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

2    ***cerevisiae* strains**

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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 at 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 autocthonous 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

## 2.2 Culture media and growth condition

Strains were routinely grown in YPD at 25°C. Fermentations were carried out in a synthetic grape must (pH 3.3) as described by Riou et al. (1997). In this medium two different nitrogen concentrations were used. In control nitrogen condition (CNC) the yeast assimilable nitrogen (YAN) content in the synthetic grape must was 440 mg N/l, ammoniacal nitrogen ($NH_4Cl$) 120 mg N/l, and amino acids 320 mg N/l (taking into account all the N present in the amino acids). To mimic grape must composition this medium also contained 426 mg/l of proline, that is not part of the assimilable nitrogen. The low nitrogen condition (LNC) contained 1/5-fold (88 mg N/l) of the YAN present in CNC at the same ammoniacal and amino-acid proportion.

## 2.3 Fermentation trials

### 2.3.1 Pre-culture preparation

Each yeast strain was grown on YPD solid medium at 25°C for 3 days. A loopful of yeast cells was used to inoculate 30 ml of YPD liquid medium. The culture was incubated for 24 hours at 25 °C to reach the stationary phase (about $10^7$-$10^8$ cells/ml). Yeast concentration was measured by counting the cells in a Thoma chamber using a phase contrast microscope (Olympus).

In pairwise-strains fermentations cell concentration present in each pre-culture was used to calculate the suitable volume of the inoculum to reach, for each strain, a final concentration of $1x10^6$ cells/ml. In co-fermentations where all the four strains were present simultaneously, the cell concentration for each strain was of $5x10^5$ cells/ml. In single-strain fermentation, for each strain, the concentration was $2x10^6$ cells/ml.

### 2.3.2 Fermentation conditions

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

162    Kinetics were monitored by daily measuring weight loss or must density. Fermentations were

163    considered completed when the daily weight loss was lower than 0.1 g within 24 hours. In the

164    single-strain fermentations where nitrogen consumption and yeast gene expression were detected,

165    due to the large number of samplings, a continuous orbital shaking (150 rpm) was performed during

166    the trial. The continuous shaking ensured an optimal collection of cell samples.  Fermentations were

167    performed in triplicate and the whole experimental set was repeated twice.  Yeast cell concentration

168    during fermentation was determined by plate counts on YPD medium. To perform real-time

169    quantitative PCR, cell samples were collected throughout the fermentation at different time points

170    and harvested by centrifugation, frozen in liquid nitrogen and stored at -80 °C. Supernatant was also

171    stored at -20 °C to analyze the content of nitrogen and some fermentation products.

172    During mixed fermentations, at three sampling points (12, 24, 48 hours) yeast isolation was carried

173    out on YPD plates.  At each sampling, a total of 20 colonies, randomly chosen from isolation plates

174    from the same dilution series, was submitted to amplification of inter-δ region.

175

176    *2.4 Nitrogen content analysis*

177    Analysis of ammonia and individual amino acids was determined by diethyl

178    ethoxymethylenemalonate (DEEMM) derivatization (Gómez-Alonso et al., 2007), using the Agilent

179    1100 Series HPLC (AgilentTechnologies, Germany). Separation was performed in an ACE HPLC

180    column (C18-HL), particle size 5 μm (250 mm × 4.6 mm) thermostatized at 20 °C. The

181    concentration of each compound was calculated using internal (L-2-aminoadipic acid, 1 g/l) and

182    external standards, and expressed as mg N/l. The software used was Agilent ChemStation Plus

183    (Agilent Technologies, Germany).

184

185    *2.5 PCR-amplification of inter-delta sequences*

186    Cell suspension for DNA Amplification was performed as described by Nardi et al. (2006). Two

187    microlitres of the cell suspension were used for PCR amplification. Oligonucleotides primers

188    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 transilumination. 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

8

216     recommended by Applied Biosystems.  Each sample had two controls, which were run in the same

217     quantitative PCR: NAC (No Amplification Control; sample without reverse transcriptase reaction)

218     to avoid the interference by contaminant genomic DNA and NTC (No Template Control; sample

219     without RNA template) to avoid interference by primer-dimer formation. Relative gene expression

220     was determined using the 2-$\Delta\Delta$Ct formula, where Ct is defined as the cycle at which fluorescence is

221     determined to be statistically significantly above background; $\Delta$Ct is the difference in Ct of the gene

222     of interest and Ct of the housekeeping gene (ACT1); and $\Delta\Delta$Ct is the difference in $\Delta$Ct at time = t

223     and $\Delta$Ct at time = 12 h in the non-limiting condition (300 mg N/L) for strain QA23, used as

224     reference. All samples were analysed in duplicate and the expression values were averaged by the

225     analysis software (Applied Biosystems, USA). (Gutiérrez et al., 2013). The coefficient of variation

226     in all samples analysed was less than 20 %.

227

228     *2.7 Chemical analyses*

229     Glucose, fructose, glycerol, acetic acid and ethanol were quantified using commercial enzymatic

230     kits (Roche Diagnostics, Germany). The pH was determined using a pH-meter Crison MicropH

231     2000 (Crison, Barcelona, Spain).

