Yeast populations associated with grapes during withering and their fate during alcoholic fermentation of high sugar must

UNIVERSITÀ DEGLI STUDI DI TORINO

19 **Yeast populations associated with grapes during drying and their fate during alcoholic**
20 **fermentation of high sugar must**
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Abstract

Background and Aims: Grape mycobiota may be a determining factor for the population dynamics that develop during alcoholic fermentation for the production of wine. For sweet wine fermentations, high sugar content grape musts are employed that represent complex microbial ecosystems. The focus of this study, the Passito di Caluso, is a sweet wine produced in the North of Italy from grapes harvested in the fall and subjected to a withering process.

Methods and Results: The withering process was studied by sampling and microbiological analysis, while the alcoholic fermentation was followed by both culture–dependent and culture–independent approaches. During the withering process we observed a succession of three yeast populations associated with grapes. A high degree of species biodiversity was detected the last day of the monitoring period. The dominance of *Saccharomyces cerevisiae* in the inoculated fermentation was confirmed.

Conclusions: A succession of yeast populations was observed during grape withering; species, such as *Candida zemplinina*, *Metschnikowia fructicola* and *Hanseniaspora uvarum*, were also detected during alcoholic fermentation. Autochthonous *C. zemplinina* populations could play an important technological role in sweet wine production.

Significance of the Study: The grape mycobiota during withering was described and its fate during alcoholic fermentation determined by molecular identification methods.

Keywords: *yeast ecology, grape withering, high sugar fermentation*, Candida zemplinina, *PCR-DGGE*

Introduction

Passito wines, a particular typology of sweet wines, are produced from dehydrated grapes with high sugar concentration, and achieved through a drying or withering process, which can

be on-vine or off-vine. For the off-plant dehydration, mature grapes are harvested and subjected to drying either directly in the sun or in rooms, under controlled (temperature, relative humidity, air flow) or ambient conditions. Apart from the concentration of sugars, other physicochemical characteristics of the grapes are altered, contributing to the characteristic profile of these sweet wines (Rolle et al. 2011). Further, a contribution in the dehydration process can be provided by the development of *Botrytis cinerea*, in the form of 'noble rot'. In this last case, the resulting botrytised wines are enriched with aroma compounds produced by *B. cinerea* (Magyar 2011). The environmental conditions that develop during withering process create a particular ecological niche that may apply a selective pressure and determine the microbial ecology that evolves in the grape must. Potential problems, associated with sweet wine production, may be the sluggish initiation of fermentation (Bisson 1999) and the increased acetic acid production, due to *Saccharomyces cerevisiae* osmotic stress (Erasmus et al. 2003).

The main sources of yeast biodiversity in spontaneous alcoholic fermentation are represented by the grape ecosystem and the resident microbiota in the winery environment. The importance of the grape microbiota, especially in the first stages of the alcoholic fermentation, is well documented (Fleet 2003, Prakitchaiwattana et al. 2004, Barata et al. 2012). This aspect connects well with a new trend arising in winemaking, represented by the use of cultures of *S. cerevisiae* mixed with other non-*Saccharomyces* species. This strategy has been proven to add complexity to the final product or improve fermentation performance (Bely et al. 2008, Ciani et al. 2010, Rantsiou et al. 2012,). The study of the grape microbiota and of the evolution of yeast populations during subsequent alcoholic fermentation provides valuable information regarding species distribution and persistence. It is the first step in evaluating potential candidates for development of cultures, of autochthonous origin, ideally adapted in specific environments, such as high sugar content musts.

With this perspective, in this study we focused on the microbiological aspects of the withering process of Erbaluce grapes. This variety is autochthonous to the Piedmont region of Italy and is used for the production of the traditional Passito di Caluso DOCG (Denomination of Origin Controlled and Guaranteed) wine (Rolle et al. 2012). Withering takes place in the autumn—winter months, in relatively cold conditions. An alcoholic fermentation was followed in order to describe the main yeast populations involved. For this purpose we employed a culture-dependent approach, based on plating and molecular identification of isolates and a culture–independent approach. Polymerase Chain Reaction Denaturing Gradient Gel Electrophoresis (PCR-DGGE) was applied on DNA and RNA extracted directly from fermenting must, in order to detect the yeast populations present (DNA) and metabolically active (RNA) during the fermentations.

