments (Rolle, Torchio, Giacosa, & Gerbi, 2009). The production parameters for authentic icewines are in fact set by the International Organization of Vine and Wine and, according to these regulations, harvesting and pressing should be performed at a temperature below or equal to -7°C , to obtain juice which should have a minimum °Brix value of 25.3 prior to fermentation (OIV, 2008a).

During the pressing of the frozen grapes, much of the water is retained within the grape skins as ice, while a juice that has high concentrations of sugars, acids and aroma compounds is extracted (Nurgel, Pickering, & Inglis, 2004). The alcoholic fermentation of the must usually ends when there is still a considerable amount of residual sugar present, which imparts the sweetness of the wine.

Because of the considerable interest shown by consumers, in recent years different studies have been focused on the sensory and chemical characteristic of these kinds of sweet wines (Cliff, Yuksel, Girard, & King, 2002; Nurgel et al., 2004; Kilmartin, Reynolds, Pagay, Nurgel, & Johnson, 2007; Soleas & Pickering, 2007) but, to the authors' knowledge, very little is known about the mycobiota present on the grapes during the withering period and in the grape must and their relative contributions to wine fermentation. The microbial communities on grapes may be affected by several factors, including the high osmotic pressure generated during withering. Climatic conditions, including the effect of temperature, UV exposure, rainfall, sunlight and winds, influence

the diversity and quantity of microbial populations (Barata, Malfeito-Ferreira, & Loureiro, 2012; Chamberlain, Husnik, & Subden, 1997). The importance of the presence of autochthonous populations on grapes during fermentation is well known and has been reported in various studies (Barata et al., 2012; Fleet, 2003).

In this paper, a typical icewine production has been simulated, considering Italian weather conditions. In particular, attention has been focused on yeast ecology, during an on-vine withering period, of Mondeuse grapes, an autochthonous variety from the Savoy mountain region in France, which has recently been introduced into the Susa Valley, in the Italian Western Alps. Laboratory fermentations have been investigated in order to describe the yeast populations involved in both spontaneous and inoculated fermentations. A culture-dependent approach, based on plating and the molecular identification and characterization of *S. cerevisiae* isolates, has been employed together with culture-independent methods using Polymerase Chain Reaction- Denaturing Gradient Gel Electrophoresis (PCR-DGGE) on nucleic acids extracted from the fermenting must. The total DNA and RNA were extracted directly from each sampling point and PCR-DGGE was carried out to establish the composition of the mycobiota during the fermentation. Finally, in order to evaluate the ability of inoculated starters to take over the natural yeast microbial communities and conduct the fermentation, *S. cerevisiae*, isolated from the starter cultures and during the fermentations, has been subjected to minisatellites analysis.

2. Material and methods

2.1. Sampling during grape withering and must fermentation

Natural on-vine grape withering occurred on the Mondeuse black cultivar winegrapes (*Vitis vinifera* L.) in winter in an experimental vineyard located at 750 m a.s.l. in Chiomonte (the Susa Valley, Piedmont, NW Italy, 45°7'28.29"N 6°58'59,51"E), during 2007. The environmental climatic

conditions (temperature, relative humidity, rain) of the vineyard were monitored using a Vantage PRO2 weather station (Davis Instruments, Hayward, CA, USA).

The withering process was considered to have started when the grape juice sugar concentration reached about 180 g/L, and it continued for about 120 days until both a sugar concentration and temperatures suitable for the grape ($< -7^{\circ}\text{C}$) were reached. Three samples of 200 randomly picked grape berries were selected, placed in sterile stomacher bags and transported to the laboratory in order to monitor the withering process, which started on 19 September. The monitoring was conducted every 3 weeks. The grapes were pressed inside the stomacher bag and the obtained grape juice was subjected to chemical and microbiological analysis.

After 120 days, at the end of the withering process, about 10 kg of grapes were picked and subdivided into three replicates. The harvest was carried out in the early morning and the berries were transported and pressed frozen. Different sets of laboratory fermentations were conducted, each performed in triplicate and in 250 ml flasks containing 100 ml of must at 25°C in static conditions. One trial was conducted spontaneously (S fermentation), while two different *Saccharomyces cerevisiae* active dried yeasts (ADY) were added (initial inoculum of about 10^6 cells/ml final concentration) for the others. The ADY were Ba11 (BA fermentation) and the EC1118 (EC fermentation), both of which came from Lalvin, Lallemend (Montreal, Canada).

Samplings were performed on grape juices and at 1, 2, 5, 7, 14 and 21 days of fermentation. At each sampling point, aliquots of fermenting must (1 ml each) were collected for chemical analysis and DNA and RNA extraction. The aliquots for DNA and RNA extraction were centrifuged for 5 min at 13,400 rpm, the supernatant was removed and 0.2 ml of RNA later (Ambion, Milan, Italy) was added to the RNA aliquot.

2.2. Traditional microbiological analysis

The grapes, after being crushed in a sterile bag during dessication using a stomacher machine, and the musts, were serially diluted in a Ringer solution (Oxoid Milan, Italy) and plated on a

Wallerstein Laboratory Nutrient medium (Oxoid, Milan, Italy), which is able to differentiate yeast populations on the basis of the colour and morphology of the colony (Urso et al., 2008). In the case of inoculated fermentations, a Lysine medium (Oxoid, Milan, Italy), which is selective towards non- *Saccharomyces* yeasts, was also used. The plates were incubated at 25°C for 4 days. After counting, the means and standard deviations were calculated.

During grape withering, at least 10 yeast colonies from each sampling point were selected and isolated. In the case of the must fermentations, 15 colonies were randomly isolated from each sample in order to obtain a representative yeast population during the fermentations. Regarding inoculated fermentations, 10 colonies were isolated from Lysine Agar and 5 from WL. Five colonies were also isolated from the starter cultures used in the study for comparison purposes. Purification was carried out on WL and the pure cultures were stored at -80°C in yeast-peptone-dextrose broth (YPD, 2% (wt/vol) glucose, 2% (wt/vol) peptone and 1% (wt/vol) yeast extract, all of which came from Oxoid (Milan, Italy) after the addition of glycerol (30%) (Sigma-Aldrich, Milan, Italy).

