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

21

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Abstract

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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

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

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

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

97

98 **Materials and methods**

99 *Grape withering*

100 Grape clusters of the Erbaluce white cultivar (*Vitis vinifera* L.) from a vineyard located in
101 Caluso (Piedmont, NW Italy) were carefully harvested in the 2007 season at a soluble solids
102 content (SSC) of 25°Brix. About 10000 kg of grapes were placed in perforated boxes (60 × 40
103 × 15 cm, 6 kg of grapes in each box) in a single layer. For the natural, off-vine dehydration
104 process, the boxes were placed inside a typical room called *fruttaio* without control of
105 temperature, relative humidity or air flow (ambient conditions). The fruit was dehydrated for
106 139 days, from 12 September 2007 to 29 January 2008 (autumn–winter thermohygro-metric
107 drying conditions) in accordance with the Erbaluce di Caluso DOCG wine production rules
108 (Rolle et al. 2012). The participating winery in this study undertakes one dehydration process
109 per year on the grapes destined for the production of Passito di Caluso; thereby one batch was
110 followed in this work.

111 During this off-vine withering period, grapes were sampled approximately every 15 days
112 (0, 17, 30, 49, 63, 93, 124 and 139 days after harvest). At each sampling time, three lots of
113 about 500 sound berries were sampled randomly, placed in sterile stomacher bags and
114 transported to the laboratory within an hour. The grapes were manually crushed inside the
115 stomacher bag, and the grape juice obtained was subjected to chemical and microbiological
116 analysis.

117

118 *Must fermentation and sampling*

119 At the beginning of February 2008, the dried grapes were ready for vinification. They were
120 crushed resulting in a must with a pH value of 3.23 and a titratable acidity of 8.12 g/L
121 expressed as tartaric acid. . The must was fermented in triplicate (approximately 1200 L per
122 replicate) by a winery in the Caluso area; a starter culture (Lalvin EC118, Lallemand, Montreal,
123 Canada) was added and the fermentation lasted 14 days. Before inoculation of the starter
124 culture, 25 mg/L SO₂ was added. The fermentation was conducted at a controlled
125 temperature of 23±1°C and was sampled at 1, 3, 7 and 14 days. At each sampling point 50 mL
126 of the must and fermenting must were collected in sterile screw cap tubes and stored at 4°C
127 during transportation to the lab. Microbiological analysis were carried out as described below.
128 In addition, at each sampling point, aliquots (1 mL each) of the samples were collected for
129 chemical analysis, and extraction of DNA and of RNA. The aliquot destined for chemical
130 analysis was filtered through a 0.2 µm filter and stored at -20°C until analysis. The aliquots
131 destined for DNA and RNA extraction were centrifuged for 5 min at 13400 rpm, the
132 supernatant was removed and for the RNA aliquot, 0.2 mL of RNAlater (Ambion, Applied
133 Biosystems, Italy) were added. Pellets prepared in this way were stored at -20°C.

134

135 *Microbiological analysis*

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

142

143 *Chemical analysis*

144 Glucose, fructose, malic acid, glycerol, ethanol, and acetic acid of grape juices and fermenting
145 musts, were quantified by means of an HPLC (Thermo Electron Corp., Waltham, MA, USA)
146 equipped with a UV detector (UV100), set to 210 nm, and a refractive index detector (RI-150).
147 The analyses were run isocratically at 0.8 mL/min and 65°C on a cation-exchange column
148 (300 mm by 7.8 mm inner diameter; Aminex HPX-87H) fitted with a Cation H⁺ Microguard
149 cartridge (Bio-Rad Laboratories, Hercules, CA, USA), using 0.0026 N H₂SO₄ as the mobile
150 phase (Giordano et al. 2009).

151

152 *Molecular identification of isolates*

153 At each sampling point during the grape withering process and the fermentation, yeast
154 colonies (at least 10) were randomly selected from the WLN medium, isolated and stored at -
155 80°C in Yeast-Peptone-Dextrose Broth (YPD, 2% (wt/vol) glucose, 2% (wt/vol) peptone and
156 1% (wt/vol) yeast extract, all from Oxoid, Milan, Italy), with glycerol (30% final
157 concentration). For the molecular identification, isolates were grown in YPD Broth, their DNA
158 was extracted, amplified with primers NL1GC/LS2 and subjected to DGGE Cocolin et al.
159 (2000) (as described below). Isolates were grouped according to their DGGE profile, and
160 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/ μ L. In order to eliminate DNA traces from the preparation,
170 RNA was resuspended in 50 μ L of water containing the Turbo DNase (Ambion, Milan, Italy).
171 Complete DNA digestion was confirmed by using 1 μ L 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 μ l containing: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 0.2 mM
181 deoxynucleoside triphosphates (dNTPs), 1.25 U of *Taq* Polymerase (Applied Biosystems,
182 Milan, Italy) and 0.2 μ M 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 thermocycler
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

186 was preceded by an initial denaturation at 95°C for 5 min and followed by a final extension at
187 72°C for 7 min.