Materials and methods

Grape withering

Grape clusters of the Erbaluce white cultivar (*Vitis vinifera* L.) from a vineyard located in Caluso (Piedmont, NW Italy) were carefully harvested in the 2007 season at a soluble solids content (SSC) of 25°Brix. About 10000 kg of grapes were placed in perforated boxes (60 × 40 × 15 cm, 6 kg of grapes in each box) in a single layer. For the natural, off-vine dehydration process, the boxes were placed inside a typical room called *fruttaio* without control of temperature, relative humidity or air flow (ambient conditions). The fruit was dehydrated for 139 days, from 12 September 2007 to 29 January 2008 (autumn—winter thermohygrometric drying conditions) in accordance with the Erbaluce di Caluso DOCG wine production rules (Rolle et al. 2012). The participating winery in this study undertakes one dehydration process per year on the grapes destined for the production of Passito di Caluso; thereby one batch was followed in this work.

During this off-vine withering period, grapes were sampled approximately every 15 days (0, 17, 30, 49, 63, 93, 124 and 139 days after harvest). At each sampling time, three lots of about 500 sound berries were sampled randomly, placed in sterile stomacher bags and transported to the laboratory within an hour. The grapes were manually crushed inside the stomacher bag, and the grape juice obtained was subjected to chemical and microbiological analysis.

Must fermentation and sampling

At the beginning of February 2008, the dried grapes were ready for vinification. They were crushed resulting in a must with a pH value of 3.23 and a titratable acidity of 8.12 g/L expressed as tartaric acid. ,. The must was fermented in triplicate (approximately 1200 L per replicate) by a winery in the Caluso area; a starter culture (Lalvin EC118, Lallemand, Montreal, Canada) was added and the fermentation lasted 14 days. Before inoculation of the starter culture, 25 mg/L SO_2 was added. The fermentation was conducted at a controlled temperature of 23±1°C and was sampled at 1, 3, 7 and 14 days. At each sampling point 50 mL of the must and fermenting must were collected in sterile screw cap tubes and stored at 4°C during transportation to the lab. Microbiological analysis were carried out as described below. In addition, at each sampling point, aliquots (1 mL each) of the samples were collected for chemical analysis, and extraction of DNA and of RNA. The aliquot destined for chemical analysis was filtered through a 0.2 μm filter and stored at -20°C until analysis. The aliquots destined for DNA and RNA extraction were centrifuged for 5 min at 13400 rpm, the supernatant was removed and for the RNA aliquot, 0.2 mL of RNA*later* (Ambion, Applied Biosystems, Italy) were added. Pellets prepared in this way were stored at -20°C.

Microbiological analysis

For microbiological analysis, serial dilutions in Ringer's solution were prepared and plated on WLN medium (for grapes during withering and must during fermentation), a differential medium on which yeast colonies can putatively be identified based on their topomorphology (Pallman et al. 2001, Urso et al. 2008), and on Lysine medium (for must during fermentation) to count non-*Saccharomyces* (Oxoid, Italy). Plates were incubated at 30°C for 5 days and subsequently counted.

Chemical analysis

Glucose, fructose, malic acid, glycerol, ethanol, and acetic acid of grape juices and fermenting musts, were quantified by means of an HPLC (Thermo Electron Corp., Waltham, MA, USA) equipped with a UV detector (UV100), set to 210 nm, and a refractive index detector (RI-150). The analyses were run isocratically at 0.8 mL/min and 65°C on a cation-exchange column (300 mm by 7.8 mm inner diameter; Aminex HPX-87H) fitted with a Cation H^+ Microguard cartridge (Bio-Rad Laboratories, Hercules, CA, USA), using 0.0026 N H_2SO_4 as the mobile phase (Giordano et al. 2009).

Molecular identification of isolates

At each sampling point during the grape withering process and the fermentation, yeast colonies (at least 10) were randomly selected from the WLN medium, isolated and 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 from Oxoid, Milan, Italy), with glycerol (30% final concentration). For the molecular identification, isolates were grown in YPD Broth, their DNA was extracted, amplified with primers NL1GC/LS2 and subjected to DGGE Cocolin et al. (2000) (as described below). Isolates were grouped according to their DGGE profile, and representatives of each group were amplified with primers NL1/NL4 (Kurtzman and Robnett

