Università degli Studi di Padova

Padua Research Archive - Institutional Repository

The role of nitrogen uptake on the competition ability of three vineyard *Saccharomyces cerevisiae* strains

Original Citation:

Availability:

This version is available at: 11577/3239156 since: 2017-07-21T09:06:36Z

Publisher:

Published version:

DOI: 10.1016/j.ijfoodmicro.2017.07.006

Terms of use:

Open Access

This article is made available under terms and conditions applicable to Open Access Guidelines, as described at <http://www.unipd.it/download/file/fid/55401> (Italian only)

(Article begins on next page)

1 **The role of nitrogen uptake on the competition ability of three vineyard *Saccharomyces***
2 ***cerevisiae* strains**

3
4 Chiara Vendramini^a, Gemma Beltran^c, Chiara Nadai^a, Alessio Giacomini^{ab}, Albert Mas^c, Viviana
5 Corich^{ab}

6 ^a*Centro Interdipartimentale per la Ricerca in Viticoltura ed Enologia (C.I.R.V.E.), Università degli*
7 *Studi di Padova, Conegliano (TV), Italy*

8 ^b*Dipartimento di Agronomia Animali Alimenti Risorse naturali e Ambiente, Università degli Studi*
9 *di Padova, Legnaro (PD), Italy*

10 ^c*Departament de Bioquímica i Biotecnologia. Facultat d'Enologia. Universitat Rovira i Virgili.*
11 *Tarragona, Spain.*

12
13 **Abstract**

14
15 Three vineyard strains of *Saccharomyces cerevisiae*, P301.4, P304.4 and P254.12, were assayed in
16 comparison with a commercial industrial strain, QA23. The aim was to understand if nitrogen
17 availability could influence strain competition ability during must fermentation. Pairwise-strain
18 fermentations and co-fermentations with the simultaneous presence of the four strains were
19 performed in synthetic musts at two nitrogen levels: control nitrogen condition (CNC) that assured
20 the suitable assimilable nitrogen amount required by the yeast strains to complete the fermentation
21 and low nitrogen condition (LNC) where nitrogen is present at very low level.

22 Results suggested a strong involvement of nitrogen availability, as the frequency in must of the
23 vineyard strains, respect to QA23, in LNC was always higher than that found in CNC. Moreover, in
24 CNC only strain P304.4 reached the same strain frequency as QA23. P304.4 competition ability
25 increased during the fermentation, indicating better performance when nitrogen availability was
26 dropping down. P301.4 was the only strain sensitive to QA23 killer toxin. In CNC, when it was co-

27 inoculated with the industrial strain QA23, P301.4 was never detected. In LNC, P301.4 after 12
28 hours accounted for 10% of the total population. This percentage increased after 48 hours (20%).
29 Single-strain fermentations were also run in both conditions and the nitrogen metabolism further
30 analyzed. Fermentation kinetics, ammonium and amino-acid consumptions and the expression of
31 genes under nitrogen catabolite repression evidenced that vineyard yeasts, and particularly strain
32 P304.4, had higher nitrogen assimilation rate than the commercial control. In conclusion, the high
33 nitrogen assimilation rate seems to be an additional strategy that allowed vineyard yeasts successful
34 competition during the growth in grape musts.

35
36 **Keywords:** autochthonous yeast, alcoholic fermentation, killer toxin, population dynamics, wine

37
38 **Highlights:**

39 Nitrogen concentration selectively influences yeast competition.

40 Low nitrogen concentrations jeopardize fermentation conduction by wine strain QA23.

41 Yeast higher survival rate is related to fast nitrogen consumption.

42
43 **1. Introduction**

44 The population diversity of yeast species associated with spontaneous grape juice fermentation is
45 quite complex and growth dynamics are well documented (Fleet, 1993; Ribereau-Gayon et al.,
46 2006). From the perspective of practical winemaking, the relevant outcomes of these interactions
47 are whether or not they enhance or inhibit the growth of any particular species or strain, modifying
48 the final quality of wines. During the fermentation process the species number is strongly reduced
49 by the ethanol increase, changes in the fermenting must composition and microorganisms
50 interaction (Wang et al., 2015). The results of these combinations of factors determine, at the end of
51 the process, the presence of the most adapted *Saccharomyces cerevisiae* species. The number of
52 *Saccharomyces* strains present during spontaneous fermentation is reported to be strongly variable:
53 Povhe Jemec et al. (2001) identified from 1 to 18 different strains simultaneously present during

54 five fermentations of Malvasia must. Similar results were found by Santamaria (2005) that isolated
55 from 1 to 14 strains for each of 7 fermentations of Tempranillo grape variety, whereas Torija et al.
56 (2001) evidenced the presence of up to 112 different *S. cerevisiae* strains in two Spanish cellars
57 monitoring must fermentations from grapes collected during three consecutive harvests. From a
58 technological point of view spontaneous grape juice fermentations sometimes become stuck or
59 sluggish. This lack of reproducibility and predictability has favored, in the past, the use of yeast
60 starters, generally composed of single strains of *S. cerevisiae*. Selected *S. cerevisiae* strains
61 predominate during must fermentation, ensure rapid and reliable grape juice fermentation and, as
62 consequence, consistent and predictable wine quality. However, wines made with a single
63 *Saccharomyces* strain culture are less complex, producing standardized wines (Ciani and Comitini,
64 2015; Swiegers et al., 2005).

65 Therefore, to enrich the complexity of the resulting wines, the growing interest towards controlled
66 mixed fermentations that use more than one selected yeast strain, pushes strongly the knowledge
67 towards yeast-to-yeast interaction. Within the wine ecosystem, there are numerous mechanisms
68 whereby one yeast may influence the growth of another yeast, some of them are still to be fully
69 investigated (Fleet, 2003). They involve the presence of growth-inhibitory compounds, such as
70 ethanol, medium-chain fatty acids and acetic acid, or, more traditionally, killer toxin (Bauer and
71 Pretorius, 2000; Perez et al., 2001). Moreover molecule-mediated cell-to-cell interactions were
72 found to be responsible of coordinating yeast growth by means of quorum sensing-like phenomena
73 during alcoholic fermentation. In the case of yeasts, there is evidence that carbonate (Hall et al.,
74 2010; Volodyaev et al., 2013), acetaldehyde (Weber et al., 2012) ammonia (Ciani and Comitini,
75 2015; Honigberg, 2011; Joutheen et al., 2016; Palkova and Vachova, 2006), farnesol (Hornby et al.,
76 2001; Nickerson et al., 2006) and tyrosol (Barriuso, 2015; Chen et al., 2004) may act as cell
77 communicating molecules. Among abiotic factors, low temperature was demonstrated to influence
78 the growth dynamics of a mix strain population of *S. cerevisiae* during alcoholic fermentation.
79 Lowering the temperature clearly improved the development of some *Saccharomyces* strains and, as
80 a consequence, affected wine quality and characteristics (Torija et al., 2003). Furthermore, cell-to-

81 cell contact has also been shown as a mechanism for *S. cerevisiae* interaction and dominance of
82 some strains over others (Perrone et al., 2013).