232

233     *2.8 Statistical analysis*

234     The software XLSTAT, vers.7.5.2, was used to perform the analysis of variance (one-way

235     ANOVA), followed by the Tukey "post-hoc" test. The analysis was conducted by comparing the

236     averages of three independent replications and differences were considered statistically significant

237     for *p*-value less than 0.05. Visualization of the amino-acids consumption data was obtained by

238     means of the hierarchical clustering program, MULTIEXPERIMENT VIEWER (MeV, Rockville,

239     MD, USA). The clustering process was carried out with Pearson distance and the average linkage

240     method.

241

242     **3. Results**

243

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

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

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

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

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

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

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

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

condition (LNC).

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

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

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

by QA23. The $CO_2$ production and cell concentration were determined during all fermentation and

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

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

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

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

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

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

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

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

showed a lower cell concentration than the others.


*3.Strain competition during mixed fermentations*

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

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

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270 analyzed during these early periods (12, 24 and 48 h). In the CNC condition, the commercial strain

271 took over the other strains, dominating the early stages up to 48 h, when no more nitrogen was

272 available (Table 1). This was also true when the four strains were co-inoculated together, where the

273 percentage of the commercial strain was always close to 50%. Only one of the vineyard strains

274 (P304.4) seemed to be almost as competitive as QA23, reaching higher populations after 48h either

275 when pairwise fermentation or co-fermentation of the 4 strains were performed. Instead, when the

276 fermentations were in presence of limiting concentrations of nitrogen, yeast populations changed

277 considerably. All the vineyards strains improved their competition ability, as their frequencies

278 increased during the first 48 hours of fermentation. The strain sensitive to the killer toxin, P301.4,

279 although to a lesser extent, improved competition performances, as well (Table 2). This last strain

280 that immediately disappeared in CNC presented up to 20% of the total population in LNC. In the

281 mixed fermentations with all the strains, three of the strains (two vineyard and the commercial) co-

282 dominated the fermentation, with similar cell levels.

283

284 *3.3 Nitrogen consumption and expression of genes under nitrogen catabolic repression during*

285 *single-strain fermentations*

286 To better understand the ability of each strain to utilize the nitrogen source present in the synthetic

287 musts the single-strain fermentations were run again in both conditions and the nitrogen metabolism

288 analyzed further.

289 At CNC (Fig. 2A) sugar consumption during tumultuous fermentation was significantly higher (*p*-

290 values < 0.05) up to T48 sampling in vineyard strains than in the commercial strain. However, at

291 the end of fermentation the commercial strain was faster, ending the fermentation at the same time

292 as all the strains. When LNC was tested (Fig. 2B), the fermentation rate was very much slower than

293 in CNC. Although the starting of the fermentation (up to T24 sampling) was similar and without

294 significant differences in sugar consumption between strains, shortly after the start, the strain

295 P304.4 was significantly slower than the rest of the strains with a reduced viable cell population

296 during the growth stationary phase. The commercial strain was the fastest yeast to finish the

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

No significant differences among strains were found at the end of fermentation in ethanol and acetic acid production at both CNC and LNC (data not shown). However, in the latter condition, glycerol concentration was significantly higher in P254.12 and P304.4 (6.60 and 6.85 g/l respectively) than in P301.4 and QA23 (6.00 and 5.60), despite that no significant differences were found in CNC. Consumption of ammonium and yeast assimilable nitrogen (YAN) was monitored throughout these fermentations. In CNC (Fig. 3) YAN available in the must was assimilated within 24-30 hours and the commercial strain showed a slower nitrogen consumption than the vineyard strains. Ammonium was completely consumed within 21 hours by the vineyard strains whereas the commercial control needed 24 hours. After that, in the vineyard strain fermentations, YAN dropped to 4.18-4.72 g/l, a residual concentration mainly composed by cysteine. At the same sampling time the YAN concentration in the commercial strain fermentation was 82.7 mg N/l (data not shown). At CNC the consumption of each amino acid was estimated by calculating the difference with the initial concentrations. The heatmap analysis (Fig. 4A; Supplementary table S1) showed a conserved pattern among strains, with some amino acids that were generally more rapidly consumed (lysine, methionine, threonine, leucine, serine, glutamine, histidine, aspartic acid) than others. Amino-acid consumption patterns revealed that vineyard strains consumed the nitrogen sources (amino acid and ammonium) more rapidly than the commercial QA23. Several clusters in consumption patterns could be identified. The main cluster included the preferred nitrogen sources and was composed by ammonium, valine, glutamate, aspartate, phenylalanine, histidine, glutamine, serine, leucine, threonine and methionine. This group of amino acids was mostly taken by the autochthonous strains by 15-18 h, whereas the uptake by the commercial strain was after 21 h. However, a couple of subgroups can be seen in this cluster, regarding the early consumption: the subgroup formed by valine, glutamate, aspartate and phenylalanine, that are taken up after 9 h, and the rest that are consumed from the first sampling time (6 h). A second group was formed by tryptophan, tyrosine, arginine and alanine, that are consumed completely by 21-24 h by the autochthonous strains and by

324    30 h in the commercial strain. Glycine is similar to this last group, although a little bit delayed.

325    Finally cysteine remained in considerable amounts after 30 h, independently of the strain.

326    In LNC ammonium was completely depleted after 12 h in all fermentations and the amino acid

327    concentrations were notably reduced (Fig.4B; Supplementary table S2). After 12 hours vineyard

328    strains had totally consumed most of the amino acids that were early consumed in CNC (serine,

329    glutamine, histidine, valine, leucine isoleucine phenyalanine) together with glycine, alanine and the

330    most abundant arginine, that at CNC was totally consumed in 21-24 hours. Instead, the commercial

331    strain showed a lower amino-acid consumption rate as at 12 hours tryptophan, alanine, glycine,

332    phenylalanine, valine and throsine were still present in the must at high concentrations. All the

333    strains left cysteine concentration unchanged in the medium as previously found in CNC.

