1 **Title:** Hypoxia and iron requirements are the main drivers in transcriptional adaptation of
2 *Kluyveromyces lactis* during wine aerobic fermentation.

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22 Abstract

23 The respiratory metabolism of yeast species alternative to *Saccharomyces cerevisiae* has
24 been explored in recent years as a tool to reduce ethanol content in grape wine. The
25 efficacy of this strategy has been previously proven for mixed cultures of non-
26 *Saccharomyces* and *S. cerevisiae* strains. In this work, we perform a transcriptomic
27 analysis of the Crabtree-negative yeast *Kluyveromyces lactis* under tightly controlled
28 growth conditions in order to better understand physiology of non-*Saccharomyces* yeasts
29 during the fermentation of grape must under aerated conditions. Transcriptional changes
30 in *K. lactis* are mainly driven by oxygen limitation, iron requirement, and oxidative
31 stress. Oxidative stress appears as a consequence of the hypoxic conditions achieved by
32 *K. lactis* once oxygen supply is no longer sufficient to sustain fully respiratory
33 metabolism. This species copes with low oxygen and iron availability by repressing iron
34 consuming pathways and activating iron transport mechanisms. Most of the physiological
35 and transcriptomic features of *K. lactis* in aerobic wine fermentation are not shared with
36 the Crabtree-positive yeast *S. cerevisiae*.

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43 Keywords

44 *K. lactis*; RNAseq; Non-*Saccharomyces*; Aerobic fermentation; Oxidative stress

45

46 1 Introduction

47 Despite *Saccharomyces cerevisiae* is the main yeast species responsible for wine
48 fermentation, other yeasts, including *Hanseniaspora/Kloeckera*, *Pichia*, *Candida* or
49 *Metschnikowia* strains, develop during the first stages of grape must fermentation (Fleet
50 and Heard, 2004). The metabolic footprint of these alternative yeast species is now
51 recognized as a relevant contribution to wine aromatic complexity and sensory quality
52 (Jolly et al., 2014). However, an uncontrolled fermentation process driven by non-
53 *Saccharomyces* yeasts and bacteria would often result in wine spoilage. Historically, the
54 use of *S. cerevisiae* starter cultures constituted an inflection point in the microbiological
55 control of wine fermentation (Carrascosa et al., 2011), but it has been blamed for wine
56 aromatic standardization and lack of complexity. In order to recover some of the positive
57 contribution of “wild yeasts” to wine quality, while keeping a reasonable control of the
58 fermentation process, wine microbiologists have been suggesting the use of non-
59 *Saccharomyces* yeasts in mixed starter fermentations with *S. cerevisiae*, either by
60 simultaneous or sequential inoculation (Jolly et al., 2014). Indeed, several non-
61 *Saccharomyces* yeast starters are currently available in the market and their use by
62 winemakers is growing. Most of these new starters are selected according to their impact
63 on wine aromatic profile, glycerol or mannoprotein content, volatile acidity, or colour
64 stability (Gonzalez et al., 2016).

65

66 Crabtree-negative non-*Saccharomyces* yeasts have been suggested in the context of
67 climate change as an instrument to reduce ethanol content of wines (Gonzalez et al.,
68 2013). The proposal for alcohol level reduction with these alternative yeast species is
69 based on their capacity to respire sugars from grape must under aerated fermentation

70 conditions (Quirós et al., 2014). Under oxygen sufficient conditions, Crabtree-negative
71 yeasts consume all sugar by respiration, while most of the carbon flux goes to ethanol
72 production in the case of Crabtree positive species, like *S. cerevisiae*. Our research group
73 has shown the usefulness of several non-*Saccharomyces* yeasts, including *Kluyveromyces*
74 *lactis*, for this purpose (Morales et al., 2015, Rodrigues et al., 2016). These works
75 identified the overproduction of acetic acid, above commercially accepted levels, as a
76 critical point for the interest of this approach. The increase in acetic acid production
77 occurred either under aerobic conditions or after growing in aerobic conditions and then
78 switching to anaerobic conditions. A tight control of the dissolved oxygen levels reduced
79 the production of acetic acid. In addition to oxygen supply, other environmental factors,
80 namely temperature and nitrogen source availability have also been shown to impact the
81 output of aerated fermentation experiments with different non-*Saccharomyces* yeasts
82 (Rodrigues et al., 2016).

83

84 Reducing ethanol content of wines by the strategies mentioned above involves the use of
85 yeast species usually poorly characterized as well as growth conditions (aerated wine
86 fermentation) that are fairly unexplored from a biotechnological viewpoint. In order to
87 make advances in the development of fermentation procedures based on respiratory
88 metabolism of non-*Saccharomyces* yeasts, it was judged interesting to know the
89 transcriptional changes experienced by yeast cells under these previously unexplored
90 growth conditions. *K. lactis* was chosen because of its good properties for alcohol level
91 reduction by respiration (Rodrigues et al., 2016). In addition, the genome of this species
92 is well sequenced and annotated (Dujon et al., 2004; Sherman et al., 2004). The aim of
93 this work was hence using *K. lactis* as a model system to study changes, at the
94 transcription level, relevant for our understanding of yeast physiology under aerated
95 fermentation conditions, as those employed for alcohol level reduction in wine.

97 2 Methods

98 2.1 Strains and media

99 Three yeast strains were used in this work, *S. cerevisiae* EC1118, a widely used industrial
100 wine yeast strain; *K. lactis* type strain, CBS2359; and *K. lactis* AQ2166, a natural oak
101 isolate from Hungary. In order to mimic industrial wine fermentations, we used a
102 synthetic grape must recipe based on the Herwig complex synthetic media (Herwig et al.,
103 2001) with some modifications as follows (per liter): glucose 200 g; (NH₄)₂SO₄ 5 g,
104 KH₂PO₄ 3 g, MgSO₄ 7H₂O 0.5 g, trace element solution 2.67 mL, vitamin solution 2.67
105 mL, anti-foam (PPG P2000) 0.1 mL. The vitamin solution, stored in refrigerator and
106 sterilized by filtration, contained per liter: biotine 0.05 g, Ca-D(+)panthothenate 1 g,
107 nicotinic acid 1 g, myo-inositol 25 g, thiamine hydrochloride 1 g, pyridoxal
108 hydrochloride 1 g, para-amino benzoic acid 0.2 g. The autoclaved trace element solution
109 contained per liter: EDTA 15 g, ZnSO₄ · 7H₂O 45 g, MnCl₂ · 4H₂O 1 g, CoCl₂ · 6H₂O
110 0.3 g, CuSO₄ · 5H₂O 0.3 g, Na₂MoO₄ · 2H₂O 0.4 g, CaCl₂ · 2H₂O 4.5 g, FeSO₄ · 7H₂O 3
111 g, H₃BO₃ 1 g, KI 0.1 g. Fermentations in real grape must were previously performed to
112 ensure that our results were comparable to synthetic grape must (Supplementary Figure
113 S1).