83 Nutrient availability and nutrient limitation are likely factors that modulate the yeast ecology of
84 fermentation, as one yeast species or strain produces or utilizes a nutrient relevant to another species
85 or strain. Several studies clearly demonstrated that *Saccharomyces* metabolism changes due to the
86 presence of different concentration of nitrogen and that nitrogen request is strain dependent (Beltran
87 et al., 2005; Martinez-Moreno et al., 2012), but it is still not clear how nitrogen concentration can
88 modulate *S. cerevisiae* competition ability.

89 *S. cerevisiae* requires a relatively high level of nutrients to complete the fermentation of grape must,
90 typically producing 12–15% v/v ethanol. Assimilable nitrogen has been identified as a key nutrient
91 that is often suboptimal in many grape musts surveyed worldwide (Vilanova et al., 2007). A
92 minimal concentration of more than 140 mg/L is often quoted as necessary for the fermentation of
93 low-solids (filtered), low-temperature (<15°C), anaerobic musts of moderate sugar level (20%)
94 (Bell and Henschke, 2005). However, the nitrogen requirements are dependent on the sugar
95 concentration in the must (Martinez-Moreno et al., 2012). Nitrogen sources are rapidly accumulated
96 by yeast in the early stages of fermentation, during which they fill the biosynthetic pools of amino
97 acids needed for protein synthesis and growth, while the surplus is stored in the cell vacuole
98 (Vilanova et al., 2007). When nitrogen is limiting, most of the available nitrogen is consumed
99 during the first 24-36 h of alcoholic fermentation (Beltran et al., 2004; Varela et al., 2004; Vilanova
100 et al., 2007).

101 Although nitrogen concentration is a relevant factor, it is noteworthy that not all the nitrogen
102 sources support equally yeast growth. In complex mixtures of amino acids and ammonium, such as
103 grape must, wine yeasts have preference for some nitrogen sources, and the pattern of the
104 preferential uptake of the nitrogen sources is determined by different molecular mechanisms. In *S.*
105 *cerevisiae*, the mechanism is known globally as nitrogen catabolite repression (NCR) allowing the
106 detection of the presence of the best sources of nitrogen by limiting the use of those that do not
107 allow for the best growth (Ter Schure et al., 2000). The detection of the rich nitrogen sources

108 triggers a signaling chain that culminates with the activation of genes involved in the transport and
109 metabolism of these rich sources and the suppression of those genes involved in the transport and
110 use of poorer sources. Once the richest sources of nitrogen (ammonium, glutamine, and asparagine)
111 are consumed, yeast metabolism activates the utilization of the poorer sources of nitrogen (arginine,
112 glutamate, alanine, etc.) (Mas et al., 2014).

113 In this work pairwise strain fermentations in synthetic must supplemented with two different
114 nitrogen concentrations were set up. In these conditions, the competition ability of three *S.*
115 *cerevisiae* strains, isolated directly from the vineyard, and the industrial strain QA23, was
116 evaluated. The control nitrogen condition (CNC) provided the suitable nitrogen amount required by
117 the yeast strains to complete the fermentation when 200 g/l of sugar are present in the must. The
118 low nitrogen condition (LNC) is considered a limiting concentration and generally results in
119 sluggish fermentations during the last part of the process (Beltran et al., 2004, Chiva et al., 2009,
120 Deed et al., 2011). The nitrogen requirements of the strains were investigated monitoring the amino
121 acids and ammonium consumption during fermentation in synthetic must. The expression level of
122 some of the genes under nitrogen catabolite repression was monitored in order to correlate nitrogen
123 metabolism, fermentation rate and nitrogen request. Results for nitrogen demand and population
124 dynamics were used to understand the possible role of nitrogen availability in strains competition
125 during must fermentations.

126

127 **2. Materials and Methods**

128 *2.1 Yeast strains and killer assay*

129 For this study the commercial wine yeast QA23 (Lallemand S.A. Canada) and three autochthonous
130 strains, P301.4, P304.4, P254.12 were used. The autochthonous yeasts were isolated from vineyards
131 in the winemaking area of Prosecco Superiore di Conegliano Valdobbiadene DOCG (Italy). The
132 three vineyard strains were chosen within a wide pool of strains among those with good
133 fermentation performance (Treu et al., 2014 a).

134 The killer phenotype between the three strains was tested according to Cavazza et al. (1992).

135

136 2.2 Culture media and growth condition

137 Strains were routinely grown in YPD at 25°C. Fermentations were carried out in a synthetic grape
138 must (pH 3.3) as described by Riou et al. (1997). In this medium two different nitrogen
139 concentrations were used. In control nitrogen condition (CNC) the yeast assimilable nitrogen
140 (YAN) content in the synthetic grape must was 440 mg N/l, ammoniacal nitrogen (NH₄Cl) 120 mg
141 N/l, and amino acids 320 mg N/l (taking into account all the N present in the amino acids). To
142 mimic grape must composition this medium also contained 426 mg/l of proline, that is not part of
143 the assimilable nitrogen. The low nitrogen condition (LNC) contained 1/5-fold (88 mg N/l) of the
144 YAN present in CNC at the same ammoniacal and amino-acid proportion.

145

146 2.3 Fermentation trials

147 2.3.1 Pre-culture preparation

148 Each yeast strain was grown on YPD solid medium at 25°C for 3 days. A loopful of yeast cells was
149 used to inoculate 30 ml of YPD liquid medium. The culture was incubated for 24 hours at 25 °C to
150 reach the stationary phase (about 10⁷-10⁸ cells/ml). Yeast concentration was measured by counting
151 the cells in a Thoma chamber using a phase contrast microscope (Olympus).
152 In pairwise-strains fermentations cell concentration present in each pre-culture was used to calculate
153 the suitable volume of the inoculum to reach, for each strain, a final concentration of 1x10⁶ cells/ml.
154 In co-fermentations where all the four strains were present simultaneously, the cell concentration
155 for each strain was of 5x10⁵ cells/ml. In single-strain fermentation, for each strain, the concentration
156 was 2x10⁶ cells/ml.

157

158 2.3.2 Fermentation conditions

159 Fermentations were performed at 25 °C in 250 mL glass bottles containing 200 mL of synthetic
160 must and fitted with closures that enabled the carbon dioxide to escape and the samples to be
161 removed.