334    Methionine and lysine were generally less consumed than in CNC.

335    To better understand the different fermentation behaviors and nitrogen assimilation rates between

336    the strains, the expression of *MEP2* and *GAP1* genes, both controlled by nitrogen catabolite

337    repression, was investigated (Fig. 5 and 6). The ammonium permease encoded by *MEP2* is

338    activated when ammonium is very low. This allows the complete consumption of ammonium. In

339    both CNC and LNC, P304.4 and P301.4, the two vineyard strains that presented the lowest

340    fermentation rates in LNC, showed the earliest and the strongest activation of *MEP2*. In CNC this

341    was particularly evident at 72 hours, while in LNC the increased expression was after 48 hours.

342    The general amino-acid permease *GAP1* enhances the assimilation of amino acids, increasing the

343    transport of those that do not have specific permeases that are constitutively expressed.  The *GAP1*

344    gene expression trend for each strain was very similar to that observed for *MEP2*, although the

345    levels of expression were much higher than those detected for *MEP2*. The commercial strain

346    showed in all the cases the weakest increase of gene expression levels.

347

348    **4. Discussion**

349    Although most of the studies on strain competition have been done with non-*Saccharomyces* yeasts,

350    some have centered the interest in the interaction between *Saccharomyces* strains (revised by Ciani

351    et al., 2016). In fact, nutrient availability and specifically nitrogen demand has been pointed out as

352    one of the mechanisms underlying situations of dominance or taking over from some strains over

353    others (García-Rios et al., 2014).  For this reason, we used the *S. cerevisiae* strain QA23 that is well

354    known to have a low nitrogen demand and strong ethanol resistance during vinification and also

355    produce a killer toxin that improves its ability to colonize the enological environment (Beltran et al.,

356    2005; Jiménez-Martí et al., 2007). The vineyard yeasts P301.4, P304.4 and P254.12 were isolated

357    from single grape bunch fermentations. In each fermentation, co-domination of at least 3 strains was

358    found. In the case of P304.4 nine different strains were present simultaneously (Treu et al., 2014 a,

359    2014 b) P254.12 and P304.4 showed a neutral phenotype when QA23 killer toxin where present in

360    the growing medium, whereas P301.4 was sensitive. Our results, comparing the needs of the

361    different strains, suggested a strong implication of nitrogen assimilation ability on strain

362    competition during the growth in grape musts. In fact, the competition ability of the vineyard strains

363    in LNC was always higher than that found in CNC. Moreover, in CNC the strain P304.4 showed the

364    highest competition ability respect to QA23, evidenced by the same strain frequency at the end of

365    fermentation. The P304.4 competition ability increased during the fermentation, indicating better

366    performance when nitrogen was depleted. When nitrogen concentration was high only small

367    differences were found among the fermentation kinetics. Notwithstanding, the sugar consumption

368    rate of the commercial strain during exponential and early stationary phase was significantly slower

369    than those found for the other yeasts. This finding suggested that the lower nitrogen assimilation of

370    QA23, compared with vineyard strains, limits the fermentation rate when nitrogen is abundant. On

371    the contrary when low nitrogen was present strong differences in the fermentation kinetics were

372    evidenced. QA23 completed the fermentation earlier than the others. P304.4 was the slowest strain

373    evidencing the highest nitrogen demand. When ammonium and amino acids consumption was

374    determined, strong differences between vineyard strains and the commercial strain were found

375    confirming the lower nitrogen demand of the commercial strain.  In particular in CNC after 12

376    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.

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

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

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

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

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

**Fig. 6.** Expression of *GAP1* gene during single-strain fermentations in CNC (A) and LNC (B). The data were quantified by calculating the ratio between the concentration of the studied genes normalized with the concentration of the housekeeping genes *ACT1* and *IPP1*, and expressed as