161 1997) to obtain a PCR product, which was sequenced by a commercial facility (Eurofins,
162 Edersberg, Germany). Identification of each group was based on a BLAST search (Altschul et
163 al. 1990) of the sequence obtained on National Center for Biotechnology Information. Both
164 primer pairs targeted the D1-D2 loop of the 26S rRNA gene.
165
166 *Nucleic acids extraction from must*
167 The protocols described in Mills et al. (2002) were followed for extraction of DNA and RNA
168 from must; DNA was quantified with a Nanodrop ND-1000 spectrophotometer (Celbio, Milan,
169 Italy) and standardised to 100 ng/μL. In order to eliminate DNA traces from the preparation,
170 RNA was resuspended in 50 μL of water containing the Turbo DNase (Ambion, Milan, Italy).
171 Complete DNA digestion was confirmed by using 1 μL in PCR, and if a product was obtained,
172 the treatment was prolonged until negative PCR reaction was obtained from all RNA samples.
173
174 *Polymerase chain reaction (PCR) and Reverse transcription (RT)-PCR amplification*
175 Amplification of the DNA (extracted from pure cultures or directly from the must) was
176 achieved with primers NL1 (5′-GCC ATA TCA ATA AGC GGA GGA AAA G-3′) and LS2 (5′-ATT
177 CCC AAA CAA CTC GAC TC-3′) (Cocolin et al. 2000). A GC-clamp (5′-CGC CCG CCG CGC CCC GCG
178 CCC GTC CCG CCG CCC CCG CCC G -3′) was attached to the forward NL1 primer when the PCR
179 product was destined for DGGE analysis (Sheffield et al. 1989). PCR was performed in a final
180 volume of 25 μl containing: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM
181 deoxynucleoside triphosphates (dNTPs), 1.25 U of *Taq* Polymerase (Applied Biosystems,
182 Milan, Italy) and 0.2 μM of each primer. A total of 100 ng of DNA were added in the reaction.
183 Amplifications were carried out in a PTC-220 DNA Engine Dyad MJ Research thermalcycler
184 (Celbio, Milan, Italy). The amplification cycle was: denaturation at 95°C for 1 min, annealing at
185 42°C for 1 min, extension at 72°C for 1 min, and the cycle was repeated 35 times. The cycle

was preceded by an initial denaturation at 95°C for 5 min and followed by a final extension at 72°C for 7 min.

Reverse transcription was performed with the Moloney Murine Leukemia Virus reverse transcriptase of Promega (Milan, Italy). One microgram of RNA was mixed with 100 μM of primer LS2 in a final volume of 10 μL. The mix was denatured at 75°C for 5 min, immediately put on ice and then the reverse transcription reaction mix was added. The reverse transcription was performed in the 25 μL total volume containing: 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 2 mM deoxynucleoside triphosphates (dNTPs), 4 μM primer, 200 units M-MLV and 0.48-0.96 units RNasin ribonuclease inhibitor. The mix was incubated at 42°C for 1 h and it was followed by a regular PCR reaction, as described above (addition of 1 μL of RT reaction into 25 μL of PCR reaction).

Denaturing gradient gel electrophoresis (DGGE)

After agarose gel electrophoresis (2% in 1.25 X Tris-Acetate-EDTA), PCR products were analysed by DGGE, using the D-Code universal mutation detection system (Bio-Rad, Hercules, CA, USA), with a 0.8-mm-thick polyacrylamide gel (8% [wt/vol] acrylamide-bisacrylamide [37.5:1]). A 30 to 50% denaturing gradient (100 % corresponds to 7 M urea and 40 % [wt/vol] formamide), increasing in the direction of the electrophoretic run, was used. The run was undertaken at 60°C, using 130V for 270 min. Gels were stained for 20 min in 1.25 X Tris-acetate-EDTA containing 1 X SYBR Green (Sigma, Milan, Italy). They were visualised under UV light, digitally captured, and analysed with the UVIpro Platinum 1.1 Gel Software (Eppendorf, Milan, Italy) for the recognition of the bands present.

Results

Microbial counts and yeast biodiversity during grape withering

Yeast and mould counts on grapes during withering, (Table 1) followed a similar trend. They decreased to their lowest level on day 63, followed by an approximately 2 $\log_{10}$ cfu/mL increase at day 93 with no further change until the end of the monitoring period. Yeast counts fluctuated between 3.63 $\log_{10}$ cfu/mL (lowest level recorded after 49 days of withering) and 5.71 $\log_{10}$ cfu/mL (highest count reached at day 93). Overall, yeast counts at the beginning and end of the process remained constant and in the order of 4 $\log_{10}$ cfu/mL. The range for moulds was between 2.76 $\log_{10}$ cfu/mL (lowest count, day 30) and 4.72 $\log_{10}$ cfu/mL (highest count, recorded at day 0). The count at the end of the period was 4.37 $\log_{10}$ cfu/mL for yeasts and 3.18 $\log_{10}$ cfu/mL for moulds.