114

115 2.2 Synthetic must fermentations under controlled aerobic conditions and RQ 116 determination

117 Fermentation experiments were performed in triplicate in small bioreactors MiniBio
118 (Applikon Biotechnology B.V., Delft, The Netherlands) coupled to BlueInOne Cell gas
119 analyzer units (BlueSens, Germany). This setup allows to monitor different parameters:

120 temperature, pH and dissolved oxygen (DO) in the media, as well as O₂ and CO₂ from the
121 output gas.

122 Seed cultures were grown in YPD broth for 48 h, at 25 °C and 250 rpm. Bioreactors were
123 filled in with 150 mL of synthetic grape must. Temperature was set to 25 °C, stirring to
124 1000 rpm, pH to 3.5, and inoculation to approximately 0.2 initial optical density at 600
125 nm (OD₆₀₀). The cultures were sparged with air at 25 ml/min (10 gas volumes/culture
126 volume/h (vvh)). Gas flow was controlled with MFC17 mass flow controllers (Aalborg
127 Instruments and Controls, Inc., Orangeburg, NY), whose calibration was regularly
128 verified with an electronic flowmeter (Agilent Technologies, Santa Clara, CA).

129 CO₂ and O₂ readings from the gas analyzer were recorded every minute and used to
130 calculate RQ in three steps. First the contribution of gas exchanges to changes in gas
131 volume was taken into account to calculate actual amount (per minute) of CO₂ and O₂
132 coming out of the bioreactor. Then, CO₂ release and O₂ consumption rates were
133 calculated as the difference between in and out values (per minute). Finally, RQ was
134 estimated as the ratio between CO₂ production and O₂ consumption rates for each time
135 point.

136

137 2.3 Analytical methods

138 Production and consumption of the main metabolites, glucose, fructose, glycerol, and
139 ethanol, were determined in duplicate using a Surveyor Plus Liquid Chromatograph
140 (Thermo Fisher Scientific, Waltham, MA) equipped with a refraction index and a
141 photodiode array detector (Surveyor RI Plus and Surveyor PDA Plus, respectively) on a
142 300 × 7.7 mm HyperREZTM XP Carbohydrate H+ (8 µm particle size) column and guard
143 (Thermo Fisher Scientific). The column was maintained at 50 °C, and 1.5 mM H₂SO₄
144 was used as the mobile phase at a flow rate of 0.6 mL/min. Prior to injection in duplicate,

145 the samples were filtered through 0.22 μ m pore size nylon filters (Micron Analytica,
146 Madrid, Spain) and diluted 10-fold in MilliQ water.

147

148 2.4 RNA sequencing and data analysis

149 Total RNA from biological triplicates was extracted using RNeasy® mini kit (QIAGEN)
150 and subjected to DNAase treatment using the Ambion DNA-free™ kit according to the
151 manufacturers' instructions. Concentration, purity and integrity of RNA samples were
152 determined by spectrophotometric analysis considering the absorbance ratio at 260/280
153 nm and at 230/260 nm. Library preparation and sequencing of RNA was performed at
154 Institute of Biomedicine & Biotechnology of Cantabria (Santander, Spain). After poly-A
155 filtering, libraries were generated for the different time points and conditions. From these
156 libraries, 50-bp single-end sequence reads were produced with Illumina HiSeq 2000. All
157 raw RNAseq data have been deposited in NCBI under Sequence Read Archive
158 SRP064945 (BioProject PRJNA298965) accession number.

159 Alignment of reads to the S288c *S. cerevisiae* yeast reference genome assembly or to
160 CBS2359 *K. lactis* reference genome assembly was carried out using TopHat2 v.2.0.13
161 (Kim et al., 2013). Only uniquely mapped single copy, ≤ 1 polymorphism per 25bp reads
162 with quality ≥ 20 were kept for further analysis. The htseq-count tool (v.0.5.4p5) from
163 HTSeq (Anders and Huber, 2015) was used to estimate unambiguous read count per
164 genome assembly annotated transcript. Normalization following the trimmed mean of M-
165 values (TMM) method (Robinson and Oshlack, 2010), as well as a time-points DEGs
166 searches (adjusted Benjamini–Hochberg $P \leq 0.05$ and ≥ 2 -fold change) were performed in
167 edgeR v.2.2.6 (Robinson et al., 2010). Finally, fragments per kb of exon per million
168 fragments mapped (RPKM) was calculated using Cuffdiff v.2.2.1 (Trapnell et al., 2013)
169 and low-expressed transcripts were filtered out when RPKM was < 1 in both samples.

170

171 2.5 Real-time quantitative PCR

172 RNA was prepared as described above, and quantification was run in triplicate. All the
173 reactions were run in a LightCycler® 480 Real-Time PCR System. The gene expression
174 levels are shown as the changes in the concentration of the studied gene as compared to
175 the control sample and were normalized with the concentration of the housekeeping
176 *ACT1* gene and *PCAI* gene, with similar results (Teste et al., 2009) (only *ACT1* is shown
177 in the figures) using the $\Delta\Delta C_t$ method. Primers used in this study are listed in
178 Supplementary Table S1.

179

180 2.6 Statistical analysis

181 Principal component analysis of the normalized RNAseq data transcripts per million
182 (TPM) was done using MeV software (4.8v10.2). The remaining statistical analyses were
183 done using STATA-SE. Venn diagram was drawn by using Venny 2.1 on-line tool
184 software (Oliveros, J.C. (2007-2015) Venny. An interactive tool for comparing lists with
185 Venn's diagrams: <http://bioinfogp.cnb.csic.es/tools/venny/index.html>). GO term analysis
186 was performed using YeastMine (Balakrishnan et al., 2012). The *p-values* were corrected
187 for multiple testing by the Bonferroni test for functional associations and GO analyses.
188 The statistical level of significance was set at $p \leq 0.05$. Then, GO terms were grouped in
189 biomodules by GO-Module (Yang et al., 2011) to prioritize Gene Ontology terms.

190

191 3 Results and Discussion

192 3.1 Fermentation profile under aerated conditions

193 Fermentation assays of synthetic grape must were performed with two different *K. lactis*
194 yeast strains, the type strain CBS 2359, with complete annotated genome sequence
195 available (Dujon et al., 2004; Sherman et al., 2004) and *K. lactis* AQ2166. This second *K.*
196 *lactis* strain was selected as a more suitable yeast strain for winemaking applications,
197 especially considering acetic acid production under aerated conditions (Fig. 1). An
198 industrial *S. cerevisiae* wine yeast strain, EC1118, was also included in the experiments
199 in order to illustrate the impact of aerated fermentation methodology in an industrial wine
200 yeast background (Novo et al., 2009). The experimental conditions, including aeration
201 regime, were chosen according to previous results (Morales et al., 2015; Rodrigues et al.,
202 2016). A validation of the aeration regime and synthetic grape must composition was
203 done by comparing dissolved oxygen (DO) profiles in fermentations carried out in natural
204 grape must. Similar DO profiles were obtained in natural or synthetic grape must
205 (Supplementary Fig. S1).