2.3. Chemical monitoring of the grape dehydration and must fermentation

The reducing sugars, titratable acidity and pH were monitored during the withering process using official OIV methods (OIV, 2008b). The glucose, fructose, glycerol, ethanol, malic and acetic acid contents in the fermented musts were quantified using an HPLC system (P100-AS3000, Thermo Electron Corp., Waltham, MA), equipped with a UV detector (UV3000) set to 210 nm, and a refractive index detector (RI-150). The analyses were performed isocratically at 0.8 mL/min and 65°C with a cation-exchange column (Aminex HPX-87H, 300x7.8 mm internal diameter) equipped with a Cation H⁺ Microguard cartridge (Bio-Rad Laboratories, Hercules, CA), using 0.0026 N H₂SO₄ as the mobile phase (Giordano, Rolle, Zeppa, & Gerbi, 2009).

2.4. DNA extraction from the pure cultures and from the musts

Genomic DNA was extracted from 1 millilitre of an overnight culture in YPD broth (Oxoid, Milan, Italy) of each isolate and centrifuged at 13,400 rpm for 10 min at 4°C in order to pellet the cells. The pellet was subjected to DNA extraction, according to Cocolin, Bisson, & Mills (2000).

As far as the nucleic acid extractions taken directly from the must are concerned, the DNA and RNA were extracted as described in Rantsiou et al. (2013) using the DNeasy Plant Mini Kit (Qiagen, Italy) and the Concert Plant RNA reagent (Invitrogen, Italy), respectively. The DNA was quantified using a Nanodrop ND-1000 spectrophotometer (Celbio, Milan, Italy) and standardized to 100 ng/μl. Re-suspended RNA was treated with Turbo DNase (Ambion, Italy), in order to eliminate the DNA. Complete DNA digestion was confirmed using 1 μl in PCR and, in those cases in which a product was obtained, the treatment was prolonged until a negative PCR reaction was obtained from all the RNA samples.

2.5. PCR and RT-PCR amplification

The DNA extracted from the pure cultures, grape juice and fermenting musts was amplified with NL1 (5'- GCA TAT CAA TAA GCG GAG GAA AAG-3') and LS2 (5'- ATT CCC AAA CAA CTC GAC TC-3') primers, as reported by Cocolin et al. (2000). A GC-clamp (5'- CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3') was attached to the NL1 primers for DGGE analysis, as described by Sheffield, Cox, Lerman, & Myers (1989). PCR reactions were performed in a final volume of 25 μL, containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM of deoxynucleoside triphosphates (dNTP), 0.75 U of Taq Polymerase (Applied Biosystems, Milan Italy), 0.2 μM of each primer and 100 ng of template DNA. Amplifications were carried out in a PTC-200 DNA Engine MJ Research thermal cycler (Biorad, Milan, Italy), as described in Cocolin et al. (2000). Reverse transcription (RT) reactions were performed using the M-MLV reverse transcriptase (Promega, Milan, Italy). Five hundred ng of RNA was mixed with 1 μL of 100 μM of the LS2 primers, and sterile water for a final volume of 10 μL and incubated at 75°C for 5 min. The mix was placed on ice and a mixture containing 50 mM Tris- HCl (pH 8.3), 75

mM KCl, 3 mM MgCl₂, 10 mM DTT, 2 mM of each dNTP, 1 µL of 200 U/µL M-MLV and 0.96 units of RNasin ribonuclease inhibitor (Ambion) was transferred to the reaction tube. The reverse transcription was carried out at 42°C for 1 h, and 1 µL of the obtained complement DNA was added to the PCR reaction mix.

2.6. DGGE analysis

The Dcode universal mutation detection system (Biorad, Milan, Italy) was used for DGGE analysis. The PCR products were analyzed by means of DGGE, as described by Cocolin et al. (2000).

The PCR products were applied to a 8% (wt/vol) polyacrilamide gel (acrylamide-bis acrylamide 37:5:1) with a denaturing gradient of 30 to 50% (Cocolin et al., 2000) in a 1X TAE buffer (2 M Tris base, 1 M glacial acetic acid, 50 M EDTA [pH 8]). The gels were subjected to a voltage of 130 volt for 4 h at 60°C, stained for 20 min in 1X TAE containing 1x SYBR Green I (Sigma) and then analysed under UV using UVI pro platinum 1.1 Gel Software (Eppendorf, Hamburg, Germany).

2.7. Sequencing of the DGGE bands obtained from the DNA and RNA analysis of the musts

Selected DGGE bands were extracted from the gels, using sterile pipette tips, transferred into 50 µL sterile water and incubated overnight at 4°C. Two µL of the eluted DNA was re-amplified using the conditions described above and checked by means of DGGE. The PCR products that gave a single band, co-migrating with the DNA/RNA control, were then amplified with the same primers without a GC clamp and sequenced by MWG Biotech, Ebersberg, Germany. The sequences were aligned in Gene Bank using the Blast Program.

2.8. Molecular identification of the isolates and characterization of S. cerevisiae

The DGGE profiles of the isolates were grouped and representatives of each group were amplified with NL1/ NL4 primers (Kurtzman & Robnett, 1997) to amplify the partial 26S rRNA gene. The PCR products were sent to a commercial facility (MWG Biotech Ebersberg, Germany) for

sequencing and the resultant sequences were aligned with those in Gene Bank using the Blast program (Altschul, Gish, Miller, Myers, & Lipman, 1990) to determine the known relatives.

Isolates identified as *S. cerevisiae* were subjected to minisatellites analysis. Amplification reactions were carried out using forward DAN4 (5'-AGC GCT TTC AAA GGA TGG TAT TTA CA-3') and reverse DAN4 (5'-AAA GTA GAC CCG AAG GAA GAA ACA GG-3') primers, as previously described by Marinangeli, Angelozzi, Ciani, Clementi, & Mannazzu (2004). The minisatellite-PCR products were separated by electrophoresis on 1.5% (w/v) agarose gels in a 1X TBE buffer at 120V for 2 h. Pictures of the gels were digitally captured using UVIpro Platinum 1.1 Gel Software (Eppendorf), and BioNumerics Software (Applied-Maths, Sint-Martens-Latem, Belgium) was used for the pattern analysis. The calculation of the similarities of the band profiles was based on the Pearson product moment correlation coefficient. Dendrograms were obtained by means of the unweighted pair group method, using an arithmetic average (UPGMA) clustering algorithm (Vauterin & Vauterin, 1992).