188 Reverse transcription was performed with the Moloney Murine Leukemia Virus reverse
189 transcriptase of Promega (Milan, Italy). One microgram of RNA was mixed with 100 µM of
190 primer LS2 in a final volume of 10 µL. The mix was denatured at 75°C for 5 min, immediately
191 put on ice and then the reverse transcription reaction mix was added. The reverse
192 transcription was performed in the 25 µL total volume containing: 50 mM Tris-HCl (pH 8.3),
193 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 2 mM deoxynucleoside triphosphates (dNTPs), 4 µM
194 primer, 200 units M-MLV and 0.48-0.96 units RNasin ribonuclease inhibitor. The mix was
195 incubated at 42°C for 1 h and it was followed by a regular PCR reaction, as described above
196 (addition of 1 µL of RT reaction into 25 µL of PCR reaction).

197

198 *Denaturing gradient gel electrophoresis (DGGE)*

199 After agarose gel electrophoresis (2% in 1.25 X Tris-Acetate-EDTA), PCR products were
200 analysed by DGGE, using the D-Code universal mutation detection system (Bio-Rad, Hercules,
201 CA, USA), with a 0.8-mm-thick polyacrylamide gel (8% [wt/vol] acrylamide-bisacrylamide
202 [37.5:1]). A 30 to 50% denaturing gradient (100 % corresponds to 7 M urea and 40 %
203 [wt/vol] formamide), increasing in the direction of the electrophoretic run, was used. The run
204 was undertaken at 60°C, using 130V for 270 min. Gels were stained for 20 min in 1.25 X Tris-
205 acetate-EDTA containing 1 X SYBR Green (Sigma, Milan, Italy). They were visualised under UV
206 light, digitally captured, and analysed with the UVipro Platinum 1.1 Gel Software (Eppendorf,
207 Milan, Italy) for the recognition of the bands present.

208

209 **Results**

210 *Microbial counts and yeast biodiversity during grape withering*

211 Yeast and mould counts on grapes during withering, (Table 1) followed a similar trend. They
212 decreased to their lowest level on day 63, followed by an approximately 2 log₁₀ cfu/mL
213 increase at day 93 with no further change until the end of the monitoring period. Yeast counts
214 fluctuated between 3.63 log₁₀ cfu/mL (lowest level recorded after 49 days of withering) and
215 5.71 log₁₀ cfu/mL (highest count reached at day 93). Overall, yeast counts at the beginning
216 and end of the process remained constant and in the order of 4 log₁₀ cfu/mL. The range for
217 moulds was between 2.76 log₁₀ cfu/mL (lowest count, day 30) and 4.72 log₁₀ cfu/mL (highest
218 count, recorded at day 0). The count at the end of the period was 4.37 log₁₀ cfu/mL for yeasts
219 and 3.18 log₁₀ cfu/mL for moulds.

220 At each sampling point, colonies on WLN medium were randomly picked, isolated and
221 identified to the species level by PCR-DGGE grouping and sequencing of partial 26S rRNA
222 encoding gene (Table 1). A total of 133 isolates, belonging to 12 species, was identified. The
223 species most represented were *Aureobasidium pullulans*, with 40 isolates, followed by
224 *Hanseniaspora uvarum*, with 36 isolates and *Candida zemplinina*, with 22 isolates. These three
225 species, together with *Metschnikowia fructicola*, were also constantly present on the grapes.
226 *Hanseniaspora uvarum* was isolated in 7 out of 8 sampling points, while *A. pullulans* and *M.*
227 *fructicola* were isolated in 6 out of 8 time points, and *C. zemplinina* in 5 out 8. All four species
228 were present both on the first and last day of sampling. *Hanseniaspora uvarum* was
229 predominant during the first period of withering, up to day 49, and constituted almost 50% of
230 the isolates; while *A. pullulans* prevailed at the end of the period, reaching 71.4% and 51.3%
231 of the isolates at 124 and 139 days, respectively. *Rhodotorula nothofagi*, *Rhodotorula glutinis*,
232 *Candida californica* and *Issatchenkia terricola* constituted minor populations during the
233 monitoring period, since they were isolated at low proportion and unsystematically. Finally,
234 four species, *Pichia anomala*, *Lachancea thermotolerans*, *Saccharomyces cerevisiae* and
235 *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