At each sampling point, colonies on WLN medium were randomly picked, isolated and identified to the species level by PCR-DGGE grouping and sequencing of partial 26S rRNA encoding gene (Table 1). A total of 133 isolates, belonging to 12 species, was identified. The species most represented were *Aureobasidium pullulans*, with 40 isolates, followed by *Hanseniaspora uvarum*, with 36 isolates and *Candida zemplinina*, with 22 isolates. These three species, together with *Metschnikowia fructicola*, were also constantly present on the grapes. *Hanseniaspora uvarum* was isolated in 7 out of 8 sampling points, while *A. pullulans* and *M. fructicola* were isolated in 6 out of 8 time points, and *C. zemplinina* in 5 out 8. All four species were present both on the first and last day of sampling. *Hanseniaspora uvarum* was predominant during the first period of withering, up to day 49, and constituted almost 50% of the isolates; while *A. pullulans* prevailed at the end of the period, reaching 71.4% and 51.3% of the isolates at 124 and 139 days, respectively. *Rhodotorula nothofagi*, *Rhodotorula glutinis*, *Candida californica* and *Issatchenkia terricola* constituted minor populations during the monitoring period, since they were isolated at low proportion and unsystematically. Finally, four species, *Pichia anomala*, *Lachancea thermotolerans*, *Saccharomyces cerevisiae* and *Candida ishiwadae,* were isolated only on the last day of the monitoring period (day 139).

236

237 *Microbial counts and yeast biodiversity during fermentation*

238 The total yeast counts and the non-*Saccharomyces* counts during the industrial fermentation
239 are presented in Table 2. A yeast count of about 5 $\log_{10}$ cfu/mL was recorded for the grape
240 must used for the fermentation. On day 1 a 2 $\log_{10}$ increase was observed and the counts
241 stabilised to around 8 $\log_{10}$ cfu/mL for the remaining of the period (day 3 through 14). The
242 initial non-*Saccharomyces* count (in the grape must) was of the order of 4 $\log_{10}$ cfu/mL and it
243 remained stable throughout the fermentation.

244 Similarly to that described for grapes during drying, yeasts were randomly isolated and
245 identified during the fermentation. In the grape must, six species were identified, including *S.*
246 *cerevisiae*. Except for *Pichia kluyveri*, which was only detected in the grape must, all other
247 species were common between the grape sample at day 139 and the grape must sample. In
248 the fermentation, *S. cerevisiae* dominated the fermentation and represented 49 % (37 isolates
249 out of 75 in total) of the isolates. It was present from day 1 up until the last day of the
250 fermentation. Non-*Saccharomyces* yeasts were present up until day 7 of the fermentation and
251 belonged to *P. kluyveri*, *C. zemplinina*, *M. fructicola*, *H. uvarum*, *A. pullulans*, *P. anomala*, *C.*
252 *ishiwadae* and *L. thermotolerans*, in order of abundance.

253

254 *Population dynamics and evolution during alcoholic fermentation by PCR-DGGE*

255 Total DNA and RNA were extracted from the grape sample at the end of the withering period
256 (day 139), from the grape must used for fermentations as well as during the alcoholic
257 fermentation (days 1, 3, 7 and 14). The nucleic acids were used as templates for PCR-DGGE,
258 using primers NL1-LS2, in order to profile the yeast populations at each sampling point. The
259 DGGE profiles of the samples of grapes and grape must at DNA and RNA level are shown in
260 Figure 1. No differences were observed between the three replicates of the fermentation; ;