206 Under these experimental conditions total sugar consumption was achieved in a relatively
207 short time for all the strains assayed, 50 hours for both *K. lactis* strains, and slightly faster
208 for the industrial *S. cerevisiae*, 42 hours (Fig. 2). Ethanol production under these aerated
209 conditions was low as compared to regular (anaerobic) fermentation conditions; and
210 lower for both strains of *K. lactis* (7 to 8.30 % v/v ethanol) than for *S. cerevisiae* (10%
211 v/v ethanol). This is in agreement with previous results by us and other authors, under
212 both standard laboratory conditions (Quirós et al., 2014; Contreras et al., 2015) and in
213 natural grape must (Morales et al. 2015). Also, as shown previously (Morales et al. 2015),
214 oxygenation results in increased acetic acid production for *S. cerevisiae* and the type
215 strain of *K. lactis* CBS2359, from 30 hours. Although acetic acid is the primary volatile
216 acid in wine, its production over detectable levels remembers vinegar and is considered
217 as wine spoilage (Carrascosa et al. 2011). However, the second *K. lactis* strain showed
218 very low acetic acid production (Fig. 1).

219 Aeration regime, as mentioned above was based on previous results, with the main goal
220 of inducing a quick oxygen consumption by *K. lactis* strains, that will protect grape must
221 components against oxidation. Accordingly, the two *K. lactis* strains showed a high
222 consumption rate after the initial 5 hours, reaching DO values below 10% after 15 hours.
223 The decline in DO was slightly faster for the *K. lactis* strain AQ2166. On the other hand,
224 *S. cerevisiae*'s oxygen consumption was noticeable after around 12 hours with a much
225 slower decay, and never fell below 30% dissolved oxygen (Fig. 3). Oxygen demand
226 decayed after about 30 hours of culture in all instances, probably due to the depletion of
227 some essential nutrients, slow metabolic activity, and the entry of the cultures into
228 stationary phase. These differences in dissolved oxygen profiles are in agreement with
229 Respiratory Quotient (RQ) values for each strain (Fig. S2). *K. lactis* strains showed
230 steady RQ values around 1 (fully respiratory metabolism), until oxygen became a limiting
231 factor. Once the increment in biomass results in an oxygen demand that cannot be
232 fulfilled by the preset air flow (hypoxic conditions), an increase in RQ values is observed
233 in the *K. lactis* strains.

234 *S. cerevisiae*, on the contrary, showed a quick increase in RQ values from the beginning
235 of the experiment. Therefore, despite it consumes up to 30% of the oxygen available, RQ
236 values indicate that *S. cerevisiae* is mainly fermenting. This behavior is in agreement with
237 the well-known metabolic features of *S. cerevisiae*, an archetypical Crabtree-positive
238 yeast. The Crabtree-negative nature of *K. lactis* allows these yeasts to keep RQ values
239 always below those of *S. cerevisiae*, lowering the final ethanol production, as shown
240 above. Despite these important differences in respiro-fermentative metabolism, all the
241 yeast strains assayed showed similar sugar consumption kinetics (Fig. 2).

242 The two *K. lactis* strains showed slight differences in oxygen consumption. The type
243 strain showed a constant slope in dissolved oxygen decline, down to 0%, while AQ2166
244 slowed down oxygen consumption after reaching 10% DO, needing several hours to drop

245 to 0%. This behavior correlates with the increase in RQ values, starting earlier for
246 AQ2166 than for the *K. lactis* type strain, and could indicate a higher sensitivity of
247 AQ2166 to very low oxygen availability. Reaching 0% DO values is important in the
248 context of wine aerobic fermentation, in order to protect wine color and aromatic features
249 (Morales et al., 2015).

250 Sampling points for RNAseq and qPCR analyses were decided focusing on the two
251 different stages in oxygen availability for *K. lactis*. Two sample points were defined for
252 RNAseq analysis (Fig. 3). Sample point S1 (12 hours) defined by no oxygen limitation,
253 the RQ value was steady around 1 for *K. lactis* strains, indicating that glucose metabolism
254 was fully respiratory. In contrast, sample point S2 (30 hours) was taken when hypoxic
255 conditions (0% DO) had been running for several hours in *K. lactis* cultures. This point
256 was also characterized by RQ values clearly above 1 (respiro-fermentative metabolism).
257 In the case of *S. cerevisiae* S1 corresponds to the initial stages of oxygen consumption
258 while S2 corresponds to the maximum oxygen uptake, both cases with RQ ratios higher
259 than one, while there is a high oxygen availability.

260

261 3.2 Global analysis of the transcriptome

262 The main purpose of this work was to study the transcriptomic profile of *K. lactis*, in
263 synthetic grape must with an aerated regime, under two very different metabolic states
264 defined by the available dissolved oxygen. Neither *K. lactis* nor aerobic conditions are
265 commonly used in wine fermentation, but they have been lately used to lower the ethanol
266 content in wine (Morales et al., 2015; Rodrigues et al., 2016). Therefore, it is of great
267 interest, to better understand how *K. lactis* responds to this new biotechnological
268 application.

269 Samples for gene expression analysis were taken from S1 and S2 (see above), and
270 analyzed by RNAseq as described in Materials and Methods. In a first attempt to obtain a

271 global picture of the transcriptome data, a Principal Component Analysis (PCA) was ran
272 (Supplementary Fig. S3). PCA draw two very different scenarios, while samples from *K.*
273 *lactis* cluster in two groups according to its time points, in *S. cerevisiae* samples from S1
274 and S2 cluster together. The results of the PCA analysis correlate well with the number of
275 differentially expressed genes (DEG) for each species (S1 vs. S2). In *K. lactis* there is a
276 high degree of divergence between sample points, with more than 12% of the
277 transcriptome significantly modified (Fig. 4), in contrast to the 2.5% modified for *S.*
278 *cerevisiae*. Of the 623 genes showing modified expression in *K. lactis*, 337 were up-
279 regulated, while 286 genes were down-regulated. However, only 68 up-regulated genes
280 and 92 down-regulated genes were detected for *S. cerevisiae*. In addition, average fold-
281 change values for genes differentially expressed in *K. lactis* was higher than for *S.*
282 *cerevisiae*.