3. Results

3.1. Grape withering process

The environmental conditions registered during the grape withering process are reported in Figure 1. At the final date, as in many other days during the month of January, the minimum temperatures were below -7°C, thus meeting harvest requirements. A regular distribution of rainfall was observed, and, in the last 10 days, snow was present on the grapes. At the end of the withering process, the sugar content of the grape juice was higher than 25.3 °Brix, as required for the production of this type of wine (Figure 2). A decrease in acidity was observed during the slow dehydration, although a clear trend was not evident. The must used in the laboratory fermentations contained 147 g/L of glucose, 162 g/L of fructose and 4.8 g/L of glycerol. The pH was 3.15 and titratable acidity was 6.2 g/L, expressed as tartaric acid. The tartaric, malic and citric acid contents

were 3.12, 1.50 and 0.60 g/L, respectively.

3.2. Yeast counts during grape freeze-withering and during fermentation

The yeast plate counts remained pretty stable during the withering period, with about 4 log₁₀ colony forming units (cfu)/ml. It was interesting to note that an increase of 1 log₁₀ cfu/ml occurred from day 93 after which the counts remained constant until the end of the freeze-withering period (data not shown).

The grapes were collected after 120 days of withering and used to prepare the juice for the inoculated and spontaneous fermentations. The counts of the total and non- *Saccharomyces* yeast, from grape juice and during the 21 days of fermentation are reported in Table 1. The yeast counts in the grape juice were around 5 log₁₀ cfu/ml. The evolution of the two inoculated fermentations was very similar until day 14, but at the last sampling point (day 21) the EC fermentation showed a reduction in the yeast counts from 8 to 7 log₁₀ cfu/ml, while the BA fermentation reached values of 9 log₁₀ cfu/ml. The spontaneous fermentation from the initial 5 log₁₀ cfu/ml reached a similar value to the inoculated fermentations after 5 days. The yeast colonies on the Lysine medium (Table 1) were detected until day 7 in both inoculated fermentations, but already at day 5 the counts started to decrease.

3.3. Molecular identification of the yeast isolates during grape withering and fermentations

During the withering period, 166 isolates were identified. Table 2 reports the evolution of the yeasts on the grapes during the withering. A greater biodiversity emerged at the beginning of desiccation. Apart from *Aureobasidium pullulans*, which represented about 45% of the isolates, the remaining share consisted of *Rhodotorula glutinis*, and this was followed by *Kazachstania unispora*, *Hanseniaspora uvarum*, genus *Metschnikowia* with the species *M. fructicola* and *M. pulcherrima*, and *Pseudozyma graminicola*. *A. pullulans* showed the highest percentage over the entire withering period, and, until day 30, represented 40% of the isolates, reaching 70% at day 63

and only decreasing at the last sampling point. Similar results were observed for *R. glutinins* which, despite the lower number of isolates, was isolated at all six sampling points. *Cryptococcus uzbekistanensis* showed a higher prevalence at day 49 and represented 10% of the total isolates, while *Cryptococcus terrestris* was isolated in the middle of the withering period, from day 17 to day 63. *Torulaspora delbrueckii* and *Kazachstania unispora* constituted minor populations during the monitoring period. *Hanseniaspora osmophila*, and *Candida zemplinina* were only isolated at the end of the freeze-withering period. *M. fructicola* was isolated until day 30, but it again prevailed at the last sampling point.

300 isolates were randomly selected from the WL and Lysine plates and were subjected to molecular identification. The number and percentage of yeast isolates among the samplings are reported in Table 3. In the spontaneous fermentation, *H. uvarum* represented 41% of the total isolates and it was the most abundant species together with *S. cerevisiae* (33%) and *M. fructicola* (18%). As far as the inoculated fermentations are concerned, *S. cerevisiae* reached values of 56% in the BA and 39% in the EC fermentations during all the fermentations, while the second isolated species was *H. uvarum*. Both fermentations showed heterogeneity of the minor species with a predominance of *M. fructicola* in the EC fermentation.

Figure 3 presents the evolution, over time, of the yeast species diversity during fermentation. Concerning the spontaneous process (panel A), *H. uvarum* and *M. fructicola* were dominant until day 7, then decreased at day 14 and disappeared at the end of fermentation. *S. cerevisiae* appeared on the second day, but only at day 14 did it represent 60% of the isolates. It decreased slightly at day 21, when *H. osmophila* and *C. zemplinina* both reached values of 54%.

In the case of inoculated fermentations (panels B and C), *S. cerevisiae* dominated the fermentation and it was present from day 1 until the end of the fermentations. Non *Saccharomyces* yeasts were present until day 7, with a high share and dominance of *H. uvarum*. *C. zemplinina* was also found in the BA fermentation at the end of the fermentation.

3.4. Characterization of *S. cerevisiae* isolates by means of minisatellites analysis

As far as the spontaneous fermentation is concerned, 33 *S. cerevisiae* isolates were subjected to minisatellites analysis, with the amplification of gene DAN4, followed by cluster analysis using a coefficient of similarity of 70%. As reported in Figure 4 (panel A), the isolates showed a certain level of variability. It is possible to differentiate 4 clusters. Cluster 1 contains 70% of the total isolates from the entire fermentation period. Clusters 2, 3 and 4 have a smaller number of isolates and clusters 3 and 4 only feature a few isolates at the beginning of fermentation, thus showing their possible low competition power. Regarding the inoculated fermentations (Fig. 4, panels B and C), a major cluster was detected, with 90% and 75% of the total *S. cerevisiae* isolates, for the BA and EC fermentations, respectively. The main cluster also included the isolates from the starter cultures, thus highlighting the capability of the inoculated strains to carry out the fermentation process.

3.5. DGGE analysis of the fermentations

The total DNA and RNA were extracted and analysed by means of PCR-DGGE. No differences were observed between the three fermentation replicates (data not shown) and for this reason the profiles of only one has been presented.