261 only one is presented. The profiles obtained from the grapes and grape must were similar. At
262 the DNA level, the profile was characterised by the presence of *Botrytis cinerea* and another
263 fungal species, which were not possible to be identified more precisely. The other two bands
264 that were visible in these samples belonged to *C. zemplinina*. Also present at RNA level was *C.*
265 *zemplinina*, together with *A. pullulans*, in grapes and grape must. During fermentation, from
266 day 1 onwards, the profiles presented bands appertaining to *S. cerevisiae* only, at both DNA
267 and RNA level (data not shown).
268
269 *Chemical analysis during alcoholic fermentation*
270 The results of the chemical analysis of the process of grape withering and of the alcoholic
271 fermentations are shown in Figure 2 and Table 3, respectively.
272 A progressive increase of sugars content, proportionally to weight loss, was observed during
273 the grape dehydration process. In accordance with a previous study (Rolle et al. 2011) a
274 significant decrease of tartaric acid was noticed, in particular during the first 60 days of
275 withering. *Botrytis cinerea* Pers. has the property of degrading the tartaric acid (Ribéreau-
276 Gayon et al. 2000). The ambient conditions of this withering process are favorable for the
277 development of *Botrytis* at the stage of noble rot (Rolle et al. 2012). In fact, the glycerol
278 concentration in the grape must, a chemical marker of *Botrytis* infection (Ravji et al. 1988),
279 was 3.9 g/L (Table 3).
280 The final must used for the fermentations was characterised by a high sugar content (428 g/L
281 of sugars). Glucose and fructose were consumed in parallel, although at different speed.
282 During fermentation, glucose was consumed at a higher rate than that of fructose. From a
283 starting value of about 221 g/L, it reached a final value of about 76 g/L. Fructose, in contrast,
284 was reduced from about 207 g/L to 132 g/L. The ethanol content at the end of the
285 fermentation was on average 133 mL/L (Ethanol produced /consumed sugar yield

286 0.466±0.004 [g/g]). During fermentation about 11 g/L of glycerol was produced. The malic
287 content of the final wine was 1 g/L less than that of the grape must.
288
289 **Discussion**
290 Numerous authors have reported on the importance of grape yeast ecology and its influence
291 on wine quality. Grapes are the main source of yeasts that inoculate must and initiate the
292 alcoholic fermentation. Non-inoculated alcoholic fermentations rely on yeast biota,
293 indigenous to the must, while in inoculated fermentations, initial stages of the process are
294 characterised by co-existence of wild, usually non-*Saccharomyces* yeasts and those of the
295 starter culture. The purpose of this work was two-fold. First, we investigated the yeast
296 ecology on mature grapes of the Erbaluce cultivar during off-vine withering. Then, we focused
297 on monitoring the yeast dynamics and the principal chemical parameters of an inoculated
298 fermentation of the high sugar must. In this study, particular attention was given to culture-
299 independent methods, able to study DNA and RNA extracted directly from the matrix without
300 isolation of the yeasts. Studying the DNA, it is possible to define the number and identity of
301 the microbial species present in a specific sample, thereby giving a view of the microbial
302 diversity and ecology. In contrast RNA gives insight into the metabolically active portion of
303 the populations present. This is relevant for food fermentations, including alcoholic
304 fermentation for wine production, where it is necessary to study the species that are
305 responsible for the transformation (Cocolin et al. 2011). It should be emphasised that the
306 detection limit for DGGE is of the order of 10^3 cfu/mL (Cocolin et al. 2000). As a consequence,
307 microbial groups that are present and active, but their population is lower than 10^3 cfu/mL,
308 will not be taken into consideration. The determination of the detection limit in DGGE analysis
309 is not always simple to perform because it depends on the different affinity that the primers

have towards the microbial species present in one ecosystem, thereby this limit may change according to the specific group of microorganisms being studied.

Three species successively dominated the grape mycobiota. *Hanseniaspora uvarum* prevailed for about the first half of the grape withering process, and although it was present until the last day of sampling, its frequency of isolation decreased in the second half of the process. Concomitantly, *C. zemplinina* and *A. pullulans*, occasionally isolated at the beginning, became the major component of the mycobiota towards the end of the process. Among the first reports on the dominance and persistence of *A. pullulans* on wine grapes is that of Prakitchaiwattana et al. (2004), in a pioneer study of grape ecology by a combination of culture-dependent and culture–independent approaches. Subsequently, other authors have also reported on its presence (Renouf et al. 2005, Nisiotou and Nychas 2007). *Aureobasidium. pullulans* is known to possess antagonistic properties towards other yeasts and fungi, and it can be speculated that it may influence the overall grape ecology (Castoria et al. 2001, Prakitchaiwattana et al. 2004,). A signal, corresponding to *A. pullulans,* was also detected on the grapes sampled on the last day of withering, at the RNA level.