283 These gene expression changes are in agreement with the DO profiles shown in Fig. 3. *K.*
284 *lactis* shows pure respiratory metabolism (RQ values around 1; Supplementary Fig. 2S)
285 in S1, with DO values above 30%, but the oxygen nutrient limitation (0% DO) in S2 re-
286 shapes the transcriptome in response to the respiro-fermentative metabolism. The high
287 impact of growth conditions in S2 on the transcriptome reprogramming of *K. lactis* takes
288 place despite oxygen is supplied at the same rate in both S1 and S2. In contrast to other
289 studies with *K. lactis*, in this case the trigger for the observed transcriptomic changes is
290 not a deliberated shift from aerobic to anoxic conditions (Blanco et al., 2007; David and
291 Poyton 2005) but a gradual modification from aerobic to hypoxic conditions due to
292 increased biomass and metabolic activity of yeast cells. Under this conditions, *S.*
293 *cerevisiae* shows respiro-fermentative metabolism in both sample points, with a clear
294 preference for fermentation, and DO values above 30% in both cases. Accordingly,
295 changes in the expression pattern are much less pronounced than for *K. lactis*.

296

297 3.3 GO term enrichment among differentially expressed genes

298 The analysis of GO term enrichment is shown in Table 1. In order to ease the
299 interpretation of results, the GO-Module tool (Yang et al., 2011) was used to prevent
300 false positives and repeated terms. In the case of *K. lactis*, this analysis was performed by
301 using the corresponding *S. cerevisiae* orthologs. Approximately four-fifths of the genes
302 showing highly variable expression from *K. lactis* do have known orthologs in *S.*
303 *cerevisiae* (around 500 genes).

304 Analysis of GO terms among *K. lactis* genes significantly up-regulated in S2 revealed an
305 enrichment in oxidoreductase activity; iron ion transmembrane transporter activity;
306 aldehyde dehydrogenase (NAD) activity, and catalytic activity (Table 1). Regarding
307 genes in the iron ion transmembrane transporter activity category, *FET4* codes for a low-
308 affinity iron (II) permease, also involved in the transport of copper and zinc. Fet3p and
309 Ftr1p constitute the cell-surface high-affinity iron uptake system required for iron import
310 when it is present at low concentrations. Fet3p is a multicopper ferroxidase that receives
311 iron (II) ions from cell-surface iron reductases such as Fre3p and passes iron (III) ions to
312 the iron permease Ftr1p. In *K. lactis* two different genes *KLLA0E14477g* and
313 *KLLA0E05897g* show similarity to *FRE3* (also significantly up-regulated but not listed
314 under GO:0005381) from *S. cerevisiae*. Both orthologs are overexpressed in *K. lactis* in
315 S2. Also *ARN1* and *ARN2* are involved in iron transmembrane transport. They belong to a
316 family of transporters for siderophore-iron chelates, responsible in *S. cerevisiae* for the
317 uptake of iron bound to different siderophores like ferrirubin, ferrirhodin or
318 triacetylfusarinine C. Among genes up-regulated in *K. lactis*, four different genes, similar
319 to *S. cerevisiae* *ARN1* (*KLLA0A10439g*, *KLLA0E14609g*) or *ARN2* (*KLLA0C00220g*,
320 *KLLA0C19272g*) were found. In *S. cerevisiae*, the transcription of these genes is activated
321 by Atf1p, which is expressed in response to low iron conditions.

322 The identification of iron as a major nutrient requirement for *K. lactis* under these culture
323 conditions is reinforced by the finding of several other genes related to iron metabolism
324 being overexpressed in S2 for *K. lactis* cultures. These include five orthologs of *FIT1*,
325 *FIT2* and *FIT3* from *S. cerevisiae*. These genes code for GPI-anchored cell wall
326 mannoproteins involved in the retention of siderophore-iron complexes. Yap5p is an iron-
327 sensing transcription factor; while Cth1p is involved in iron homeostasis, as well as the
328 putative protein Fmp23p. The iron dependence of the *K. lactis* life style is also illustrated
329 by the number of copies found in the genome (Dujon et al., 2004; Sherman et al., 2004),
330 and overexpressed in this set of experiments, also *ARNI-2* and *FRE3* orthologs, as
331 mentioned above, not only of *FIT1-3* orthologs.

332 The overexpression of this set of genes in S2 indicates the importance of iron metabolism
333 for *K. lactis* under aerated conditions. This is related to the high oxygen consumption
334 levels at this time point, considering that the electron transport chain is the main
335 intracellular sink of iron ions. Also in agreement to the relevance of iron metabolism for
336 *K. lactis* is the observation that, at this sample point, bioreactors turned pink-red, as
337 shown for *Metschnikowia pulcherrima* under similar growth conditions. Both species are
338 known to produce pulcherrimin, an iron chelate molecule, with antimicrobial effect by
339 limiting access of other microorganisms to iron (Sipiczki, 2006). Indeed, the absorbance
340 spectrum of the *K. lactis* supernatants showed the characteristic pulcherrimin peak at 385
341 nm.

342 According to the dissolved oxygen profiles, one major change in the environmental
343 conditions between S1 and S2 for *K. lactis* cultures is the transition from oxygen
344 sufficient to hypoxic conditions. Transition from aerobic to anoxic conditions has been
345 shown to induce the production of reactive oxygen species (ROS) in *S. cerevisiae* (David
346 and Poyton, 2005). ROS production might be a consequence of a sudden redox
347 imbalance, when the excess NADH resulting from the activity of the tricarboxylic acid

348 (TCA) cycle can no longer be taken up by the electron transport chain in the absence of
 349 molecular oxygen (Murphy, 2009). Two enriched categories, oxidoreductase activity, and
 350 aldehyde dehydrogenase (NAD) activity, suggest this to be also the case for *K. lactis* cells
 351 under our experimental conditions. This happens despite oxygen is still available and
 352 being used in time point S2, and despite the gradual reduction in dissolved oxygen levels
 353 would be expected to allow for a smoother adaptation of yeast cells to oxygen depletion.
 354 A high number of significantly enriched GO terms were found from the set of genes
 355 down-regulated in *K. lactis* (Table 1). As shown in Table 1, the Key Modules (Yang et
 356 al., 2011) are iron-sulfur cluster binding; ion transmembrane transporter activity;
 357 ubiquinol-cytochrome-c reductase activity; structural constituent of ribosome and
 358 monovalent inorganic cation transmembrane transporter activity. Down-regulation of
 359 iron-sulfur cluster binding in S2 is in agreement with the above observation of iron
 360 becoming a limiting nutrient for *K. lactis* under these culture conditions. Iron-sulfur
 361 proteins are considered as regulatory elements of iron metabolism. Yeast cells exhibit
 362 loss of iron-sulfur proteins in response to iron depletion (Shakoury-Elizeh et al., 2010).
 363 The oxygen limiting conditions in *K. lactis* in S2 reduced the flux distribution towards
 364 respiration compared to S1. Therefore, the mitochondrial electron transport chain is
 365 expected to show lower relative activity in S2. Indeed, both the ion transmembrane
 366 transporter activity and ubiquinol-cytochrome-c reductase activity GO terms were
 367 significantly down-regulated (Table 1). These GO terms include, among others, genes
 368 coding for the cytochrome c oxidase (*COX12*, *COX13*, *COX4*, *COX5b*, *COX6*, *COX7*,
 369 *COX8*, and *COX9*), or other components of the electron transport chain (*ATP1*, *COR1*,
 370 *RIP1* and many QCR genes) as well as additional mitochondrial constituents (*AGC1*,
 371 *FSF1*). Also falling in this category are the down-regulated genes coding for permeases
 372 for amino acids and other nitrogen compounds (*GAP1*, *DIP5*, *DUR3*). This is probably
 373 related to the oxygen limitation observed in S2, since respiration is associated to higher