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

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

325 Since its description in 2003 (Sipiczki 2003), *C. zemplinina* has been frequently isolated or
326 detected in alcoholic fermentations (Urso et al. 2008, Suzzi et al. 2009) while, several strains,
327 isolated from grapes and previously identified as *C. stellata*, are now re-classified as *C.*
328 *zemplinina* (Csoma and Sipiczki 2008). The psychrotolerant and osmotolerant properties that
329 *C. zemplinina* strains possess can be advantageously exploited in botrytised-sweet wines
330 production, characterised by a high sugar concentration and a low fermentation temperature
331 (Sipiczki 2004). Furthermore, the possibility to combine *C. zemplinina* with *S. cerevisiae*, in
332 order to alleviate the osmotic stress imposed on *S. cerevisiae* in high-sugar musts and in this
333 way reduce acetic acid production, was recently investigated (Rantsiou et al. 2012). For these
334 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

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

362

363 **Conclusions**

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

377

378 **Acknowledgement**

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380 grapes during withering and the fermenting must for this study.

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498

499 **Table 1.** Yeast species biodiversity and evolution on Erbaluce grapes during drying. For each sampling
 500 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	
<i>Hanseniaspora uvarum</i>	8 (66.7)	6 (42.8)	7 (46.7)	4 (44.4)	5 (26.3)		4 (28.6)	2 (5.1)	36 (27.1)
<i>Aureobasidium pullulans</i>	1 (8.3)		3 (20)		4 (21)	2 (18.2)	10 (71.4)	20 (51.3)	40 (30.1)
<i>Candida zemplanina</i>		2 (14.3)	5 (20)		4 (21)	5 (45.5)		6 (15.4)	22 (16.5)
<i>Rhodotorula nothofagi</i>	1 (8.3)	2 (14.3)			4 (21)				7 (5.3)
<i>Rhodotorula glutinis</i>						1 (9.1)			1 (0.7)
<i>Candida californica</i>						2 (18.2)			2 (1.5)
<i>Isatchenkia terricola</i>	1 (8.3)								1 (0.7)
<i>Metschnikowia fructicola</i>	1 (8.3)	4 (28.6)		5 (55.6)	2 (10.5)	1 (9.1)		3 (7.7)	16 (12)
<i>Pichia anomala</i>								1 (2.5)	1 (0.7)
<i>Lachancea thermotolerans</i>								2 (5.1)	2 (1.5)
<i>Saccharomyces cerevisiae</i>								4 (10.2)	4 (3)
<i>Candida ishiwadae</i>								1 (2.5)	1 (0.7)
Total isolates for each sampling point	12	14	15	9	19	11	14	39	133

501 ^aCounts presented are the mean + standard deviation of triplicate samples at each time point

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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 1	start of 3	fermentation 7	14	Total FI isolates
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- <i>Saccharomyces</i> 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	
<i>Pichia anomala</i>		1 (4.5)	3 (10.7)			4 (5.3)
<i>Candida zemplinina</i>	4 (13.3)	1 (4.5)	4 (14.3)	5 (41.7)		10 (13.3)
<i>Metschnikowia fruticola</i>	3 (10)	1 (4.5)	3 (10.7)			4 (5.3)
<i>Kluyveromyces thermotolerans</i>			1 (3.6)			1 (1.3%)
<i>Saccharomyces cerevisiae</i>	3 (10)	7 (31.8)	11 (39.3)	6 (50)	13 (100)	37 (49.3)
<i>Hanseniaspora uvarum</i>	7 (23.3)		1 (3.6)	1 (8.3)		2 (2.7)
<i>Aureobasidium pullulans</i>		2 (9.1)	2 (7.1)			4 (5.3)
<i>Candida ishiwadae</i>	6 (20)		1 (3.6)			1 (1.3)
<i>Pichia kluyveri</i>	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 \pm 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 \pm 2	206 \pm 1	174 \pm 5	117 \pm 1	76 \pm 1
Fructose (g/L)	207 \pm 6	196 \pm 1	181 \pm 6	155 \pm 2	132 \pm 1
Ethanol (ml/L)	3.0 \pm 0.8	11.5 \pm 0.1	32.1 \pm 0.5	86.9 \pm 2.1	133.1 \pm 0.6
Glycerol (g/L)	3.9 \pm 0.1	4.0 \pm 0.1	7.8 \pm 0.1	12.7 \pm 0.2	14.2 \pm 0.1
Acetic acid (g/L)	0.06 \pm 0.03	0.06 \pm 0.01	0.38 \pm 0.01	0.75 \pm 0.02	0.88 \pm 0.01
Malic acid (g/L)	3.5 \pm 0.1	3.3 \pm 0.1	3.3 \pm 0.1	2.6 \pm 0.1	2.5 \pm 0.1