Kinetics were monitored by daily measuring weight loss or must density. Fermentations were considered completed when the daily weight loss was lower than 0.1 g within 24 hours. In the single-strain fermentations where nitrogen consumption and yeast gene expression were detected, due to the large number of samplings, a continuous orbital shaking (150 rpm) was performed during the trial. The continuous shaking ensured an optimal collection of cell samples. Fermentations were performed in triplicate and the whole experimental set was repeated twice. Yeast cell concentration during fermentation was determined by plate counts on YPD medium. To perform real-time quantitative PCR, cell samples were collected throughout the fermentation at different time points and harvested by centrifugation, frozen in liquid nitrogen and stored at -80 °C. Supernatant was also stored at -20 °C to analyze the content of nitrogen and some fermentation products. During mixed fermentations, at three sampling points (12, 24, 48 hours) yeast isolation was carried out on YPD plates. At each sampling, a total of 20 colonies, randomly chosen from isolation plates from the same dilution series, was submitted to amplification of inter- δ region.

175

2.4 Nitrogen content analysis

Analysis of ammonia and individual amino acids was determined by diethyl ethoxymethylenemalonate (DEEMM) derivatization (Gómez-Alonso et al., 2007), using the Agilent 1100 Series HPLC (Agilent Technologies, Germany). Separation was performed in an ACE HPLC column (C18-HL), particle size 5 μ m (250 mm $\times$ 4.6 mm) thermostated at 20 °C. The concentration of each compound was calculated using internal (L-2-amino adipic acid, 1 g/l) and external standards, and expressed as mg N/l. The software used was Agilent ChemStation Plus (Agilent Technologies, Germany).

184

2.5 PCR-amplification of inter-delta sequences

Cell suspension for DNA Amplification was performed as described by Nardi et al. (2006). Two microlitres of the cell suspension were used for PCR amplification. Oligonucleotides primers delta12 (TCAACAATGGAATCCCAAC) and delta21 (CATCTTAACACCGTATATGA) were

189 used to amplify total genomic DNA between the repeated interspersed delta sequences as
190 previously described (Legras et al., 2003). PCR products were separated in 1.5% agarose gels in 0.5
191 X TBE buffer. The molecular marker 100 bp DNA ladder (Promega, Madison, USA) was used as
192 molecular size standard. Electrophoresis gels were stained with ethidium bromide (5 µg/ml),
193 visualized by UV transillumination. Digital images were acquired with an EDAS290 image
194 capturing system (Kodak, Rochester, NY).

195

196 *2.6 Real-time quantitative PCR*

197 Total RNA was isolated from yeast samples as described by Sierkstra et al. (1992) and resuspended
198 in 50 µl of diethyl pyrocarbonate-treated water. cDNA was synthesised from total RNA using
199 SuperscriptTM II RNase H- Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) in a GenAmp
200 PCR System 2700 (Applied Biosystems, Foster City, CA, USA). The protocol provided by the
201 manufacturer was used. The PCR primers used in this study were ACT-F,
202 TGGATTCCGGTGATGGTGTT, and ACT-R, CGGCCAAATCGATTCTCAA (ACT, for actine
203 gene); IPP1-F, GACACCCCAACCTACTCCAA and IPP1-R, GAACCGGAGATGAAGAACCA
204 (IPP1, for inorganic pyrophosphatase gene); GAP1-F, CTGTGGATGCTGCTGCTTCA, and
205 GAP1-R, CAACACTTGGCAAACCCTTGA (GAP1, for general amino acid permease gene); and
206 MEP2-F, GGTATCATCGCTGGCCTAGTG, and MEP2-R, CAACGGCTGACCAGATTGG
207 (MEP2, for ammonium permease gene). The real-time quantitative PCR reaction was performed
208 using SYBR® Green I PCR (Applied Biosystems, USA). Gene expression data were normalized
209 using the expression value of the actin gene and the inorganic pyrophosphatase gene, chosen as
210 housekeeping genes. In the PCR reaction, the final reaction volume was 25 µl, the final
211 concentration of each primer was 300 nM, together with 1 µl of the cDNA previously synthesized
212 from total RNA. All PCR reactions were mixed in 96-well optical plates and cycled in a GeneAmp
213 5700 Sequence Detection System (Applied Biosystems, USA) under the following conditions: 50
214 °C for 2 min, 95 °C for 10 min, and 40 cycles at 95 °C for 15 s and at 60 °C for 60 s. The threshold
215 was positioned to intersect the exponential part of the amplification curve of positive reactions, as

recommended by Applied Biosystems. Each sample had two controls, which were run in the same quantitative PCR: NAC (No Amplification Control; sample without reverse transcriptase reaction) to avoid the interference by contaminant genomic DNA and NTC (No Template Control; sample without RNA template) to avoid interference by primer-dimer formation. Relative gene expression was determined using the $2^{-\Delta\Delta C_t}$ formula, where C_t is defined as the cycle at which fluorescence is determined to be statistically significantly above background; ΔC_t is the difference in C_t of the gene of interest and C_t of the housekeeping gene (ACT1); and $\Delta\Delta C_t$ is the difference in ΔC_t at time = t and ΔC_t at time = 12 h in the non-limiting condition (300 mg N/L) for strain QA23, used as reference. All samples were analysed in duplicate and the expression values were averaged by the analysis software (Applied Biosystems, USA). (Gutiérrez et al., 2013). The coefficient of variation in all samples analysed was less than 20 %.

227

228 *2.7 Chemical analyses*

Glucose, fructose, glycerol, acetic acid and ethanol were quantified using commercial enzymatic kits (Roche Diagnostics, Germany). The pH was determined using a pH-meter Crison MicroPH 2000 (Crison, Barcelona, Spain).

232

233 *2.8 Statistical analysis*

The software XLSTAT, vers.7.5.2, was used to perform the analysis of variance (one-way ANOVA), followed by the Tukey "post-hoc" test. The analysis was conducted by comparing the averages of three independent replications and differences were considered statistically significant for p -value less than 0.05. Visualization of the amino-acids consumption data was obtained by means of the hierarchical clustering program, MULTIEXPERIMENT VIEWER (MeV, Rockville, MD, USA). The clustering process was carried out with Pearson distance and the average linkage method.

241

242 **3. Results**

243

244 *3.1 Mixed fermentations: strain growth at control and low nitrogen content*

245 To analyze the effects of nitrogen availability upon yeast population dynamics during must

246 fermentation, three vineyard strains of *Saccharomyces cerevisiae*, P301.4, P304.4 and P254.12,

247 were assayed in competition with a commercial industrial wine strain (QA23).