374 biomass production rates. Also in agreement is the down-regulation of genes coding for
375 structural components of the ribosomes, highlighted in the structural constituent of
376 ribosome GO-term category (Table 1).

377 Under this experimental conditions, for the industrial *S. cerevisiae* yeast strain, the GO
378 terms that appeared to be significant from the up-regulated set of genes, were all of them
379 associated with rRNA and rRNA pseudouridylation (Table 1). Almost half of the genes
380 significantly over-expressed in S2 are small nucleolar RNAs (snoRNAs). These stable
381 RNAs are found within small nucleolar ribonucleoprotein complexes (snoRNPs) and
382 localize to the nucleoli of eukaryotic cells. The majority of the snoRNAs are involved in
383 ribosomal RNA processing, including pseudouridylation, a frequent posttranscriptional
384 modification of uridine in RNAs. Pseudouridine (Ψ), when incorporated into RNA, can
385 modify its secondary structure by increasing base stacking, improving base pairing and
386 rigidifying sugar-phosphate backbone. As a consequence, it alters the chemical and
387 physical properties of RNA molecules (Zhao and He, 2015).

388 Pseudouridylation can induce different stress factors (Wu et al., 2011; Ge and Yu, 2013;
389 Schwartz et al., 2014; Karijolic et al., 2015) suggesting a regulatory role for Ψ . The
390 replacement of multiple U sites with Ψ in synthetic RNA molecules results in an
391 increased protein expression level (reviewed in Zhao and He, 2015). The high expression
392 of genes involved in pseudouridylation observed in our work could be related to oxidative
393 stress due to respiratory metabolism. Indeed, RNA post-transcriptional modifications
394 have been previously shown to be important for recovery after an environmental stress
395 (Biggar and Storey, 2015). In addition, Tronchoni et al. (2014) described the role of RNA
396 maturation and transcription stability after cold shock in wine yeast strains. No significant
397 enrichment was found for genes down-regulated in *S. cerevisiae* (Table 1).

398

399 *3.4 Genes similarly regulated in both species point to nutrient requirements*

400 Only seven differentially expressed genes (three up- and four down-regulated) were
401 found to behave the same way for *K. lactis* and *S. cerevisiae* (Fig. 4). This low similarity
402 in their transcriptomic responses is explained by the evolutionary divergence in aerobic
403 fermentation between both species. While, according to RQ values, *K. lactis* is
404 exclusively respiring until the lack of oxygen forces it to initiate fermentation, *S.*
405 *cerevisiae* under the Crabtree effect maintains the main metabolic flux towards
406 fermentation. Despite these differences, several genes show a common regulation when
407 comparing both time points.

408 The three genes up-regulated are *INO1*, *THI2* and *FET3*. *INO1* (Fig. 4), coding for the
409 first enzyme in the inositol biosynthesis pathway, is induced by inositol requirement
410 (Culbertson and Henry, 1975). It has been previously shown that inositol might be one of
411 the limiting nutrients in some yeast culture media (Hanscho et al., 2012; Novo et al.,
412 2013). The synthetic must medium used in this work contained three times more inositol
413 than standard synthetic grape must. However, the aeration of the media allows yeast to
414 respire and probably, this increases biomass production and makes inositol to become a
415 limiting compound (Quirós et al., 2014). Respiro-fermentative metabolism and higher
416 biomass production take place in both species under aerated conditions, despite the
417 Crabtree effect of *S. cerevisiae*. The other two commonly up-regulated genes seem also
418 related to the demand of specific nutrients for biomass production. *THI2*, the
419 transcriptional activator of thiamine biosynthetic genes, responds to thiaminediphosphate
420 demand, and *FET3* (discussed above) is induced by low iron availability (Askwith et al.,
421 1994). Among the four genes commonly down-regulated in S2 we found *ERG25* (Fig. 4).
422 Erg25p, a di-ferric protein, is essential for the synthesis of ergosterol, an oxygen-
423 dependent process. Down-regulation of *ERG25* in S2, might be related to iron
424 requirement, as discussed above for *K. lactis* electron transport chain genes and genes
425 coding for other mitochondrial constituents.

426

427 3.5 Validation of RNAseq data by qPCR analysis

428 A selection of highly Differentially Expressed Genes (log fold change above 1),
429 representative of the main GO terms discussed above, were chosen to validate the
430 RNAseq data and were analyzed across the fermentation to obtain a more detailed
431 expression profile (Supplementary Fig. S4 and S5). The qPCR data for sample points S1
432 and S2 confirmed the RNAseq expression in all cases but two. In both cases gene
433 expression was very low and no solid conclusions can be drawn. For the remaining genes,
434 results from the qPCR analysis largely confirmed the expression pattern observed for S1
435 and S2 in *K. lactis* and *S. cerevisiae*.

436 Besides S1 and S2 sample points, additional sample points were included: sample point
437 R, used as a reference for relative quantification by qPCR; and sample point F,
438 representative of an advanced stage of fermentation, with dissolved oxygen levels clearly
439 above sample point S2; finally, given the important differences in metabolic state
440 between S1 and S2 for *K. lactis*, sample point I (Intermediate time point) was included, in
441 order to characterize this observed transition step (Supplementary Fig. S5). This sample
442 point corresponds to approximately 30 minutes after the culture reached 0% DO.

443 The qPCR characterization of sample point I shows an interesting result, expression
444 levels at this intermediate time point did not follow the trend between S1 and S2. On the
445 contrary, the genes analyzed show a clear drop in expression at this sample point.
446 Probably, this indicates a general reduction in gene transcription, in order to allow the
447 cell to adapt its metabolism to the new growth conditions, with limiting oxygen
448 availability.