The DNA and RNA gels are shown in Figure 5, while the results of the band identification are presented in Table 4. Variability during the fermentation period was observed at a DNA level (panel A) in the spontaneous fermentations. *A. pullulans* and *Cryptococcus victoriae* were found in the grape must together with *H. uvarum*, whose presence was observed until the end of fermentation. *S. cerevisiae* only appeared at day 14. As far as the RNA is concerned (panel B), the presence of the species detected in the grape must was confirmed over the entire sampling period: *H. uvarum* and *A. pullulans* were detected as at the DNA level, but *S. cerevisiae* was not detected. The RNA of *Vitis aestivalis* emerged as band I. Only the profile of the starter emerged for the inoculated fermentations from the beginning to the end of the fermentation at both a DNA and an RNA level. No other species were found in the DGGE analysis (data not shown).

3.6. Chemical analysis of the fermentations

The chemical composition of the wines at the end of the fermentations is shown in Table 5. The ethanol yield was the same in all the fermentation trials: although this can be considered an important result for the spontaneous fermentation, the fermentation behavior should be attributed to the ethanol production and residual sugar concentration, which it was highly different from trial to trial. The spontaneous fermentation showed a rather good ethanol yield, but the complete sugar transformation was scarce, leaving more than 130 g/L in the must and less than 10 % v/v produced ethanol. The BA inoculated fermentation also left more than 37 g/L (about 12 % of the starting must content) of sugars in the must, but 15.4 % of alcohol was reached. All the samples showed differences in the acidic composition, with the citric, tartaric and malic acid concentrations showing the most relevant differences and highest variability between repetitions. The acetic acid production was lower in the inoculated fermentations, especially in the EC inoculated trials. Glycerol production was higher in the inoculated fermentations.

4. Discussion

This study provides an overall picture of the analysis of the yeast communities in grapes during winter withering and of the subsequent fermentation, conducted with grape juice obtained after 120 days of on-vine dehydration. The natural drying of grapes is a complex process that induces several changes in the chemical - physical characteristic of the berries. The grapes are left on the vine for a long period during which the berries progressively wither, losing their water content and consequently provoking a different chemical content of the must, which becomes richer in sugars with a substantial change in the phenolic and aromatic composition (Kilmartin et al., 2007; Rolle et al., 2009).

As reported by different authors, grapes together with the winery environment, are the primary source of yeast biota (Fleet, 2003). In this study, the evolution of yeasts on grapes has been

described at maturation throughout withering, from September until the middle of January, a period that is characterized by changing weather conditions and decreasing temperatures. A multiphasic approach has been used to investigate the ecology of the yeasts during the withering period and in the wine fermentations.

The WLN medium has proved useful for wine yeast differentiation, since colonies of different species grow with a distinguishable morphology and color (Pallmann et al., 2001). During the withering period, the predominant species was *A. pullulans*. This result is in agreement with previous studies which showed that *A. pullulans* is the main species isolated from immature, mature and both damaged and undamaged grapes (Prakitchaiwattana, Fleet, & Heard, 2004), as it is an ubiquitous species found mainly in soil, water and wood, as well as in osmotically stressed environments (Gunde-Cimerman, Zalar, de Hoog, & Plemenitas, 2000). On the basis of the classification of different authors (Loureiro & Malfeito-Ferreira, 2003; Malfeito-Ferreira, 2011), it can be included in the innocent species category as it is a common, yeast-like, technologically irrelevant species which does not have the ability to spoil wine. The second species isolated during the withering period, as far as the number of isolates is concerned, was *R. glutinis*. The behaviour of this species at low temperatures, together with another 12 commonly found yeast species in Riesling grapes grown in the Niagara peninsula, Canada, was studied in a previous study where, after 20 days, a decrease of 1 log₁₀ was observed, after exposing the grapes to -25°C (Chamberlain et al., 1997). Here, *A. pullulans* and *R. glutinis* were detected for all of the 120 days of withering, thus underlining their capacity to resist on grapes at low temperature and during the other weather conditions registered in this season. A decrease in the temperature was detected during the monitored withering period after the first 60 days, when a temperature of 0°C was reached. The temperature then remained around or under 0°C until the end of the withering period.

S. cerevisiae was not isolated at any sampling point during withering. As soon as the fermentation started, *A. pullulans* and *R. glutinis* disappeared, in correspondence to the development of other species, such as *M. fructicola* and *H. uvarum*, weakly fermentative ascomycetous species that are

present on the grapes at the end of withering period when a temperature of -7°C was reached, and which are able to survive during the first days of fermentation. The minor species were represented by the *Cryptococcus* genus with *C. terrestris* and *C. uzbekistanensis* species, *K. unispora*, *T. delbrueckii*, *P. graminicola* which were randomly isolated during the withering; this emphasizes the high biodiversity in the vineyard during the long monitoring period. Some species were only found in specific withering days, thus indicating a possible influence of the environmental variability or differences in susceptibility to freeze-desiccation (Chamberlain et al., 1997). *C. zemplanina* appeared at the last withering sampling with only one isolate. It was detected in a low percentage during the fermentations, but its presence underlines the capacity of this species to resist in high osmotic stress conditions (Sipiczki, 2004). It was isolated at day 21 in the spontaneous fermentation, while, in the inoculated ones, it was present at days 7 and 21 and in the middle of fermentation in the BA and EC trials, respectively. It is interesting to note that the ethanol content was 15.4 % v/v at day 21 in the BA fermentation. The presence and the resistance of *C. zemplanina* in high sugar fermentations could offer an important contribution to the alcoholic fermentation and production of sweet wine, due to the capacity of this species to control acetic acid production through *S. cerevisiae*, as reported in (Rantsiou et al., 2013).

ADY only took over the fermentation process at day 14 in the inoculated fermentations: until day 7, the colony counts highlighted a high share of *H. uvarum*, which was found randomly during the withering period but with high prevalence levels throughout all the fermentations. In this respect, the growth and survival of *H. uvarum* might not be suppressed easily by the inoculated *S. cerevisiae* (Mora, Barbas, & Mulet, 1990). In the spontaneous fermentation process, it remained until day 14, and disappeared at the end of fermentation, when *H. osmophila* become predominant together with *S. cerevisiae*.