24

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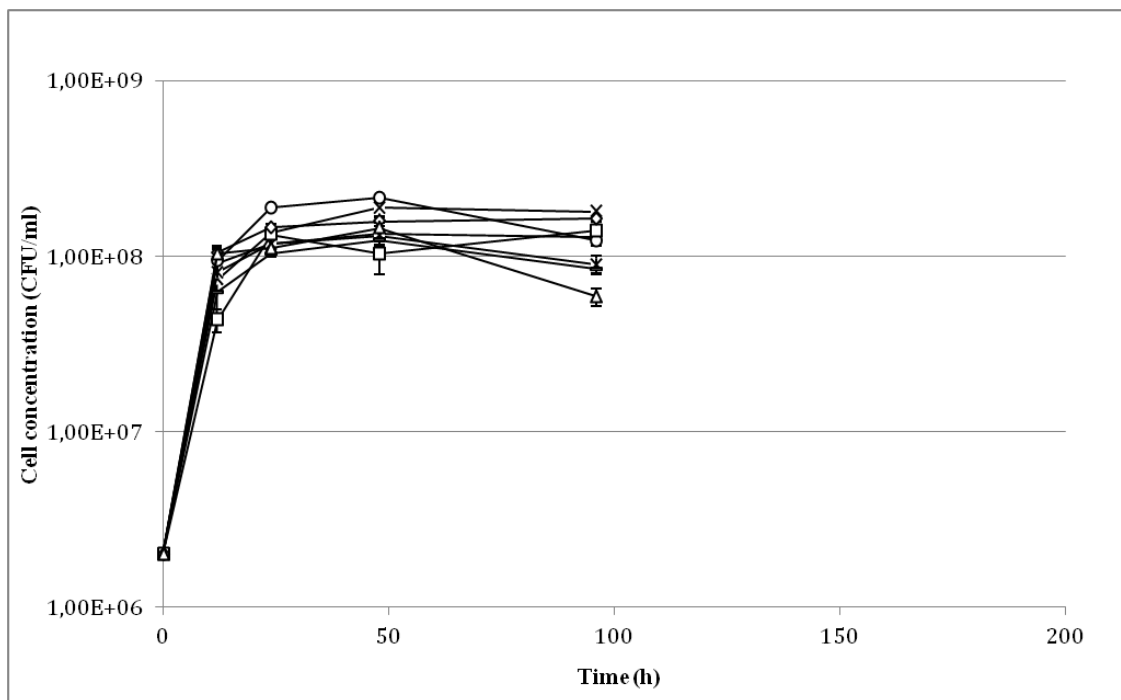
641 at t=12 in CNC as 100%. ■ P301.4, ■ P304.4, ■ P254.12, ■ QA23.

642

643 **FIGURES AND TABLES**
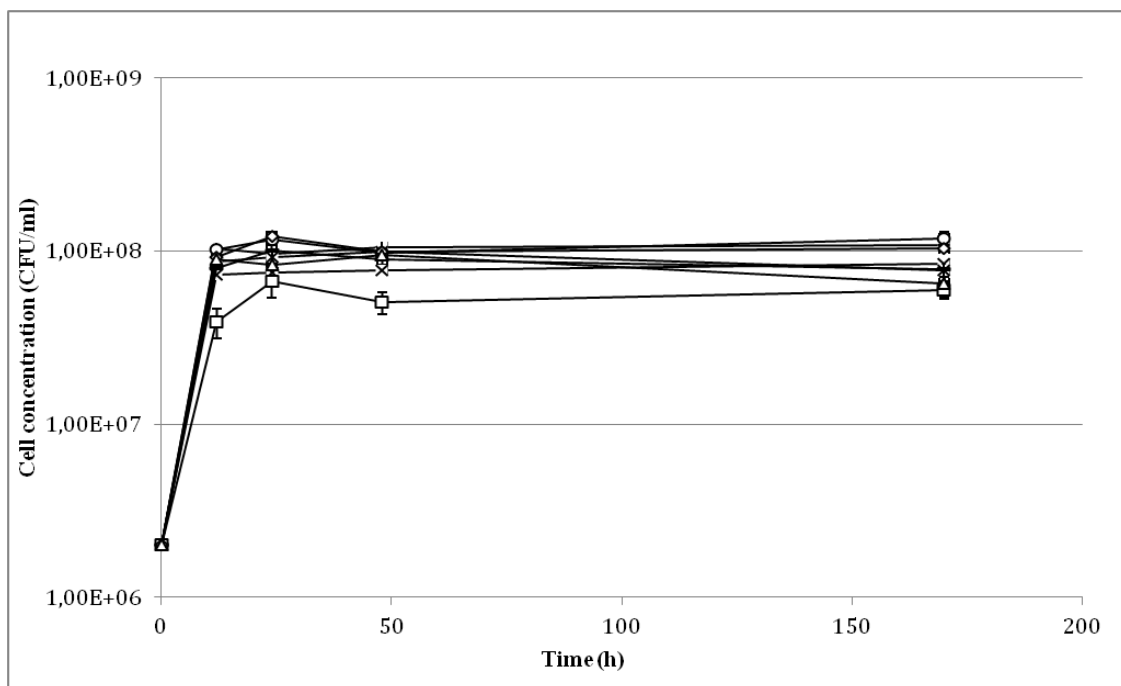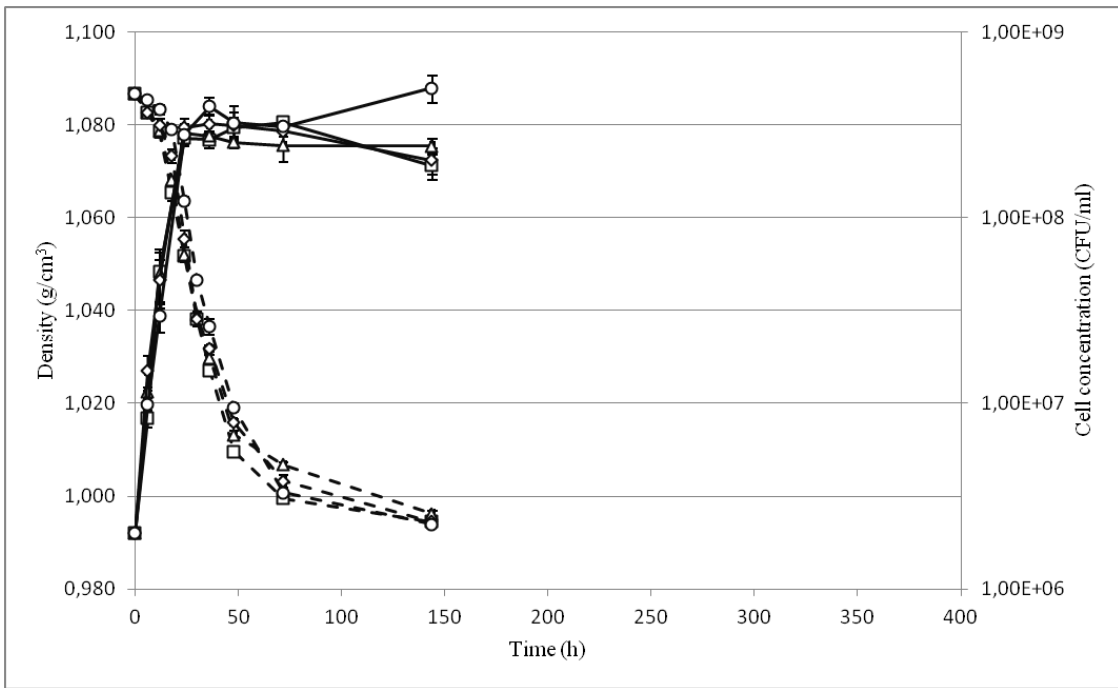
644 **Figure 1**