449 As mentioned above, preliminary experiments showed a slightly different fermentation
450 profile for *K. lactis* AQ2166, as compared to the *K. lactis* type strain. The main
451 differential feature is a slower transition step for *K. lactis* AQ2166. For this reason, qPCR

analysis was performed for this strain from sample point I, for the same genes than the type strain. As shown by the trend lines in Fig. 5, the general behavior of both *K. lactis* strains is similar, concerning relative expression levels between sample points S2 and I, and between sample points F and S2. However, the general trend towards reduced expression levels during the transition step (sample point I), previously observed for the type strain, is less pronounced for *K. lactis* AQ2166 (with the noticeable exception of *ARR2*). This weaker response to the transition step is in agreement with the slower slope cultures of this strain reach 0% DO. This suggests *K. lactis* AQ2166 is able to perceive and anticipate oxygen limitation more efficiently than the type strain, and gradually adapt transcription levels, before oxygen availability becomes a limiting factor for growth.

3.6 Oxidative stress

A transitory oxidative stress response due to hypoxic conditions has been described for *S. cerevisiae* (Becerra et al., 2002) and *K. lactis* (Blanco et al., 2007). Despite some similarities, the hypoxic induced oxidative stress response seems to be different between both yeast species, probably due to the preference for respirative metabolism in *K. lactis*, in contrast to respiro-fermentative metabolism in *S. cerevisiae*. González-Siso and Cerdán (2012) proposed a set of gene types to be explored in order to characterize these transcriptional responses, including heme biosynthesis, ergosterol biosynthesis and supply, NAD(P)H consuming oxidative defense reactions, other oxidative defense reactions, NAD(P)-dehydrogenases from the inner membrane of mitochondria, heme/respiration-related transcriptional factors, sterol-related transcriptional factors, peroxide-related transcriptional factors, life span-related proteins, and mitophagy-related proteins.

Given the hypoxic conditions identified in S2 for *K. lactis* cultures, we paid attention to changes in the expression levels of genes assigned by González-Siso and Cerdán (2012)

478 to each of the above mentioned gene types in both *K. lactis* and *S. cerevisiae*. Even
479 though time point S2 cannot be compared between yeast species given the important
480 metabolic differences, it was worth to study if oxidative stress response genes appeared
481 significantly induced in *S. cerevisiae*. As shown in Table 2 and 3, the number of these
482 genes differentially expressed between S1 and S2 is higher for *K. lactis* than for *S.*
483 *cerevisiae*. This number of DEG is higher than observed by Blanco et al. (2007),
484 probably due to differences in the experimental conditions and to the lower throughput
485 techniques available at that time for *K. lactis*.

486 Differences between *K. lactis* and *S. cerevisiae* for the expression of this set of genes are
487 in agreement with the general trend in gene expression discussed above, related to the
488 more limited physiological changes observed for each species (Fig. 3). Genes up-
489 regulated in *K. lactis* under hypoxic conditions belong to the types “other oxidative
490 defence reactions”, “mitophagy-related proteins”, and “NAD(P)H consuming oxidative
491 defence reactions”, while genes down-regulated belong to “NAD(P)-dehydrogenases
492 from the inner membrane of mitochondria and “ergosterol biosynthesis and supply”, as
493 well as two genes for “NAD(P)H consuming oxidative defence reactions” (Table 2). This
494 expression pattern would confirm the adaptation responses already hypothesised by
495 González-Siso and Cerdán (2012), that can be summarized as a reduction in
496 mitochondrial activity through mitophagy, the down-regulation of oxygen consuming
497 biosynthetic processes, like ergosterol or heme biosynthesis, and tuning of oxidative
498 defence mechanisms (with more genes up-regulated than down-regulated). Down-
499 regulation of genes typically related with ROS detoxification, like *CTA1* or *PRX1*, or
500 mitochondrial NADH dehydrogenases, like *NDE1* and *NDII*, seems to be paradoxical,
501 but it is explained by a general reduction in mitochondrial synthesis in S2.

502 Oxidative stress by hypoxia has been associated to the sudden oxygen deficiency,
503 resulting in leakage of electrons from the electron transport chain, mainly at the level of

504 complex I (Murphy, 2009), leading to a drastic increase in the rate of formation of
505 superoxide anion ($O_2^{\cdot-}$). This oxygen radical is readily converted to H_2O_2 by the activity
506 of superoxide dismutases (encoded in *S. cerevisiae* by *SOD1* and *SOD2*). The expression
507 of these genes is shown in Fig. 6 as normalized RPKM values for *K. lactis* and *S.*
508 *cerevisiae*. It shows that there is not a significant change in the expression of *SOD1* and
509 *SOD2* between S1 and S2 in *K. lactis*. Interestingly, this is because this species already
510 shows high levels of expression for these genes related to active respiratory metabolism.
511 The final detoxification step is catalyzed by catalases, Ctt1p and Cta1p. Similar to *SOD*
512 genes, expression levels of *CTA1* and *CTT1* are again higher for *K. lactis* than for *S.*
513 *cerevisiae* (Fig. 6). An intriguing possibility to explain increased acetic acid production
514 would be that the detoxification of $O_2^{\cdot-}$ produced by hypoxic conditions and reduction of
515 H_2O_2 by catalases could be coupled to the oxidation of ethanol to acetaldehyde, as has
516 been shown in human cells (Zimatkin et al., 2006). BioCyc (Caspi et al., 2016) data base
517 collection for metabolic pathways predicts that the same reaction could be catalysed by
518 Ctt1p or Cta1p in *S. cerevisiae* (Reaction: EC1.11.1.6) (Caspi et al., 2016). Further
519 oxidation of acetaldehyde, catalysed by aldehyde dehydrogenases, would result in acetate
520 production.

521

522 In conclusion, gene expression analysis has confirmed the metabolic shift observed in DO
523 and also the differences observed for *S. cerevisiae* and *K. lactis* under aerobic conditions.
524 Two main drivers appear to be responsible in *K. lactis* for this transcriptomic remodeling.
525 By one side, this species appears as a respiration and iron specialist, also indicated by the
526 high copy number of genes required for iron uptake, or the high constitutive expression
527 levels of genes involved in ROS detoxification. This species seems to activate two
528 complementary mechanisms in order to cope with increasing iron requirements,
529 activation of iron uptake mechanisms; and repression of the biosynthesis of heme

530 containing proteins. A second driver to downregulation of genes coding for heme
531 containing proteins (e.g. *COX* genes) is oxygen starvation (hypoxia), which would limit
532 the capacity of the electron transfer chain. Finally, oxidative stress caused by hypoxia
533 might be counteracted by also repressing the synthesis of genes coding for this latter
534 group of proteins, as well as mitochondrial activity, or oxygen consuming biosynthetic
535 processes, like ergosterol or heme biosynthesis. The response of *K. lactis* during adaption
536 to hypoxic conditions is also characterized by a transient general downregulation of the
537 transcriptional activity.