Since its description in 2003 (Sipiczki 2003), *C. zemplinina* has been frequently isolated or detected in alcoholic fermentations (Urso et al. 2008, Suzzi et al. 2009) while, several strains, isolated from grapes and previously identified as *C. stellata*, are now re-classified as *C. zemplinina* (Csoma and Sipiczki 2008). The psychrotolerant and osmotolerant properties that *C. zemplinina* strains posses can be advantageously exploited in botrytised-sweet wines production, characterised by a high sugar concentration and a low fermentation temperature (Sipiczki 2004). Furthermore, the possibility to combine *C. zemplinina* with *S. cerevisiae*, in order to alleviate the osmotic stress imposed on *S. cerevisiae* in high-sugar musts and in this way reduce acetic acid production, was recently investigated (Rantsiou et al. 2012). For these reasons, the consistent presence of *C. zemplinina* during the withering process of the grapes

335 studied here is important. The grapes are carriers of this microbial species and may have an
336 important technological impact for the alcoholic fermentation and the production of Passito di
337 Caluso sweet wine. This aspect was confirmed during the monitoring of the fermentation,
338 where *C. zemplinina* strains were isolated up to day 7.
339 An interesting finding of this study, that regarded the grape ecology, was the detection at
340 different time points of the withering process, of a recently described species, *M. fructicola*.
341 *Metschnikowia fructicola* is a close relative of *M. pulcherrima*, a species that is associated with
342 grapes (Barata et al. 2012), may persist in grape must during the first days of fermentation
343 and has been described to possess anti-fungal activity (De Curtis et al. 1996). Recently,
344 *Metschnikowia* strains, isolated from botrytised grapes were tested and resulted to be
345 antagonistic towards fungal and bacterial growth (Sipiczki 2006). This finding suggests that
346 the presence of *Metschnikowia* species, specifically *M. pulcherrima* and *M. fructicola*, may play
347 a protective role, against growth of filamentous fungi on the grapes during withering.
348 The largest biodiversity, in terms of species detected, was observed on the grapes the last day
349 of withering. Then, once the grapes were crushed, there was a decrease in the number of
350 species detected, from eight in the grapes to six in the must. *Candida zemplinina, H. uvarum, M.*
351 *fructicola*, *S. cerevisiae* and *C. ishiwadae* were species common to the grapes and the grape
352 must. Differences were observed also in the population dynamics, as determined by the
353 culture-dependent method, during the course of the fermentation. *Saccharomyces cerevisiae*
354 was the dominant population, representing 49% of the isolates, and was isolated from the
355 first to the last day of fermentation. It is surprising that although the must was inoculated
356 with a starter culture, several other species could be detected, up to day 7 of fermentation
357 (when the alcohol content was 87 mL/L), indicating a persistence of yeast populations, other
358 than the starter, as reported previously (Urso et al. 2008, Rantsiou et al. 2012). Among them,
359 *C. zemplinina* and *H. uvarum* were constant. It should be noted that by culture-independent

analysis, a signal corresponding only to *S. cerevisiae* was detected as reported above (data not shown).

Conclusions

To our knowledge, this is the first time that the mycobiota has been studied throughout the grape withering process and that its fate has been followed during alcoholic fermentation. Although a single batch of grapes was followed during withering process, due to the fact that all grapes used for the Passito di Caluso wine were treated at the same time, it is not possible to assess if the ecology found would be confirmed in additional studies, however, it is important to underline the importance of the results obtained in terms of contribution of yeasts developed on the grapes to the fermentation. During the withering process a succession of three populations associated with grapes was observed, while a high degree of species biodiversity was detected at the end of the monitoring period. The significance of *C. zemplinina* in sweet wine fermentations was confirmed; it was a numerically important part of the yeast mycobiota during fermentation and its provenance was the grapes. Generally the results between culture-dependent and culture–independent approaches used in this study compared well.

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Table 1. Yeast species biodiversity and evolution on Erbaluce grapes during drying. For each sampling point, the prevalence of each species is presented as a proportion (%) of the total in the parenthesis.