645 **A**



646

647 **B**

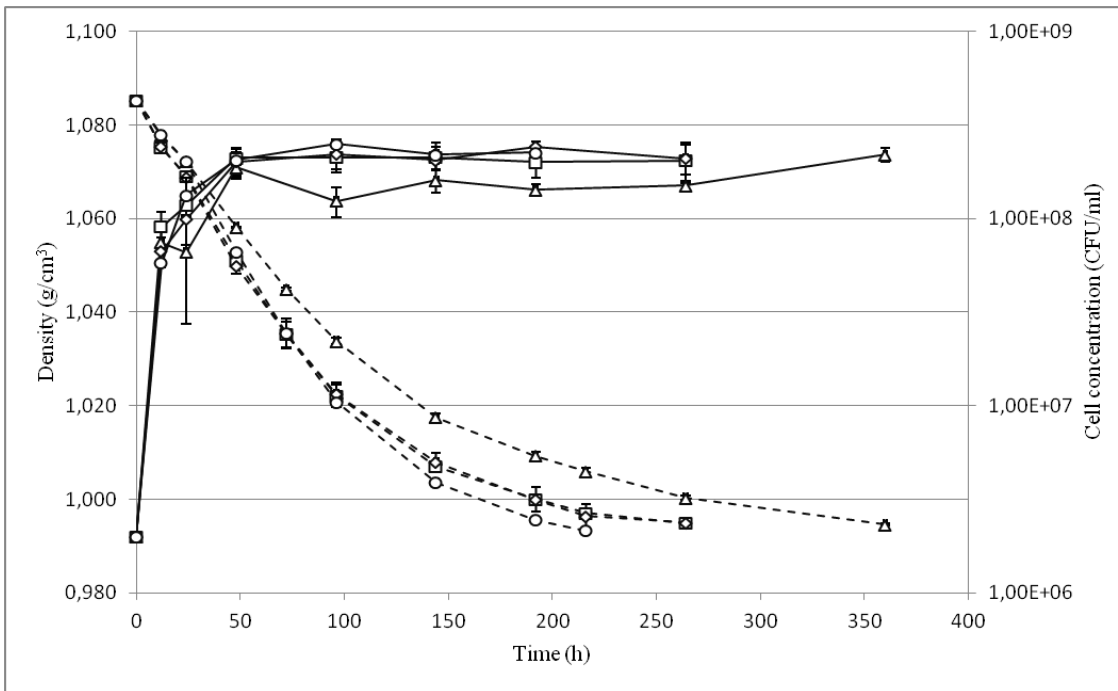

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649    **Figure 2**

650    **A**



651

652    **B**
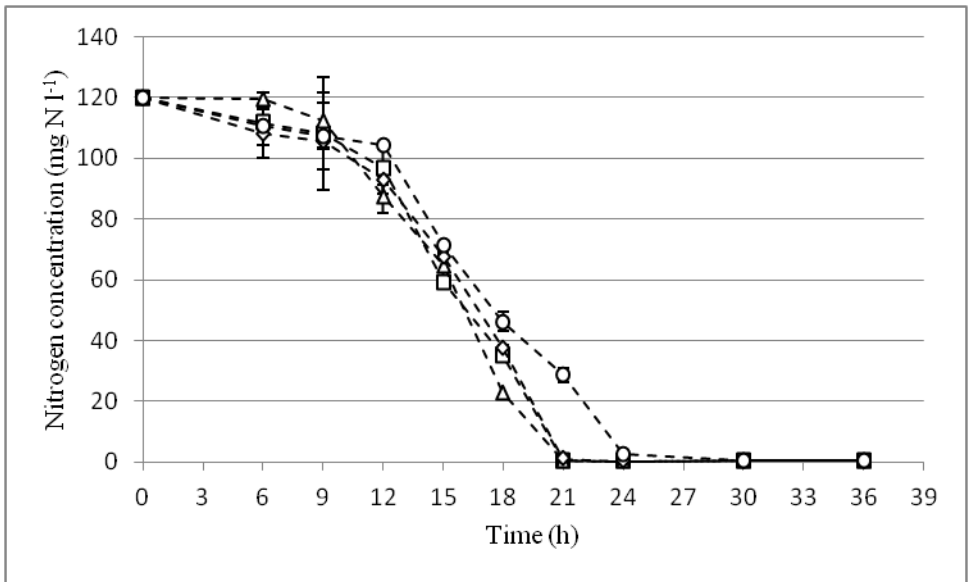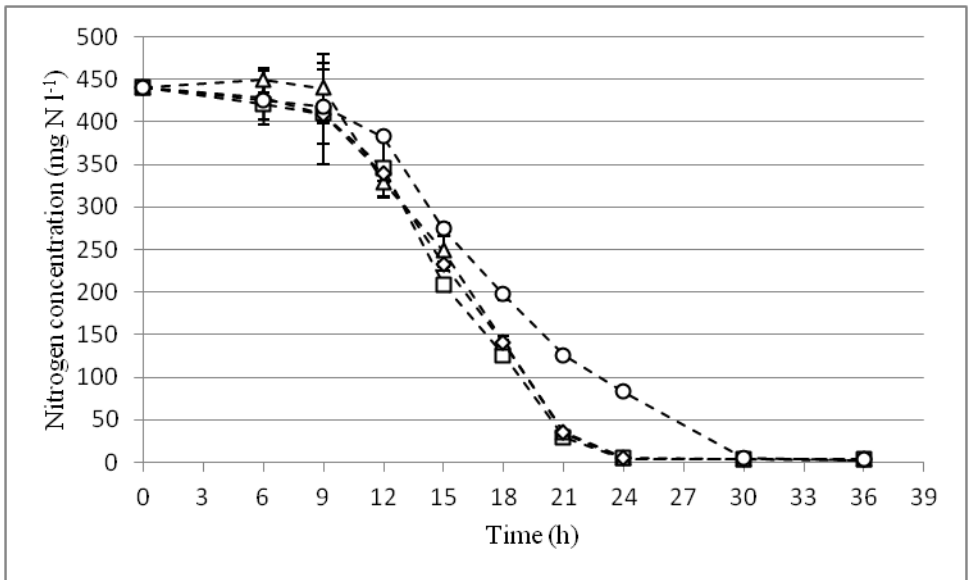