As previously reported, culture-dependent methods were associated with culture-independent methods, through which the RNA and DNA extracted directly from the musts were analysed. RNA and DNA analysis were performed, as it is known that DNA persists long after microorganism

death while RNA is easily degraded after cell death, thus allowing the live and metabolically active microorganisms to be recognised (Cocolin, Campolongo, Alessandria, Dolci, & Rantsiou, 2011). The results obtained by means of the DGGE analysis correlated partially with those from the plate counts. In the spontaneous fermentation, *H. uvarum* was detected at both DNA and RNA levels and its activity was observed until day 21. This aspect was in agreement with the results of the plating from which it emerged that *S. cerevisiae* was not able to predominate and, at the end of fermentation, as much as 50% of the total isolates did not belong to *Saccharomyces* yeasts. *A. pullulans* was only observed in the grape juice from the plates, but in the DGGE analysis, it was found at a DNA level until day 2 and surprisingly throughout the entire fermentation at an RNA level. This result is in contrast with other studies in which *A. pullulans* was found to be one of the main species isolated from grapes (Prakitchaiwattana et al., 2004), but not during fermentation. *S. cerevisiae* was only detected by means of DGGE at day 14 of fermentation, while its presence was identified from the plates from the second day. This evidence could be explained considering that the detection limit of DGGE is about 10^3 cfu/ml (Cocolin et al., 2000).

Regarding the inoculated fermentations, DGGE at both DNA and RNA only showed the starter profiles, while different results emerged from the culture dependent methods. As described above, 50% of the isolates from the plates were represented by minor species, such as *H. uvarum*, *M. fructicola* and *C. zemplinina*, until day 7, thus underlining the time needed for *S. cerevisiae* to take over the native population, as already reported in Urso et al., (2008) and Rantsiou et al., (2013). Non-*Saccharomyces* yeasts were only detected in the plates because of the Lysine agar that was used, which is a synthetic medium with glucose, vitamins, inorganic salts and lysine as the sole nitrogen source, where *Saccharomyces* spp. are unable to grow (Angelo & Siebert, 1987; Di Maro, Ercolini, & Coppola, 2007). During inoculated fermentations, the yeast diversity detected by means of PCR-DGGE was significantly lower than that found by isolation and further identification; this could be explained by the masking effect produced by the nucleic acids from the starter cultures, which prevented amplification of any other yeasts during fermentation. As reported by different

authors, both culture-dependent and-independent approaches should be employed to obtain a complete picture of the dynamic changes in the main yeast populations during fermentation (Cocolin et al., 2011; Di Maro et al., 2007).

In order to study *S. cerevisiae* intraspecies variability in spontaneous fermentation and to verify the capability of ADY to dominate the inoculated fermentations, minisatellites analysis was carried out. As described by Marinangeli et al. (2004), gene DAN4 shows a good level of resolution for the separation of *S. cerevisiae* strains. In spontaneous fermentation, a main cluster composed of 24 isolates was observed from the beginning to the end of fermentation, thus underlining its capability to conduct and dominate the fermentation. In both of the inoculated fermentations, one major cluster was composed of isolates obtained from the starter and from the entire fermentation period to the end of fermentation, thereby indicating the ability of the inoculated culture to dominate the native *Saccharomyces* population in both fermentations.

The results of the chemical analysis have shown how the yeasts in the spontaneous fermentation had a similar ability to produce ethanol from sugar as the inoculated ones, although an incomplete sugar transformation was observed. However, this aspect should not be considered negative for this type of wine; a high residual sugar content is in fact typical of icewines (Soleas & Pickering, 2007; Cliff et al., 2002; Nurgel et al., 2004) and other passito wines (Giordano et al., 2009; Urso et al., 2008). The BA inoculated fermentation left more than 37 g/L (about 12 %) of sugar in the must, but it reached 15.4 % of alcohol, which is high for icewines (Soleas & Pickering, 2007; Cliff et al., 2002; Nurgel et al., 2004). The EC fermentation also reached high ethanol values (16.6 % v/v), but only 3.1 g/L of the sugars remained. These data underline the correct course of the fermentation, although the residual sugars are far too low for an icewine. Therefore, the use of ADY yeasts might imply the need to stop the alcoholic fermentation when the desired residual sugar concentration is reached. The spontaneous fermentation showed higher levels of acetic acid than the inoculated fermentations, but which were lower than those reported in scientific literature for industrial products (Soleas & Pickering, 2007; Nurgel et al., 2004).

To the authors' knowledge, this is the first time mycobiota have been studied throughout the grape withering process in winter conditions for a period of 120 days on the vine. A recent paper has focused on the yeast populations that are present on grapes during an Erbaluce grape withering process in a typical room, called *fruttaio*, without control conditions (Rantsiou et al., 2013). A succession of yeast populations was reported, underlining the possibility of withering having a possible affect on yeast populations.

The wide heterogeneity and biodiversity that have emerged in this study have shown that icewine fermentation is driven by complex mycobiota, and that the environmental conditions that characterize the withering process promote an ecological niche which evolves in the microbial ecology of high sugar grape musts, thus influencing the yeast ecology during the following fermentation process.

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Table 1. Microbial counts on WLN and Lysine media in grape juice and during the 21 days of fermentation, expressed as the means of \log_{10} cfu/ml \pm standard deviation of the three independent repetitions.

WLN	Grape juice	1	2	5	7	14	21
S	5.45 \pm 0.03	5.61 \pm 0.10	6.73 \pm 0.11	7.62 \pm 0.10	7.43 \pm 0.30	8.11 \pm 0.10	8.30 \pm 0.04
EC	5.45 \pm 0.03	7.15 \pm 0.09	7.71 \pm 0.03	7.84 \pm 0.12	7.31 \pm 0.35	8.39 \pm 0.57	7.36 \pm 0.59
BA	5.45 \pm 0.03	7.13 \pm 0.08	7.61 \pm 0.04	7.67 \pm 0.04	7.31 \pm 0.38	8.33 \pm 0.74	9.08 \pm 0.46
Lysine							
EC		3.99 \pm 0.23	4.40 \pm 0.17	3.86 \pm 0.07	1.54 \pm 0.09	<10	<10
BA		4.07 \pm 0.42	4.50 \pm 0.13	4.49 \pm 0.02	1.92 \pm 0.36	<10	<10

Table 2. Molecular identification of yeasts isolated during the withering period. The prevalence of each species for each sampling point is presented as a % of the single sampling points and of the total isolates.