248 In mixed fermentations (pairwise and co-fermentation of the four strains) all the yeasts had the

249 same inoculum size. Single-strain fermentations were run, as control. The assays were performed in

250 synthetic must containing two nitrogen concentrations. The yeast-assimilable nitrogen (YAN)

251 content was 440 mg N/l in control nitrogen condition (CNC) and 88 mg N/l in low nitrogen

252 condition (LNC).

253 Before starting the fermentation trials killer toxin production was tested as this trait influences the

254 competition ability. Results indicated that the industrial wine strain QA23 had a killer phenotype,

255 P234.15 and P304.4 had a neutral phenotype and P301.4 was sensitive to the killer toxin produced

256 by QA23. The CO₂ production and cell concentration were determined during all fermentation and

257 residual sugar, acetic acid and glycerol production were analyzed at the end of the process. As

258 expected fermentations at low nitrogen condition (LNC) were slower than those at CNC. At LNC

259 all the fermentations were run within 230 hours (10 days), whereas at CNC within 160 hours (7

260 days). No residual sugar were detected after 160 hours at CNC, whereas at LNC only P304.4 left 7g

261 /l sugar. No significant differences were detected at the end of fermentation in ethanol and acetic

262 acid production at both CNC and LNC (data not shown).

263 The total population dynamics is reported in Fig. 1. No significant differences were detected in the

264 growth trends at CNC, at LNC during the first 48 hours the single-strain fermentation with P304.4

265 showed a lower cell concentration than the others.

266

267 *3. Strain competition during mixed fermentations*

268 During mixed fermentation processes the strain frequency was determined. As the available

269 nitrogen is mainly consumed before 48 hours, the changes in the yeast population structure were

analyzed during these early periods (12, 24 and 48 h). In the CNC condition, the commercial strain took over the other strains, dominating the early stages up to 48 h, when no more nitrogen was available (Table 1). This was also true when the four strains were co-inoculated together, where the percentage of the commercial strain was always close to 50%. Only one of the vineyard strains (P304.4) seemed to be almost as competitive as QA23, reaching higher populations after 48h either when pairwise fermentation or co-fermentation of the 4 strains were performed. Instead, when the fermentations were in presence of limiting concentrations of nitrogen, yeast populations changed considerably. All the vineyards strains improved their competition ability, as their frequencies increased during the first 48 hours of fermentation. The strain sensitive to the killer toxin, P301.4, although to a lesser extent, improved competition performances, as well (Table 2). This last strain that immediately disappeared in CNC presented up to 20% of the total population in LNC. In the mixed fermentations with all the strains, three of the strains (two vineyard and the commercial) co-dominated the fermentation, with similar cell levels.

283

3.3 Nitrogen consumption and expression of genes under nitrogen catabolic repression during single-strain fermentations

To better understand the ability of each strain to utilize the nitrogen source present in the synthetic musts the single-strain fermentations were run again in both conditions and the nitrogen metabolism analyzed further.

At CNC (Fig. 2A) sugar consumption during tumultuous fermentation was significantly higher (p -values < 0.05) up to T48 sampling in vineyard strains than in the commercial strain. However, at the end of fermentation the commercial strain was faster, ending the fermentation at the same time as all the strains. When LNC was tested (Fig. 2B), the fermentation rate was very much slower than in CNC. Although the starting of the fermentation (up to T24 sampling) was similar and without significant differences in sugar consumption between strains, shortly after the start, the strain P304.4 was significantly slower than the rest of the strains with a reduced viable cell population during the growth stationary phase. The commercial strain was the fastest yeast to finish the

297 fermentation that was completed 140 hours earlier than P304.4 that showed a fermentation length of
298 360 hours.

299 No significant differences among strains were found at the end of fermentation in ethanol and acetic
300 acid production at both CNC and LNC (data not shown). However, in the latter condition, glycerol
301 concentration was significantly higher in P254.12 and P304.4 (6.60 and 6.85 g/l respectively) than
302 in P301.4 and QA23 (6.00 and 5.60), despite that no significant differences were found in CNC.

303 Consumption of ammonium and yeast assimilable nitrogen (YAN) was monitored throughout these
304 fermentations. In CNC (Fig. 3) YAN available in the must was assimilated within 24-30 hours and
305 the commercial strain showed a slower nitrogen consumption than the vineyard strains. Ammonium
306 was completely consumed within 21 hours by the vineyard strains whereas the commercial control
307 needed 24 hours. After that, in the vineyard strain fermentations, YAN dropped to 4.18-4.72 g/l, a
308 residual concentration mainly composed by cysteine. At the same sampling time the YAN
309 concentration in the commercial strain fermentation was 82.7 mg N/l (data not shown). At CNC the
310 consumption of each amino acid was estimated by calculating the difference with the initial
311 concentrations. The heatmap analysis (Fig. 4A; Supplementary table S1) showed a conserved
312 pattern among strains, with some amino acids that were generally more rapidly consumed (lysine,
313 methionine, threonine, leucine, serine, glutamine, histidine, aspartic acid) than others. Amino-acid
314 consumption patterns revealed that vineyard strains consumed the nitrogen sources (amino acid and
315 ammonium) more rapidly than the commercial QA23. Several clusters in consumption patterns
316 could be identified. The main cluster included the preferred nitrogen sources and was composed by
317 ammonium, valine, glutamate, aspartate, phenylalanine, histidine, glutamine, serine, leucine,
318 threonine and methionine. This group of amino acids was mostly taken by the autochthonous strains
319 by 15-18 h, whereas the uptake by the commercial strain was after 21 h. However, a couple of
320 subgroups can be seen in this cluster, regarding the early consumption: the subgroup formed by
321 valine, glutamate, aspartate and phenylalanine, that are taken up after 9 h, and the rest that are
322 consumed from the first sampling time (6 h). A second group was formed by tryptophan, tyrosine,
323 arginine and alanine, that are consumed completely by 21-24 h by the autochthonous strains and by