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655    **Figure 3**
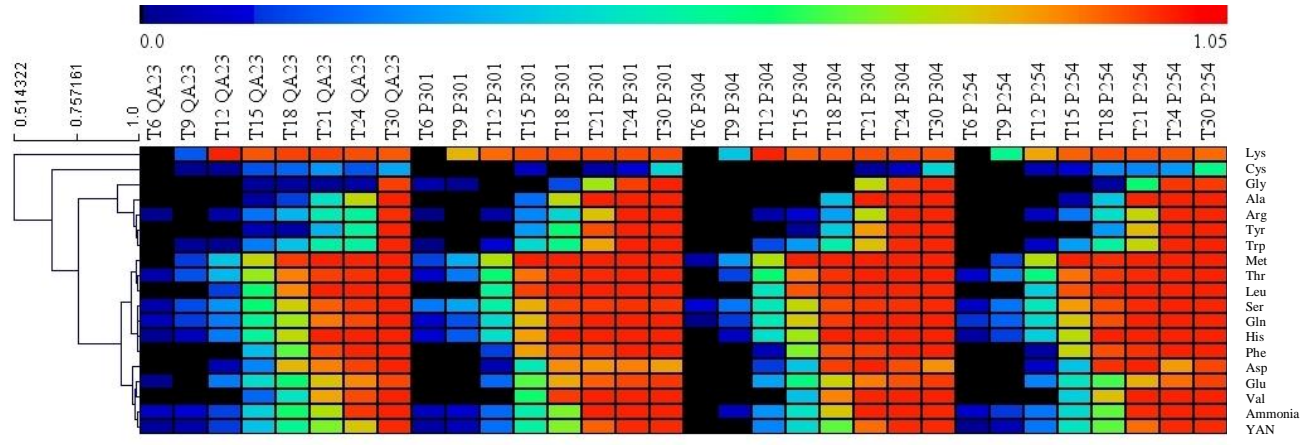
656    **A**

657

658 **B**



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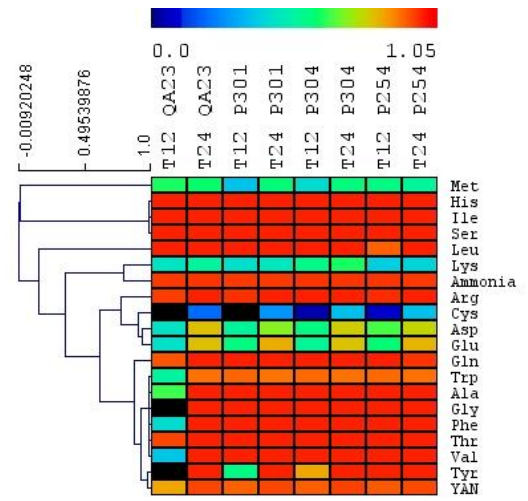
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663     **Figure 4**