	Day 0		Day 17		Day 30		Day 49		Day 63		Day 93		Day 120			
	19/09/2007		05/10/2007		18/10/2007		06/11/2007		20/11/2007		20/12/2007		16/01/2008		Total	
	N.	%	N.	%	N.	%	N.	%	N.	%	N.	%	N.	%	N.	%
<i>Aereobasidium pullulans</i>	9	45.0	12	40.0	9	36.0	17	56.7	21	70.0	8	61.5	4	22.2	80	49.7
<i>Pseudozyma graminicola</i>	1	5.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	1	0.6
<i>Cryptococcus terrestris</i>	0	0.0	1	3.3	1	4.0	0	0.0	3	10.0	0	0.0	0	0.0	5	3.1
<i>Hanseniaspora osmophila</i>	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	1	5.6	1	0.6
<i>Hanseniaspora uvarum</i>	0	0.0	5	16.7	3	12.0	0	0.0	0	0.0	0	0.0	4	22.2	12	7.3
<i>Cryptococcus uzbekistanensis</i>	0	0.0	1	3.3	2	8.0	9	30.0	1	3.3	3	23.1	0	0.0	16	9.9
<i>Rhodotorula glutinis</i>	6	30.0	2	6.7	7	28.0	4	13.3	5	16.7	1	7.7	2	11.1	27	16.8
<i>Kazachstania unispora</i>	2	10.0	7	23.3	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	9	5.6
<i>Rhodotorula mucilaginosa</i>	0	0.0	0	0.0	1	4.0	0	0.0	0	0.0	0	0.0	0	0.0	1	0.6
<i>Metschnikowia fructicola</i>	1	5.0	1	3.3	1	4.0	0	0.0	0	0.0	0	0.0	6	33.3	9	5.6
<i>Torulaspora delbrueckii</i>	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	1	7.7	0	0.0	1	0.6
<i>Metschnikowia pulcherrima</i>	1	5.0	1	3.3	1	4.0	0	0.0	0	0.0	0	0.0	0	0.0	3	1.9
<i>Candida zemplinina</i>	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	1	5.6	1	0.6
Total isolates for each sampling point	20	100.0	30	100.0	25	100.0	30	100.0	30	100.0	13	100.0	18	100.0	166	100.0

Table 3. Molecular identification of yeasts isolated during the spontaneous and inoculated fermentations. The prevalence of each species is presented as a % of the total isolates.

	Grape juice		S		BA		EC	
	N.	%	N.	%	N.	%	N.	%
<i>Saccharomyces cerevisiae</i>	0	0.0	33	33.0	47	56.0	42	39.3
<i>Metschnikowia fructicola</i>	6	40.0	18	18.0	3	3.6	14	13.1
<i>Hanseniaspora osmophila</i>	1	6.7	6	6.0	1	1.2	5	4.7
<i>Hanseniaspora uvarum</i>	4	26.7	41	41.0	29	34.5	39	36.4
<i>Candida zemplinina</i>	1	6.7	2	2.0	4	4.8	4	3.7
<i>Rhodotorula graminis</i>	2	13.3	0	0.0	0	0.0	2	1.9
<i>Aureobasidium pullulans</i>	1	6.7	0	0.0	0	0.0	1	0.9
Total	15	100.0	100	100.0	84	100.0	107	100.0

Table 4. Identification of the PCR-DGGE bands retrieved from Gene Bank

Band ^a	Closest relative	% identity	Source ^b
A	<i>Aureobasidium pullulans</i>	99%	FM212450.1
B	<i>Cryptococcus victoriae</i>	99%	EU287882.1
C	<i>Hanseniaspora uvarum</i>	100%	EU359819.1
D	<i>Saccharomyces cerevisiae</i>	98%	AB435076.1
E	<i>Saccharomyces cerevisiae</i>	99%	EU386725.1
F	<i>Hanseniaspora uvarum</i>	99%	EF116914.1
G	<i>Hanseniaspora uvarum</i>	100%	HQ149311.1
H	<i>Aureobasidium pullulans</i>	99%	GU585248.1
I	<i>Vitis aestivalis</i>	98%	AF274672.1

^a Bands numbered as indicated on the DGGE gels shown in Fig. 5

^b Accession number of the sequence closest relative found with the BLAST search

1 **Table 5.** Chemical composition of the wines at the end of the fermentations.

Fermentation	Glucose (g/L)	Fructose (g/L)	Ethanol (% vol.)	Glycerol (g/L)	Acetic acid (g/L)	Citric acid (g/L)	Tartaric acid (g/L)	Malic acid (g/L)	Yield (g ethanol/g consumed sugar)
S	57.4±2.6b	75.6±3.7c	9.93±0.45a	11.2±0.6a	0.63±0.03c	0.12±0.04a	2.79±0.53b	1.02±0.05a	0.45±0.04
BA	2.33±0.04a	35.3±0.5b	15.4±0.0b	13.8±0.1b	0.57±0.03b	0.39±0.03b	1.70±0.12a	1.46±0.01c	0.45±0.00
EC	1.37±0.09a	1.77±0.56°	16.6±1.0b	13.0±0.9b	0.34±0.01a	0.57±0.03c	1.16±0.13a	1.21±0.09b	0.43±0.03
<i>Sig.^a</i>	***	***	***	**	***	***	**	***	ns

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3 All values are expressed as triplicate fermentations averages ± standard deviation (n=3). The different Latin letters within the same column indicate significant differences (^a) among the samples
4 (*Tukey-b* test; $p < 0.05$). ^a: **, *** and ns indicate significance at $p < 0.01$, 0.001 and no significance, respectively. Fermentation yield calculated using 309 g/L as initial glucose and fructose sum
5 concentration.

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Figure legends

Fig. 1. Main climatic conditions corresponding to the on-vine grape withering period.

Fig. 2. Changes in the pH, titratable acid, glucose and fructose concentrations during grape withering. The mean and standard deviation of the chemical analysis performed on triplicate samples are shown for each sampling point.