30 h in the commercial strain. Glycine is similar to this last group, although a little bit delayed. Finally cysteine remained in considerable amounts after 30 h, independently of the strain. In LNC ammonium was completely depleted after 12 h in all fermentations and the amino acid concentrations were notably reduced (Fig.4B; Supplementary table S2). After 12 hours vineyard strains had totally consumed most of the amino acids that were early consumed in CNC (serine, glutamine, histidine, valine, leucine isoleucine phenylalanine) together with glycine, alanine and the most abundant arginine, that at CNC was totally consumed in 21-24 hours. Instead, the commercial strain showed a lower amino-acid consumption rate as at 12 hours tryptophan, alanine, glycine, phenylalanine, valine and threonine were still present in the must at high concentrations. All the strains left cysteine concentration unchanged in the medium as previously found in CNC. Methionine and lysine were generally less consumed than in CNC. To better understand the different fermentation behaviors and nitrogen assimilation rates between the strains, the expression of *MEP2* and *GAP1* genes, both controlled by nitrogen catabolite repression, was investigated (Fig. 5 and 6). The ammonium permease encoded by *MEP2* is activated when ammonium is very low. This allows the complete consumption of ammonium. In both CNC and LNC, P304.4 and P301.4, the two vineyard strains that presented the lowest fermentation rates in LNC, showed the earliest and the strongest activation of *MEP2*. In CNC this was particularly evident at 72 hours, while in LNC the increased expression was after 48 hours. The general amino-acid permease *GAP1* enhances the assimilation of amino acids, increasing the transport of those that do not have specific permeases that are constitutively expressed. The *GAP1* gene expression trend for each strain was very similar to that observed for *MEP2*, although the levels of expression were much higher than those detected for *MEP2*. The commercial strain showed in all the cases the weakest increase of gene expression levels.

347

348 **4. Discussion**

Although most of the studies on strain competition have been done with non-*Saccharomyces* yeasts, some have centered the interest in the interaction between *Saccharomyces* strains (revised by Ciani

et al., 2016). In fact, nutrient availability and specifically nitrogen demand has been pointed out as one of the mechanisms underlying situations of dominance or taking over from some strains over others (García-Rios et al., 2014). For this reason, we used the *S. cerevisiae* strain QA23 that is well known to have a low nitrogen demand and strong ethanol resistance during vinification and also produce a killer toxin that improves its ability to colonize the enological environment (Beltran et al., 2005; Jiménez-Martí et al., 2007). The vineyard yeasts P301.4, P304.4 and P254.12 were isolated from single grape bunch fermentations. In each fermentation, co-domination of at least 3 strains was found. In the case of P304.4 nine different strains were present simultaneously (Treu et al., 2014 a, 2014 b) P254.12 and P304.4 showed a neutral phenotype when QA23 killer toxin where present in the growing medium, whereas P301.4 was sensitive. Our results, comparing the needs of the different strains, suggested a strong implication of nitrogen assimilation ability on strain competition during the growth in grape musts. In fact, the competition ability of the vineyard strains in LNC was always higher than that found in CNC. Moreover, in CNC the strain P304.4 showed the highest competition ability respect to QA23, evidenced by the same strain frequency at the end of fermentation. The P304.4 competition ability increased during the fermentation, indicating better performance when nitrogen was depleted. When nitrogen concentration was high only small differences were found among the fermentation kinetics. Notwithstanding, the sugar consumption rate of the commercial strain during exponential and early stationary phase was significantly slower than those found for the other yeasts. This finding suggested that the lower nitrogen assimilation of QA23, compared with vineyard strains, limits the fermentation rate when nitrogen is abundant. On the contrary when low nitrogen was present strong differences in the fermentation kinetics were evidenced. QA23 completed the fermentation earlier than the others. P304.4 was the slowest strain evidencing the highest nitrogen demand. When ammonium and amino acids consumption was determined, strong differences between vineyard strains and the commercial strain were found confirming the lower nitrogen demand of the commercial strain. In particular in CNC after 12 hours P304.4 and P254.12 showed the highest ammonium consumption (around 90% depletion)

377 compared to P301.4 (70%) and QA23 (56%). Thus, this quick nitrogen consumption could explain
378 the increase in the competition ability in LNC.

379 Regarding amino acid assimilation the pattern seems to be very conserved among the strains,
380 indicating that the main difference between QA23 and the vineyard group is the assimilation rate.
381 These results are very close to what was observed by García-Rios et al. (2014), because the most
382 competitive strain used in that study (PDM) is genetically very close to the QA23 and also its
383 capacity to take over the other strains is enhanced by higher nitrogen availability.

384 All the strains showed the same preference in amino-acid consumption. At CNC methionine,
385 threonine, leucine, serine, glutamine were consumed faster. Glycine consumption started later and
386 cysteine contents remained unchanged. These results are very similar to what has been observed in
387 other conditions and studies (Beltran et al., 2004, 2005; Jiranek et al., 1995). To evaluate the effect
388 of different assimilation rate on nitrogen metabolism the expression of *MEP2* (ammonium
389 permease) and *GAP1* (general amino acids permease) genes was investigated. Amino acids are
390 transported into the cell by general and specific transport systems. The general high-capacity
391 permeases like *GAP1* and *AGP1* or the specific proline permease *PUT4* are nitrogen-regulated and
392 become down-regulated at the transcriptional as well as the posttranslational level, in response to
393 high-quality nitrogen sources like ammonium (Forsberg and Ljungdahl, 2001). However, specific
394 permeases like the histidine permease (*HIP1*), the lysine permease (*LYP1*) and the basic-amino-acid
395 permease *CAN1* are expressed constitutively (Ter Schure et al., 2000). The *MEP* genes related to
396 ammonium uptake are also subjected to nitrogen control. These genes are expressed when low
397 ammonium concentrations are present in the growth medium, but at high concentration of a good
398 nitrogen source (including ammonium) all three *MEP* genes are repressed. With a poor nitrogen
399 source, *MEP2* expression is much higher than *MEP1* and *MEP3* expression (Beltran et al., 2004;
400 Marini et al., 1997). The data agreed with the differences in the amino acid assimilation rate among
401 strains. In particular in CNC the strains that grew and fermented faster during exponential grow
402 phase, namely P304.4 and P301.4, were those removing earlier the nitrogen catabolite repression.
403 Thus, our results suggested that in the case of the vineyard strains tested in this work the strategy

that these yeasts adopted to colonize the must included the ability to assimilate nitrogen. In fact the increased competitiveness found in vineyard strains when nitrogen was limited could be related to the higher nitrogen assimilation rate in these yeasts. In particular, P304.4, the strain with the highest nitrogen demand, codominated the fermentation with the commercial strain. As in natural must several strains can be present simultaneously during fermentation (this was the case when the vineyard strains were isolated), those with the highest nitrogen demand could quickly remove nitrogen that is no longer available, reducing cell growth and fermentation performance of the less demanding yeasts. Finally, many strategies are known to be adopted by yeasts to dominate enological environments, among them the killer toxin production is one of the most studied (de Ullivarri et al., 2014; Fleet, 2003; Marquina et al., 2002). Strain P301.4 was sensitive to the killer toxin produced by the commercial strain; therefore, when it was co-inoculated with QA23 in CNC, it was not detected during fermentation. Notwithstanding, when LNC was tested, P301.4 partially recovered the competition ability reaching up to 20% of the total population after 48 h. In conclusion, our findings indicate that a high nitrogen assimilation rate is an additional strategy to prevail during fermentations where multiple strains are present. Thus, this is an aspect to take into consideration when selecting yeast that have to outgrow natural microbiota that could be present. Furthermore, considering some current winemaking tendencies of inoculating different strains of *Saccharomyces* the presence of nitrogen in natural musts as well as the different yeast requirements are clearly a characteristic that has to be determined before its use. This aspect is probably more important than other aspects such as killer phenotype, for instance. In fact, this last aspect is only relevant in strains that are killer sensitive, as in our case was strain 301.4.