538 The most striking feature of the transcriptional response of *S. cerevisiae* to continued
539 growth under aerated conditions is the activation of genes involved in RNA
540 pseudouridylation, which suggests this growth condition triggers a response based on
541 RNA posttranscriptional modification, similar to what has been described for other stress
542 conditions. The lack of a specific response involving genes related to respiration in this
543 species correlates with low oxygen consumption throughout the experiment, in turn
544 related to the Crabtree-positive character of *S. cerevisiae*.

545 The ability for continuous growth in grape must and to maintain a strong respiratory
546 metabolism under hypoxic and respiratory conditions, is a remarkable feature of *K. lactis*
547 strains, which contributes to the usefulness of this species for alcohol level reduction in
548 wine. Our data (both transcriptomic and physiological) indicate that, *K. lactis* is actively
549 and almost exclusively respiring sugars, until oxygen becomes a limiting factor. This
550 suggest that, increasing oxygen availability during the first fermentation stages would
551 help improve results in terms of sugar consumed by respiration and, consequently,
552 alcohol level reduction. The only drawback to take into account is the eventual impact of
553 increased oxygenation on volatile acidity, at least for some *K. lactis* strains (like the type
554 strain).

555 Metabolic diversity of *K. lactis* strains is revealed by different physiological and
556 transcriptomic responses to progressive oxygen starvation. The yeast strain showing the
557 smoother adaptation to hypoxic conditions is also the one producing the lowest acetate
558 levels therefore being the strongest candidate to low ethanol production by respiration at
559 initial stages of fermentation following this new methodology.

560

561 **Declarations**

562 **Abbreviations**

563 DO: Dissolved Oxygen

564 RQ: Respiratory Quotient

565 PCA: Principal Component Analysis

566 PC: Principal Component

567 DEG: Differentially Expressed Genes

568 S1: RNAseq Sample Point 1

569 S2: RNAseq Sample Point 2

570 ROS: Reactive Oxygen Species

571 TCA: Trycarboxilic Acid Cycle

572 snoRNAs: small nucleolar RNAs

573 snoRNPs: small nucleolar ribonucleoprotein complexes

574 Ψ : Pseudouridine

575 R: qPCR Reference time point

576 I: qPCR Intermediate time point

577 F: qPCR Final time point

578 $O_2^{\cdot -}$: superoxide anion TPM: Transcripts per million

579 RPKM: Reads Per Kilobase per Million mapped reads

580 GO: Gene Ontology

581 FDR: False Discovery Rate

582 LogFC: Log Fold Change

583 OD₆₀₀: Optical Density measured at a wavelength of 600 nm

584

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588

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593

594 **Availability of data and material**

595 The data set supporting the results of this article is available in the NCBI repository under

596 Sequence Read Archive SRP064945 (BioProject PRJNA298965) accession number

597 (<http://www.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?view=announcement>). The data set

598 supporting the results of this article is included in the article (and its Additional files)

599

600 **Competing interests**

601 The authors declare that they have no competing interests.

602

603 **Author's contributions**

604 RG, PM and JT, conceived the study; JT, AJR and JAC conducted the experiments; JT

605 analysed the data, JT and RG wrote the manuscript. All the authors read and approved the

606 final manuscript.

607

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 745

746 **Figure legends**

747 **Figure 1.** Acetic acid production under aerated conditions.

748 **Figure 2.** Evolution of main fermentation related metabolites (glucose: diamonds;
749 ethanol: triangles; glycerol: squares) under aerated conditions.

750 **Figure 3.** Evolution of dissolved oxygen levels under aerated conditions. RNAseq
751 sampling time points are shadowed in grey. qPCR sampling time points are shown as
752 dotted lines.

753 **Figure 4.** Analysis of the differentially expressed genes. (A) Percentage of transcriptome
754 significantly modified for each species: Blue circle represents *K. lactis*; Red circle
755 represents *S. cerevisiae*. (B) Venn diagram showing the number of differentially
756 expressed genes for each species (S1 vs. S2).

757 **Figure 5.** Comparative gene expression profile between *K. lactis* strains after reaching
758 zero per cent of dissolved oxygen. Gene expression trends are shown by schematic lines.
759 Continuous line: same gene expression trend; dotted lines: different expression trend.

760 **Figure 6.** Gene expression of the enzymes possibly involved in acetic acid production
761 linked to ROS detoxification. Expression values are shown as RPKM. S1: Sample time
762 point 1; S2: Sample time point 2. *: mitochondrial enzymes.

763

764 **Supplementary material**

765 **Supplementary File 1:** Figure S1. Evolution of dissolved oxygen levels under aerated
766 conditions in synthetic or natural grape must. (PDF 121 kb)

767 **Supplementary File 2:** Figure S2. Evolution of respiration quotient under aerated
768 conditions in natural must. RNAseq sampling time points are shadowed in grey.
769 Discontinuous horizontal line shows $RQ = 1$ (Full Respiratory Metabolism).

Supplementary File 3: Figure S3. Principal Component Analysis for RNAseq data (TPM from biological triplicates) from *K. lactis* and *S. cerevisiae*.

Supplementary File 4: Figure S4. qPCR of *S. cerevisiae* selected genes. Sample points are described in Fig. 3. Arrows represent the direction of gene expression between S2 and S1: Red arrows for genes showing higher expression in S2 compared to S1; Green arrows for genes showing lower expression in S2 compared to S1. Empty arrows represent a discrepancy between the RNAseq data and the qPCR data.

Supplementary File 5: Figure S5. qPCR of *K. lactis* selected genes. Sample points are described in Fig. 3. Arrows represent the direction of gene expression between S2 and S1: Red arrows for genes showing higher expression in S2 compared to S1; Green arrows for genes showing lower expression in S2 compared to S1. Empty arrows represent a discrepancy between the RNAseq data and the qPCR data.

Supplementary File 6: Table S1. Primers used in this study.

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788 TABLES

Table 1. GO term enrichment among differentially expressed genes for each species. GO terms were grouped in biomodules by GO-Module.