	Day 0 (harvest)	Day 17	Day 30	Day 49	Day 63	Day 93	Day 124	Day 139	Total
Log $_{10}$ yeast cfu/mL ± s.d. [a]	4.68±0.62	4.75± 1.52	4.31± 1.73	3.63± 0.86	3.81± 1.02	5.71± 1.56	4.36± 0.82	4.37± 0.56	
Log $_{10}$ mould cfu/mL ± s.d.[a]	4.72±0.15	4.10± 0.62	2.51± 0.06	3.15± 0.6	2.76± 0.18	4.68± 0.32	3.67± 0.51	3.18± 0.19	
Hanseniaspora uvarum	8 (66.7)	6 (42.8)	7 (46.7)	4 (44.4)	5 (26.3)		4 (28.6)	2 (5.1)	36 (27.1)
Aureobasidium pullulans	1 (8.3)		3 (20)		4 (21)	2 (18.2)	10 (71.4)	20 (51.3)	40 (30.1)
Candida zemplinina		2 (14.3)	5 (20)		4 (21)	5 (45.5)		6 (15.4)	22 (16.5)
Rhodotorula nothofagi	1 (8.3)	2 (14.3)			4 (21)				7 (5.3)
Rhodotorula glutinis						1 (9.1)			1 (0.7)
Candida californica						2 (18.2)			2 (1.5)
Isatchenkia terricola	1 (8.3)								1 (0.7)
Metschnikowia fructicola	1 (8.3)	4 (28.6)		5 (55.6)	2 (10.5)	1 (9.1)		3 (7.7)	16 (12)
Pichia anomala								1 (2.5)	1 (0.7)
Lachancea thermotolerans								2 (5.1)	2 (1.5)
Saccharomyces cerevisiae								4 (10.2)	4 (3)
Candida ishiwadae								1 (2.5)	1 (0.7)
Total isolates for each sampling point	12	14	15	9	19	11	14	39	133

[a]Counts presented are the mean + standard deviation of triplicate samples at each time point

513 **Table 2.** Yeast species biodiversity and evolution during fermentation of Erbaluce must. For
514 each sampling point, the prevalence of each species is presented as proportion (%) of the total
515 in the parenthesis.

	Grape must	Days after start of fermentation				Total FI isolates
		1	3	7	14	
Log_{10} yeast CFU/ml ± s.d. [a]	4.99±0.27	7.17±0.33	8.30±0.15	8.96±0.05	8.54±0.29	
Log_{10} non-*Saccharomyces* yeast CFU/ml ± s.d. [a]	4.39±0.11	5.43±0.6	5.78±0.08	5.22±0.04	4.12 ±0.33	
Pichia anomala		1 (4.5)	3 (10.7)			4 (5.3)
Candida zemplinina	4 (13.3)	1 (4.5)	4 (14.3)	5 (41.7)		10 (13.3)
Metschnikowia fruticola	3 (10)	1 (4.5)	3 (10.7)			4 (5.3)
Kluyveromyces thermotolerans			1 (3.6)			1 (1.3%)
Saccharomyces cerevisiae	3 (10)	7 (31.8)	11 (39.3)	6 (50)	13 (100)	37 (49.3)
Hanseniaspora uvarum	7 (23.3)		1 (3.6)	1 (8.3)		2 (2.7)
Aureobasidium pullulans		2 (9.1)	2 (7.1)			4 (5.3)
Candida ishiwadae	6 (20)		1 (3.6)			1 (1.3)
Pichia kluyveri	7 (23.3)	10 (45.5)	2 (7.1)			12 (16)
Total of isolates for each sampling point	30	22	28	12	13	75

516 [a] Counts are the mean ± standard deviation of the three independent fermentations followed. Yeast counts were
517 determined on WLN plates, while non-*Saccharomyces* on Lysine medium.
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526 **Table 3.** Composition (mean ± standard deviation) of the grape must and sampled 1, 3, 7 and
527 14 days after the start of the alcoholic fermentation (days).
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	Grape must	1	3	7	14
Glucose (g/L)	221±2	206±1	174±5	117±1	76±1
Fructose (g/L)	207±6	196±1	181±6	155±2	132±1
Ethanol (ml/L)	3.0±0.8	11.5±0.1	32.1±0.5	86.9±2.1	133.1±0.6
Glycerol (g/L)	3.9±0.1	4.0±0.1	7.8±0.1	12.7±0.2	14.2±0.1
Acetic acid (g/L)	0.06±0.03	0.06±0.01	0.38±0.01	0.75±0.02	0.88±0.01
Malic acid (g/L)	3.5±0.1	3.3±0.1	3.3±0.1	2.6±0.1	2.5±0.1