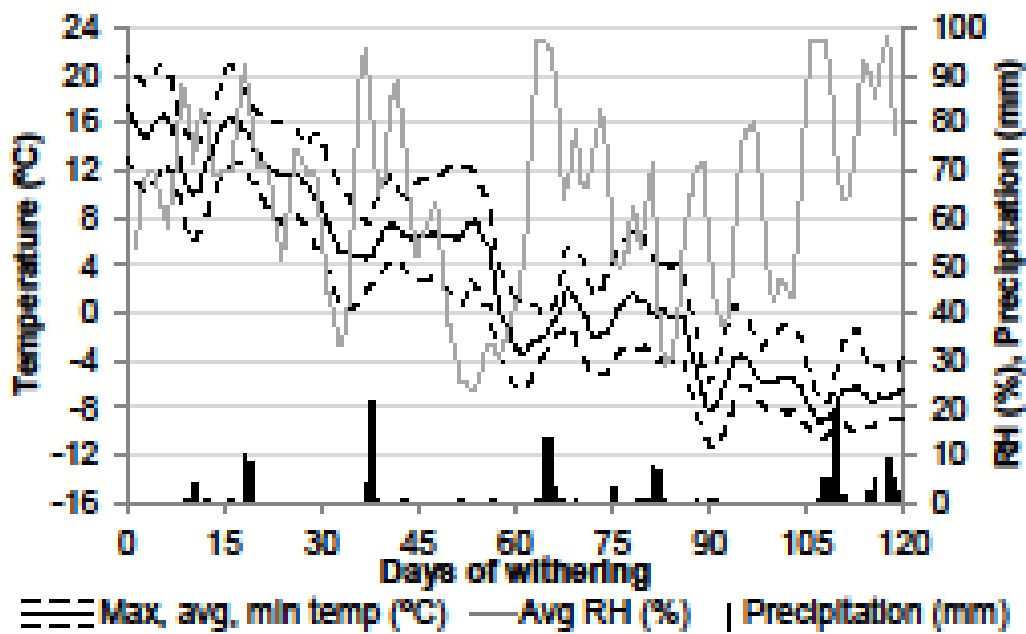
Fig. 3. Prevalence of yeast species among isolates randomly selected on the WLN and Lysine agar plates during the spontaneous (panel A), BA (panel B) and EC (panel C) fermentations.

Fig. 4. DAN4 minisatellites cluster analysis of *S. cerevisiae* isolated during the spontaneous (panel A), BA (panel B) and EC (panel C) fermentations.

Fig. 5. DGGE profiles of the DNA (panel A) and RNA (panel B) extracted directly from the grape juice (Gj) at the end of withering and after 1, 2, 5, 7, 14 and 21 days of fermentation. The bands indicated by letters were excised and after re-amplification (as described in Material and Methods) subjected to sequencing. The identification of the bands is reported in Table 4.

23 Fig. 1

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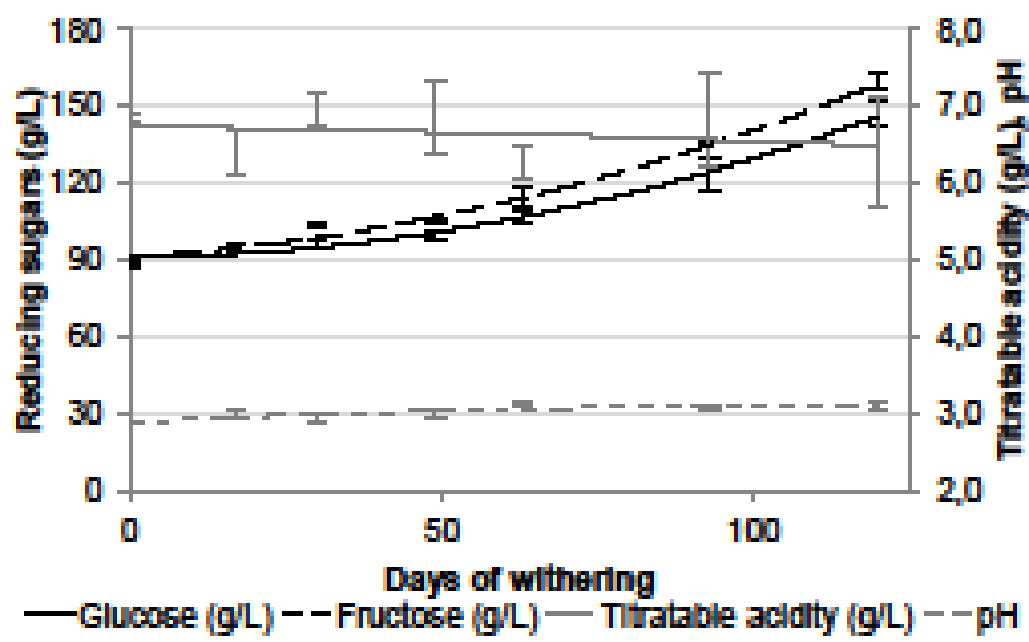
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41 Fig. 2

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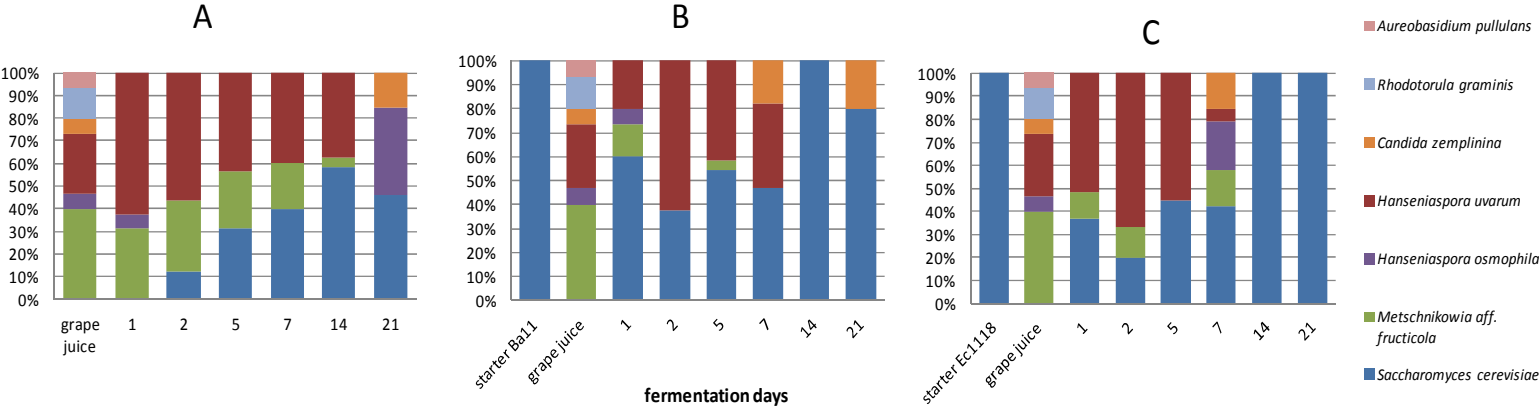
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1 **Fig. 3**