664     **A**



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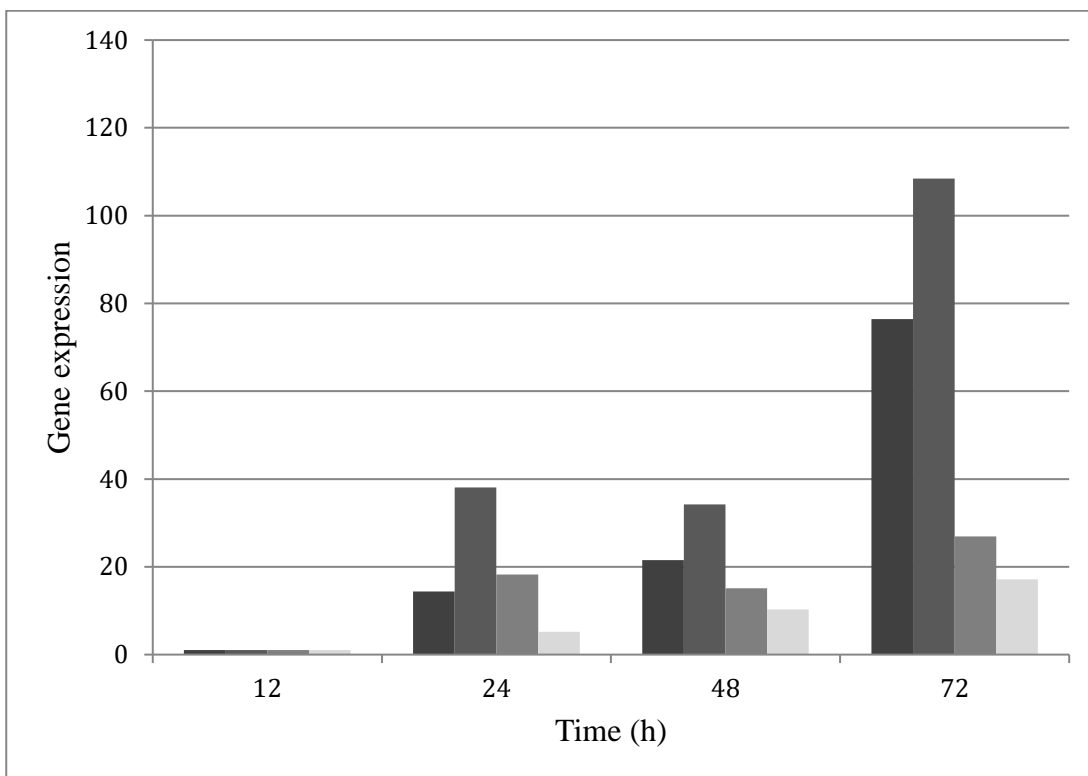
666     **B**