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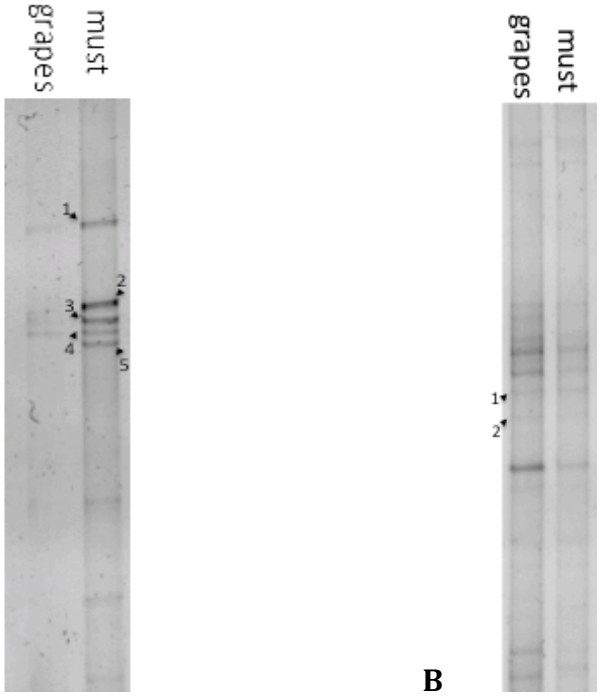
Figure legends

Figure 1. DNA (Panel A) and RNA (Panel B) DGGE profiles of samples from grapes at the end of the drying period (day 139) and grape must. Bands marked with a number were excised and sequenced (as described in the materials and methods section). **Band identification for panel A**: 1 and 3, *Botrytis cinerea*; 2 and 5, *Candida zemplinina*; 4, Fungal *sp.* **Band identification for panel B**: 1, *Candida zemplinina*; 2, *Aureobasidium pullulans.*

Figure 2. The concentration of glucose (♦), fructose (■), malic acid (×) and tartaric acid (▲) during grape drying. For each sampling point the mean and standard deviation of the chemical analysis performed on triplicate samples are presented.

555 **Figure 1**



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559 **Figure 2.**
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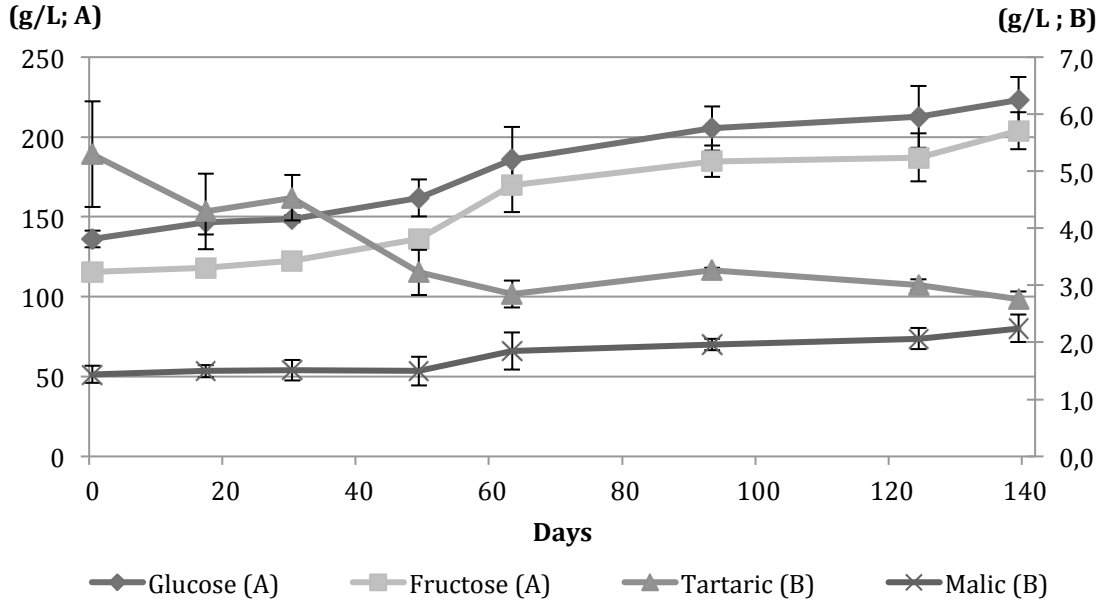

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