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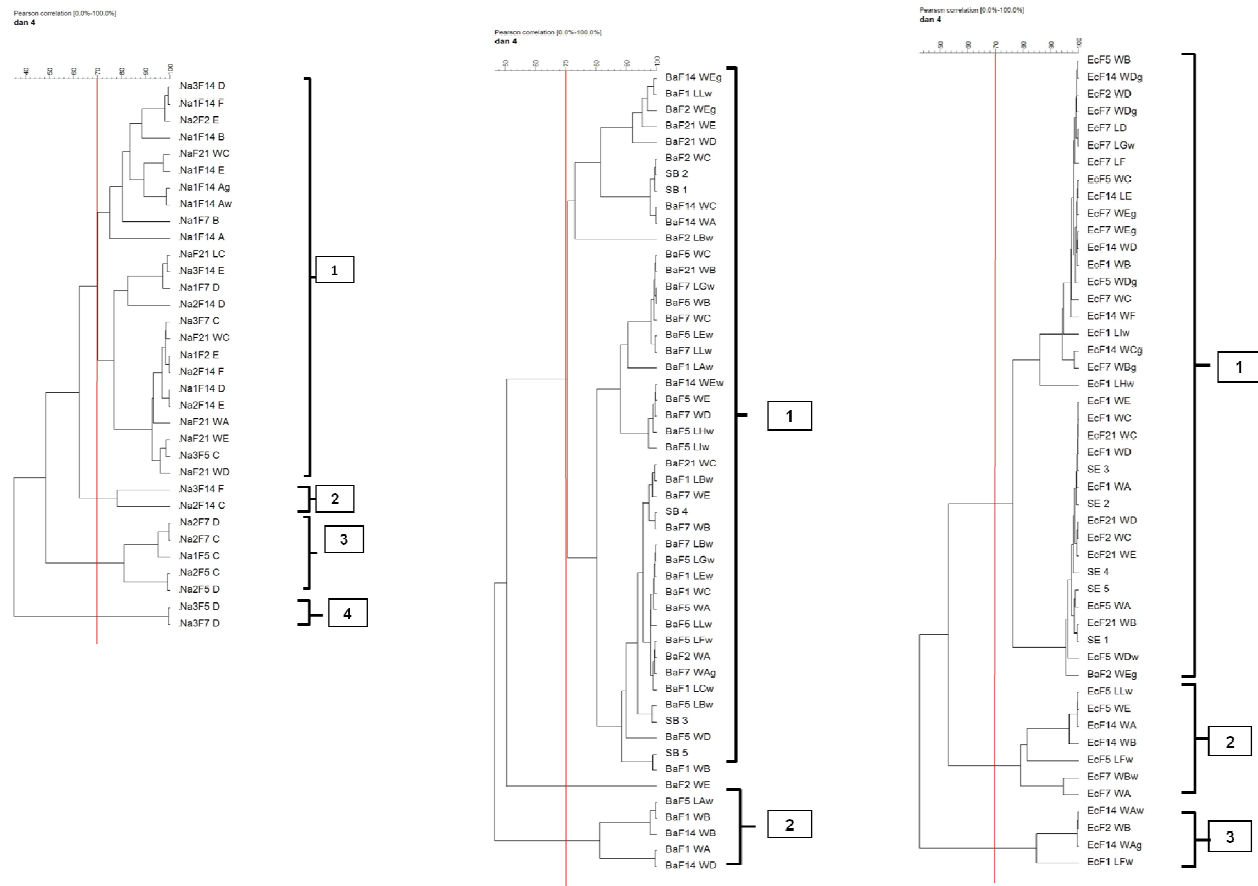
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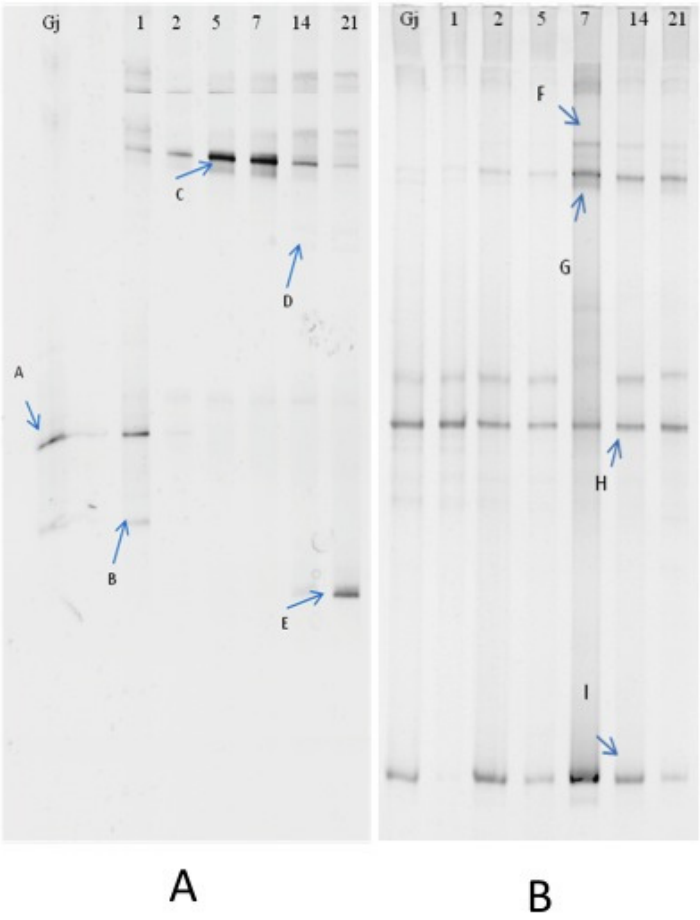
14 **Fig. 4**

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1 **Fig. 5**



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