425

426 **Acknowledgements**

427 The research financial support was provided by Provincia di Treviso, project PSR BIODILIEVITI
428 (Regione Veneto, 2013-2015), MIUR (Ministero dell'Istruzione, dell'Università e della Ricerca)
429 project number 60A08-4840/13. Dr Chiara Vendramini's fellowship was partially provided by
430 OMJ/MOY (2013-2014) OENODOC-MED.

431 **References**

- 432 Barriuso, J., 2015. Quorum sensing mechanisms in fungi. AIMS Microbiol. 1 (1), 37-47.
- 433
- 434 Bauer, F. F., Pretorius, I. S., 2000. Yeast stress response and fermentation efficiency: how to
435 survive the making of wine - A review. S. Afr. J. Enol. Vitic. 21, 27–51.
- 436
- 437 Bell, S. J., Henschke, P. A., 2005. Implications of nitrogen nutrition for grapes, fermentation and
438 wine. Aust. J. Grape Wine Res. 11, 242–295. doi:10.1111/j.1755-0238.2005.tb00028.x
- 439
- 440 Beltran, G., Novo, M., Rozès, N., Mas, A., Guillamón, J. M., 2004. Nitrogen catabolite repression
441 in *Saccharomyces cerevisiae* during wine fermentations. FEMS Yeast Res. 4, 625–632.
- 442
- 443 Beltran, G., Esteve-Zarzoso, B., Rozès, N., Mas, A., Guillamón, J. M., 2005. Influence of the
444 timing of nitrogen additions during synthetic grape must fermentations on fermentation kinetics and
445 nitrogen consumption. J. Agr. Food Chem. 53(4), 996-1002.
- 446
- 447 Cavazza, A., Grando, M., Zini, C., 1992. Rilevazione della flora microbica di mosti e vini.
448 Vignevisini 9, 17-20.
- 449
- 450 Chen, H., Fujita, M., Feng, Q., Clardy, J., & Fink, G. R., 2004. Tyrosol is a quorum-sensing
451 molecule in *Candida albicans*. P. Natl. Acad. Sci. USA 101 (14), 5048–5052.
452 <http://doi.org/10.1073/pnas.0401416101>
- 453
- 454 Chiva, R., Baiges, I., Mas, A., Guillamon, J. M., 2009. The role of *GAP1* gene in the nitrogen
455 metabolism of *Saccharomyces cerevisiae* during wine fermentation. J. Appl. Microbiol. 107 (1),
456 235-44. doi: 10.1111/j.1365-2672.2009.04201.x.
- 457

458 Ciani, M., Comitini, F., 2015. Yeast interactions in multi-starter wine fermentation. Curr. Opin.
 459 Food Sci. 1, 1–6.

460

461 Ciani, M., Capece, A., Comitini, F., Canonico, L., Siesto, G., Romano, P., 2016. Yeast interactions
 462 in inoculated wine fermentation. Front. Microbiol. 7, 555. doi:10.3389/fmicb.2016.00555.

463

464 Deed, N. K., van Vuuren, H. J., Gardner, R. C., 2011. Effects of nitrogen catabolite repression and
 465 di-ammonium phosphate addition during wine fermentation by a commercial strain of *S. cerevisiae*.
 466 Appl. Microbiol. Biotechnol. 89 (5), 1537-1549. doi: 10.1007/s00253-011-3084-y.

467

468 de Ullivarri, M. F., Mendoza, L. M., Raya, R. R., 2014. Killer activity of *Saccharomyces cerevisiae*
 469 strains: partial characterization and strategies to improve the biocontrol efficacy in winemaking.
 470 Anton. van Leeuwenhoek. 106, 865-878. doi:10.1007/s10482-014-0256-7

471

472 Fleet, G. H., 2003. Yeast interactions and wine flavor. Int. J. Food Microbiol. 86, 11 – 22.

473

474 Fleet, G. H., Heard, G. M., 1993. Yeasts-growth during fermentation. In: Fleet, G.H. (Ed.), Wine
 475 Microbiology and Biotechnology. Harwood Academic Publishers, Chur, Switzerland, pp. 27–55.

476

477 Forsberg, H., Ljungdahl, O., 2001. Sensors of extracellular nutrients in *Saccharomyces cerevisiae*.
 478 Curr. Genet. 40, 91–109.

479

480 García-Ríos, E., Gutiérrez, A., Salvadó, Z., Arroyo-López, F. N., Guillamon, M., 2014. The fitness
 481 advantage of commercial wine yeasts in relation to the nitrogen concentration, temperature, and
 482 ethanol content under microvinification conditions. Appl. Environ. Microbiol. 80, 704–713.
 483 10.1128/AEM.03405-13

484

485 Gómez-Alonso, S., Hermosín-Gutiérrez, I., García-Romero, E., 2007. Simultaneous HPLC analysis
 486 of biogenic amines, amino acids, and ammonium ion as aminoenone derivatives in wine and beer
 487 samples. J. Agric. Food Chem. 55 (3), 608–613.

488

489 Gutiérrez, A., Beltran, G., Warringer, J., Guillamón, J. M., 2013. Genetic basis of variations in
 490 nitrogen source utilization in four wine commercial yeast strains. PLoS ONE 8 (6), e67166.
 491 <http://doi.org/10.1371/journal.pone.0067166>

492

493 Hall, R. A., De Sordi, L., Maccallum, D. M., Topal, H., Eaton, R., Bloor, J. W., Robinson, G. K.,
 494 Levin, L. R., Buck, J., Wang, Y., Gow, N. A., Steegborn, C., Mühlischlegel, F. A., 2010. CO₂ acts
 495 as a signalling molecule in populations of the fungal pathogen *Candida albicans*. PLoS Pathog. 6
 496 (11), e1001193. doi: 10.1371/journal.ppat.1001193.