Expression	Strain	GO IDs	P-value	Significance*	GO terms	GO-Module IDs
Up-regulated	<i>K. lactis</i>	GO:0016491	0.000	K	oxidoreductase activity	1
		GO:0004029	0.006	K	aldehyde dehydrogenase (NAD) activity	2
		GO:0005381	0.002	K	iron ion transmembrane transporter activity	3
	<i>S. cerevisiae</i>	GO:0000944	0.000	K	base pairing with rRNA	1
		GO:0030556	0.000	T	rRNA modification guide activity	1
		GO:0030559	0.000	K	rRNA pseudouridylation guide activity	2

Down-regulated	<i>K. lactis</i>	GO:0051536	0.002	K	iron-sulfur cluster binding	1
		GO:0051539	0.003	T	4 iron, 4 sulfur cluster binding	1
		GO:0015075	0.000	K	ion transmembrane transporter activity	2
		GO:0008324	0.000	T	cation transmembrane transporter activity	2
		GO:0022890	0.000	T	inorganic cation transmembrane transporter activity	2
		GO:0008121	0.000	K	ubiquinol-cytochrome-c reductase activity	3
		GO:0003735	0.000	K	structural constituent of ribosome	4
		GO:0015077	0.000	K	monovalent inorganic cation transmembrane transporter activity	5
		GO:0004129	0.000	T	cytochrome-c oxidase activity	5
		GO:0015078	0.000	T	hydrogen ion transmembrane transporter activity	5

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792 *‘K’ refers to the key terms of GO biomodules, ‘T’ refers to the truly significant

793 hierarchical descendents of the key terms.

794 **Table 2.** Gene types responding to oxidative conditions due to hypoxia in *K. lactis*.

Gene	Orthologs	FC*	Category
KLLA0F15037g	<i>TRR1</i>	4.78	other oxidative defense reactions
KLLA0A00660g	<i>ATG32</i>	1.88	mitophagy-related proteins
KLLA0B07975g	<i>GRX8</i>	1.60	other oxidative defense reactions
KLLA0C17160g	<i>ATG1</i>	1.20	mitophagy-related proteins
KLLA0E14939g	<i>UTH1</i>	1.18	mitophagy-related proteins
KLLA0E20593g	<i>ATG8</i>	0.97	mitophagy-related proteins
KLLA0F20009g	<i>AHP1</i>	0.81	NAD(P)H consuming oxidative defense reactions
KLLA0D14333g	<i>DOT5</i>	0.79	NAD(P)H consuming oxidative defense reactions
KLLA0A00264g	<i>GTT1</i>	0.77	NAD(P)H consuming oxidative defense reactions
KLLA0B12133g	<i>ATG11</i>	0.61	mitophagy-related proteins
KLLA0E04181g	<i>BCY1</i>	0.53	life span-related proteins
KLLA0A09383g	<i>MTM1</i>	0.53	NAD(P)H consuming oxidative defense reactions
KLLA0E18547g	<i>MOT3</i>	0.52	heme/respiration-related transcriptional factors
KLLA0F26917g	<i>CCS1</i>	0.52	NAD(P)H consuming oxidative defense reactions
KLLA0F07557g	<i>GSH2</i>	0.46	NAD(P)H consuming oxidative defense reactions
KLLA0B03586g	<i>SCH9</i>	-0.42	life span-related proteins
KLLA0F22880g	<i>HAP1</i>	-0.57	heme/respiration-related transcriptional factors
KLLA0A05071g	<i>ERG4</i>	-0.59	ergosterol biosynthesis and supply
KLLA0E17733g	<i>GRX6</i>	-0.60	other oxidative defense reactions
KLLA0B11495g	<i>ROX1</i>	-0.68	heme/respiration-related transcriptional factors
KLLA0F15224g	<i>ERG1</i>	-0.74	ergosterol biosynthesis and supply
KLLA0F10285g	<i>MVD1</i>	-0.82	ergosterol biosynthesis and supply
KLLA0C15147g	<i>HEM3</i>	-0.83	heme biosynthesis
KLLA0D11242g	<i>ERG5</i>	-0.84	ergosterol biosynthesis and supply
KLLA0E03653g	<i>ERG11</i>	-0.92	ergosterol biosynthesis and supply
KLLA0C12265g	<i>ERG24</i>	-0.93	ergosterol biosynthesis and supply
KLLA0B09636g	<i>GRX5</i>	-0.95	other oxidative defense reactions
KLLA0E21891g	<i>NDE1</i>	-1.07	NAD(P)-dehydrogenases from the inner membrane of mitochondria
KLLA0B08085g	<i>ERG25</i>	-1.13	ergosterol biosynthesis and supply
KLLA0E20285g	<i>PRX1</i>	-1.17	NAD(P)H consuming oxidative defense reactions
KLLA0C06336g	<i>NDI1</i>	-1.19	NAD(P)-dehydrogenases from the inner membrane of mitochondria
KLLA0F06336g	<i>ERG2</i>	-1.28	ergosterol biosynthesis and supply
KLLA0D11660g	<i>CTA1</i>	-2.23	NAD(P)H consuming oxidative defense reactions

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796 *FC, fold change

797 **Table 3.** Gene types responding to oxidative conditions due to hypoxia in *S. cerevisiae*.

Gene	FC*	Category
<i>SOD1</i>	0.92	NAD(P)H consuming oxidative defense reactions
<i>NCP1</i>	-0.46	ergosterol biosynthesis and supply
<i>MGA2</i>	-0.47	heme/respiration-related transcriptional factors
<i>SUT2</i>	-0.47	sterol-related transcriptional factors
<i>DOT5</i>	-0.48	NAD(P)H consuming oxidative defense reactions
<i>ERG26</i>	-0.49	ergosterol biosynthesis and supply
<i>ROX1</i>	-0.51	heme/respiration-related transcriptional factors
<i>TPK2</i>	-0.52	life span-related proteins
<i>ERG7</i>	-0.55	ergosterol biosynthesis and supply
<i>HAP1</i>	-0.59	heme/respiration-related transcriptional factors
<i>ERG5</i>	-0.61	ergosterol biosynthesis and supply
<i>HOG1</i>	-0.61	mitophagy-related proteins
<i>GRX6</i>	-0.63	other oxidative defense reactions
<i>GTT1</i>	-0.70	NAD(P)H consuming oxidative defense reactions
<i>CTT1</i>	-0.72	NAD(P)H consuming oxidative defense reactions
<i>ECM22</i>	-0.72	sterol-related transcriptional factors
<i>NDE1</i>	-0.76	NAD(P)-dehydrogenases from the inner membrane of mitochondria
<i>ERG24</i>	-0.90	ergosterol biosynthesis and supply
<i>ERG1</i>	-0.93	ergosterol biosynthesis and supply
<i>SLT2</i>	-1.10	mitophagy-related proteins
<i>ERG4</i>	-1.12	ergosterol biosynthesis and supply
<i>HEM13</i>	-1.17	heme biosynthesis
<i>UPC2</i>	-1.19	sterol-related transcriptional factors
<i>ERG11</i>	-1.24	ergosterol biosynthesis and supply
<i>ERG3</i>	-1.30	ergosterol biosynthesis and supply
<i>ERG25</i>	-1.43	ergosterol biosynthesis and supply

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799 *FC, fold change

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