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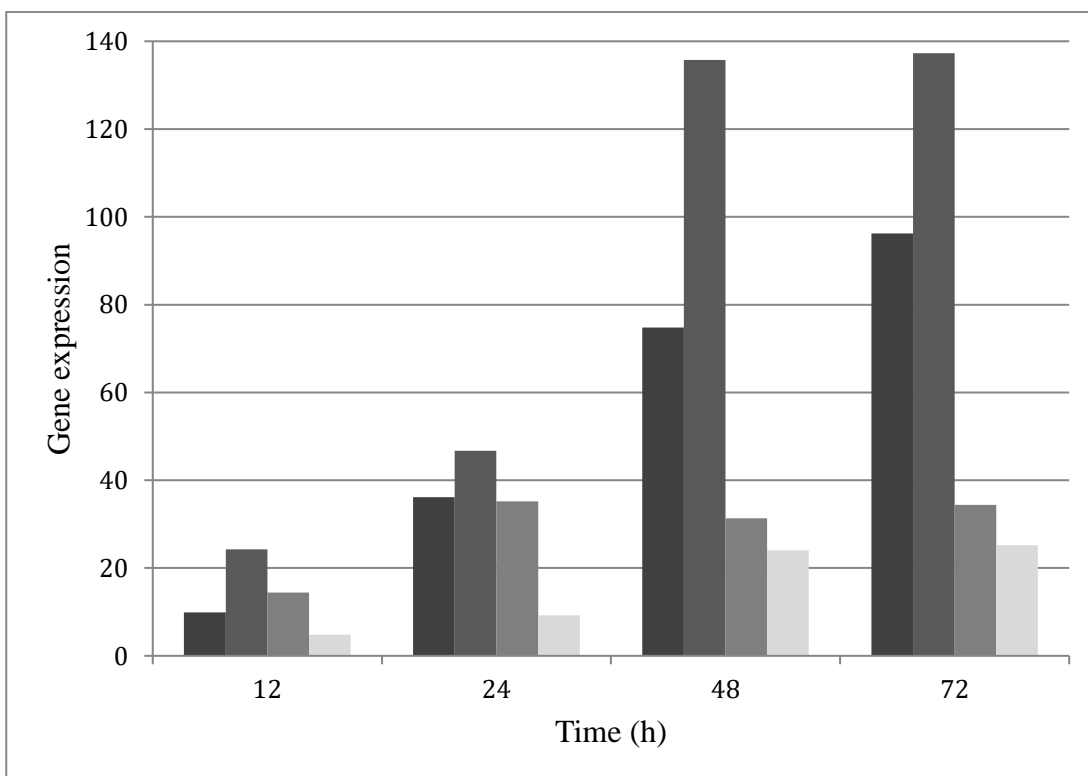668    **Figure 5**
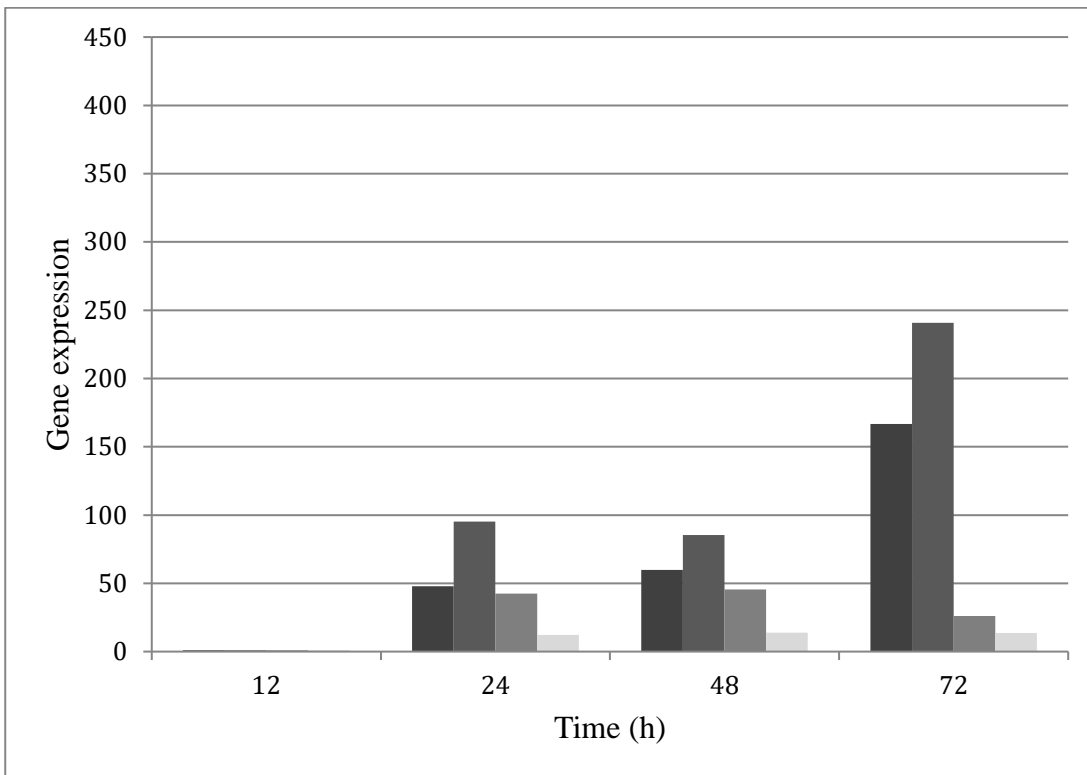
669    **A**
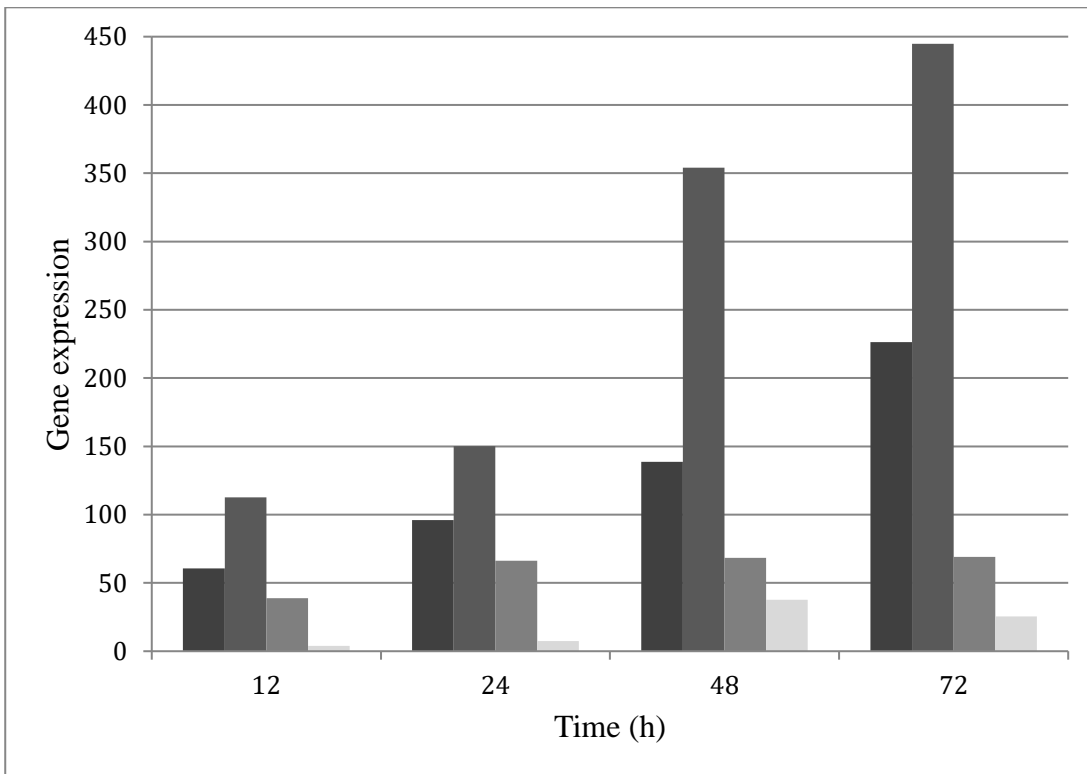


670

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.

696

697