497

498 Honigberg, S. M., 2011. Cell signals, cell contacts, and the organization of yeast
 499 communities. Eukaryot. Cell 10(4), 466–473. <http://doi.org/10.1128/EC.00313-10>

500

501 Hornby, J. M., Jensen, E. C., Lisec, A. D., Tasto, J. J., Jahnke, B., Shoemaker, R., Dussault,
 502 P., Nickerson, K. W., 2001. Quorum sensing in the dimorphic fungus *Candida albicans* is
 503 mediated by farnesol. Appl. Environ. Microbiol. 67 (7), 2982–2992.
 504 <http://doi.org/10.1128/AEM.67.7.2982-2992.2001>

505

506 Jiménez-Martí, E., Aranda, A., Mendes-Ferreira, A., Mendes-Faia, A., Í del Olmo, M., 2007. The
 507 nature of the nitrogen source added to nitrogen depleted vinifications conducted by a
 508 *Saccharomyces cerevisiae* strain in synthetic must affects gene expression and the levels of several
 509 volatile compounds. Anton. van Leeuwenhoek. 92(1), 61-75.

510

511 Jiranek, V., Langridge, P., Henschke, P. A., 1995. Amino acid and ammonium utilization by
 512 *Saccharomyces cerevisiae* wine yeasts from a chemically defined medium. Am. J. Enol. Viticult. 46
 513 (1), 75-83.
 514
 515 Jouhten, P., Ponomarova, O., Gonzalez, R., Patil, K. R., 2016. *Saccharomyces cerevisiae*
 516 metabolism in ecological context. FEMS Yeast Res. 16 (7), fow080.
 517 <http://doi.org/10.1093/femsyr/fow080>
 518
 519 Legras, J. L., Karst, F., 2003. Optimisation of interdelta analysis for *Saccharomyces*
 520 *cerevisiae* strain characterisation. FEMS Microbiol. Lett. 221, 249–255. doi:10.1016/S0378-
 521 1097(03)00205-2
 522
 523 Marini, A. M., Soussi-Boudekou, S., Vissers, S., Andre, B., 1997. A family of ammonium
 524 transporters in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 17, 4282–4293.
 525
 526 Marquina, D., Santos, A., Peinado, J. M., 2002. Biology of killer yeasts. Int. Microbiol. 5 (2), 65-
 527 71.
 528
 529 Martínez-Moreno, R., Morales, P., Gonzalez, R., Mas, A., Beltran, G., 2012. Biomass production
 530 and alcoholic fermentation performance of *Saccharomyces cerevisiae* as a function of nitrogen
 531 source. FEMS Yeast Res. 12 (4), 477-485. doi: 10.1111/j.1567-1364.2012.00802.x
 532
 533 Mas, A., Guillamon, J. M., Torija, M. J., Beltran, G., Cerezo, A. B., Troncoso, A. M., Garcia-
 534 Parrilla, M.C., 2014. Bioactive compounds derived from the yeast metabolism of aromatic amino
 535 acids during alcoholic fermentation. BioMed. Res. Int. 2014, 898045.
 536 <http://doi.org/10.1155/2014/898045>.
 537

538 Nardi, T. Carlot, M. De Bortoli, E., Corich, V. Giacomini, A., 2006. A rapid method for
539 differentiating *Saccharomyces sensu stricto* strains from other yeast species in an enological
540 environment. FEMS Microbiol. Lett., 264, 168-173. doi: 10.1111/j.1574-6968.2006.00450.x
541

542 Nickerson, K. W., Atkin, A. L., Hornby, J.M., 2006. Quorum sensing in dimorphic fungi: farnesol
543 and beyond. Appl. Environ. Microbiol. 72, 3805–3813.
544

545 Palková, Z., Váchová, L., 2006. Life within a community: benefit to yeast long-term survival.
546 FEMS Microbiol. Rev. 30 (5), 806-824.
547

548 Pérez, F., Ramírez, M., Regodón, J. A., 2001. Influence of killer strains of *Saccharomyces*
549 *cerevisiae* on wine fermentation. Anton. Van Leeuwenhoek. 79 (3), 393-399.
550

551 Perrone, B., Giacosa, S., Rolle, L., Cocolin, L., Rantsiou, K., 2013. Investigation of the dominance
552 behavior of *Saccharomyces cerevisiae* strains during wine fermentation. Int. J. Food Microbiol. 165
553 (2), 156-162. doi: 10.1016/j.ijfoodmicro.2013.04.023.
554

555 Povhe Jemec , K., Cadez , N., Zagorc, T., Bubic, V., Zupec, A., Raspor P., 2001. Yeast population
556 dynamics in five spontaneous fermentations of Malvasia must. Food Microbiol. 18 (3), 247-259.
557

558 Ribereau-Gayon, P., Dubourdieu, D., Doneche, B., Lonvaud, A., 2006. Handbook of enology. Vol.
559 1. The microbiology of wine and vinifications, 2nd edn. John Wiley and Sons, New York, NY.
560

561 Riou, C., Nicaud, J. M., Barre, P., Gaillardin, C., 1997. Stationary-phase gene expression in
562 *Saccharomyces cerevisiae* during wine fermentation. Yeast 13 (10), 903–915.
563

564 Santamaría, P., Garijo, P., López, R., Tenorio, C., Gutiérrez, A. R., 2005. Analysis of yeast
 565 population during spontaneous alcoholic fermentation: effect of the age of the cellar and the
 566 practice of inoculation. *Int. J. Food Microbiol.* 103 (1), 49–56.

567

568 Sierkstra, L. N., Verbakel, J. M., Verrips, C. T., 1992. Analysis of transcription and translation of
 569 glycolytic enzymes in glucose-limited continuous cultures of *Saccharomyces cerevisiae*. *J. Gen.*
 570 *Microbiol.* 138(12), 2559-2566.

571

572 Swiegers, J. H., Bartowsky, E. J., Henschke, P. A., Pretorius, I. S., 2005. Yeast and bacterial
 573 modulation of wine aroma and flavour. *Aust. J. Grape Wine R.* 11 (2), 139-173.

574

575 Ter Schure, E. G., van Riel, N. A. W., Verrips, C.T., 2000. The role of ammonia metabolism in
 576 nitrogen catabolite repression in *Saccharomyces cerevisiae*. *FEMS Microbiol. Rev.* 24, 67–83.

577

578 Torija, M. J., Rozes, N., Poblet, M., Guillamón, J. M., Mas, A., 2001. Yeast population dynamics in
 579 spontaneous fermentations: comparison between two different wine-producing areas over a period
 580 of three years. *Anton. van Leeuwenhoek.* 79 (3), 345-352.

581

582 Torija, M. J., Beltran, G., Novo, M., Poblet, M., Guillamón, J. M., Mas, A., Rozès, N., 2003.
 583 Effects of fermentation temperature and *Saccharomyces* species on the cell fatty acid composition
 584 and presence of volatile compounds in wine. *Int. J. Food Microbiol.* 85 (1-2), 127-136.