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531 **Figure legends**

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

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

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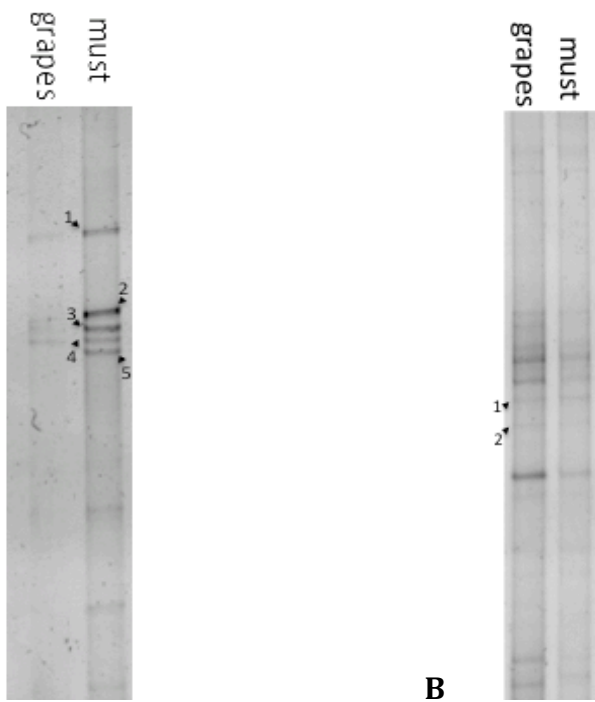
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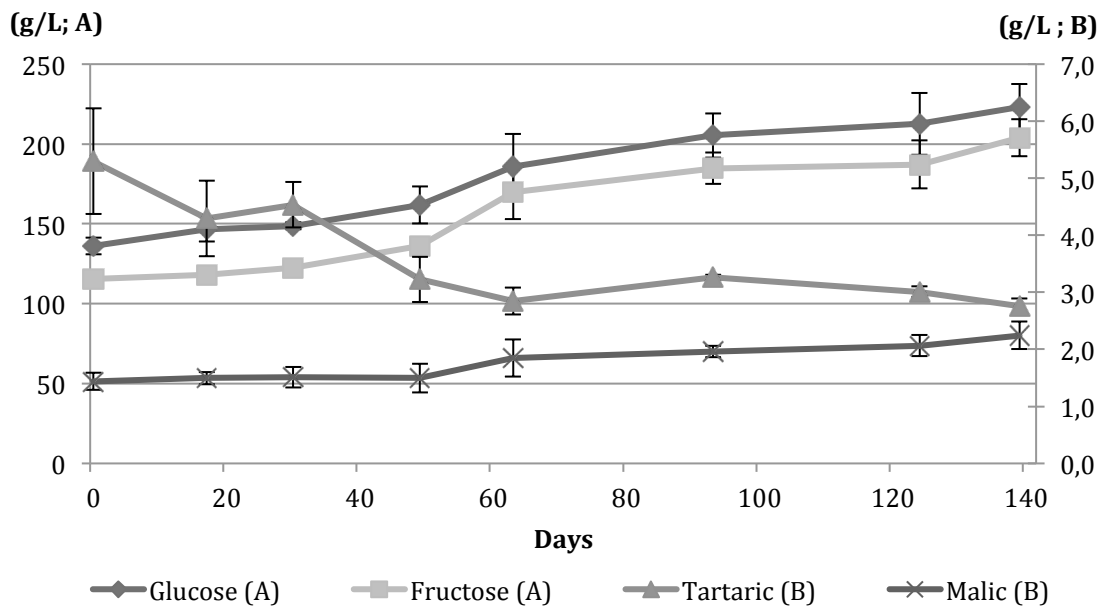
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555 **Figure 1**



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559 **Figure 2.**
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