585

586 Treu, L., Toniolo, C., Nadai, C., Sardu, A., Giacomini, A., Corich, V., Campanaro, S., 2014 a. The
 587 impact of genomic variability on gene expression in environmental *Saccharomyces cerevisiae*
 588 strains. *Environ. Microbiol.* 16, 1378–1397.

589

590 Treu, L., Campanaro, S., Nadai, C., Toniolo, C., Nardi, T., Giacomini, A., Valle, G., Blondin, B.,
591 Corich, V., 2014 b. Oxidative stress response and nitrogen utilization are strongly variable in
592 *Saccharomyces cerevisiae* wine strains with different fermentation performances. Appl Microbiol
593 Biotechnol 98 (9), 4119–4135. doi:10.1007/s00253-014-5679-6.

594

595 Varela, C., Pizarro, F., Agosin, E., 2004. Biomass content governs fermentation rate in nitrogen-
596 deficient wine musts. Appl. Environ. Microbiol. 70 (6), 3392–3400.

597

598 Vilanova, M., Ugliano, M., Varela, C., Siebert, T., Pretorius, I.S., Henschke, P.A., 2007.
599 Assimilable nitrogen utilisation and production of volatile and non-volatile compounds in
600 chemically defined medium by *Saccharomyces cerevisiae* wine yeasts. Appl. Microbiol.
601 Biotechnol. 77 (1), 145-157.

602

603 Volodyaev, I.V., Krasilnikova, E.N., Ivanovsky, R.N., 2013. CO₂ mediated interaction in yeast
604 stimulates budding and growth on minimal media. PLoS One 8(4), e62808. doi:
605 10.1371/journal.pone.0062808.

606

607 Wang, C., Mas, A., Esteve-Zarzoso, B., 2015. Interaction between *Hanseniaspora uvarum* and
608 *Saccharomyces cerevisiae* during alcoholic fermentation. Int. J. Food Microbiol. 206, 67-74.

609

610 Weber, A., Prokazov, Y., Zuschratter, W., Hauser, M. J. B., 2012. Desynchronisation of glycolytic
611 oscillations in yeast cell populations. PLoS One 7 (9), e43276.

612

613 **FIGURE LEGENDS**

614 **Fig. 1.** Population evolution during single-strain and mixed fermentations in CNC (A) and LNC
615 (B). □ P301.4, △ P304.4, ◇ P254.12, ○ QA23, × QA23-P301.4, + QA23-P304.4, * QA23-
616 P254.12, – QA23-P301.4-P304.4-P254.12.

617

618 **Fig. 2.** Must density and population evolution during single-strain fermentations in CNC (A) and
619 LNC (B). Continuous lines describe the evolution of the cell concentration (CFU/ml), dashed lines
620 must density. □ P301.4, △P304.4, ◇P254.12, ○ QA23.

621

622 **Fig. 3.** Ammonium (A) and yeast assimilable nitrogen (YAN) (B) concentration during single-strain
623 fermentations in CNC. □ P301.4, △P304.4, ◇P254.12, ○ QA23.

624

625 **Fig. 4.** Ammonium and amino acids consumption visualized by means of the hierarchical clustering
626 program MULTIEXPERIMENT VIEWER at different sampling points during single-strain
627 fermentations in CNC (A) and LNC (B). In the heat map, for each nitrogen source, each value is the
628 ratio between the consumed and the initial concentration. Color scale is reported on top of the
629 figure: black = no consumption, blue =low consumption, red = total consumption.

630

631 **Fig. 5.** Expression of *MEP2* gene during single-strain fermentations in CNC (A) and LNC (B). The
632 data were quantified by calculating the ratio between the concentration of the studied genes
633 normalized with the concentration of the housekeeping genes *ACT1* and *IPP1*, and expressed as
634 percentage (the quantity ratio 1 was set as 100%) setting the expression obtained by the strain QA23
635 at t=12 in CNC as 100%. ■ P301.4, ■ P304.4, ■ P254.12, ■ QA23.

636

637 **Fig. 6.** Expression of *GAPI* gene during single-strain fermentations in CNC (A) and LNC (B). The
638 data were quantified by calculating the ratio between the concentration of the studied genes
639 normalized with the concentration of the housekeeping genes *ACT1* and *IPP1*, and expressed as

640 percentage (the quantity ratio 1 was set as 100%) setting the expression obtained by the strain QA23
641 at t=12 in CNC as 100%. ■ P301.4, ■ P304.4, ■ P254.12, ■ QA23.

642

643 **FIGURES AND TABLES**

644 **Figure 1**

645 **A**

646

647 **B**

648

649 **Figure 2**

650 **A**

652 **B**

655 **Figure 3**

656 **A**

657

658 **B**

659

660

661

662

663 **Figure 4**

664 **A**

665

666 **B**

667

668 **Figure 5**

669 **A**

670

671 **B**

672

673

674 **Figure 6**

675 **A**

676

677 **B**

678

679

680 **Table 1** Strains frequency evaluated at three sampling points (12, 24 and 48 hours) during mixed
681 fermentations in CNC.

Co-fermentation	Strain presence (percentage)			
	T0	T12	T24	T48
QA23/P301.4	50/50	100/0	100/0	100/0
QA23/P304.4	50/50	70/30	70/30	50/50
QA23/P254.12	50/50	75/25	70/30	95/5
QA23/P301.4/P304.4/P254.12	25/25/25/25	44/0/30/26	44/0/32/24	48/4/8/40

682
683 **Table 2** Strains frequency evaluated at three sampling points (12, 24 and 48 hours) during mixed
684 fermentations in LNC.

Co-fermentation	Strain presence (percentage)			
	T0	T12	T24	T48
QA23/P301.4	50/50	90/10	95/5	80/20
QA23/P304.4	50/50	60/40	45/55	40/60
QA23/P254.12	50/50	40/60	50/50	45/55
QA23/P301.4/P304.4/P254.12	25/25/25/25	30/0/30/40	40/0/25/35	35/10/25/30

685
686 **SUPPLEMENTARY MATERIAL LEGENDS**

687
688 **Supplementary Table S1.** Concentrations of amino acids, ammonia and YAN in fermenting musts
689 (expressed as mg N/l) during single-strain fermentations in CNC measured after 6 hours (T6), 9
690 hours (T9), 12 hours (T12), 15 hours (T15), 18 hours (T18), 21 hours (T21), 24 hours (T24) and 30
691 hours (T30) from fermentation start.

692
693 **Supplementary Table S2.** Concentrations of amino acids, ammonia and YAN in fermenting musts
694 (expressed as mg N/l) during single-strain fermentations in LNC measured after 12 hours (T12) and
695 24 hours (T24) from fermentation start.