1   **Sterol uptake analysis in *Saccharomyces* and non-**
2   ***Saccharomyces* wine yeast species**

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11Abstract

12Sterols are essential components of the yeast membrane and their synthesis requires
13oxygen. Yet, *Saccharomyces cerevisiae* has developed the ability to take up sterols
14from the medium under anaerobiosis. Here we investigated sterol uptake efficiency
15and the expression of genes related to sterol import in *Saccharomyces* and *non-*
16*Saccharomyces* wine yeast species fermenting under anaerobic conditions.

17The sterol uptake efficiency of 39 strains was evaluated by flow cytometry (with 25-
18NBD Cholesterol, a fluorescent cholesterol probe introduced in the medium) and we
19found an important discrepancy between *Saccharomyces* and non-*Saccharomyces*
20wine yeast species that we correlated to a lower final cell population and a lower
21fermentation rate. A high uptake of sterol was observed in the various *Saccharomyces*
22strains. Spot tests performed on 13 of these strains confirmed the differences between
23*Saccharomyces* and non-*Saccharomyces* strains, suggesting that the presence of the
24sterol uptake transporters *AUS1* and *PDR11* could cause these discrepancies. Indeed,
25we could not find any homologue to these genes in the genome of *Hanseniaspora*
26*uvvarum*, *H. guilliermondii*, *Lachancea thermotolerans*, *Torulaspora delbreueckii*,
27*Metschnikowia pulcherrima*, or *Starmarella bacillaris* species. The specialization of
28sterol import function for post genome-duplication species may have favored growth
29under anaerobiosis.

30

31Introduction

32Sterols are essential lipids that determine membrane properties such as permeability,
33transport, resistance to stress. In *Saccharomyces cerevisiae*, a higher sterol content
34can change membrane fluidity and thus resistance to ethanol stress (Tesnière, 2019).
35*S. cerevisiae* cells are able to synthesize sterols as ergosterol only in aerobic
36conditions, this ergosterol being a typical, main sterol of the membranes (Weete *et al.*,
372010). Indeed, oxygen is required for the epoxidation of squalene by squalene
38epoxidase *Erg1p*, which is a limiting step for the biosynthesis of ergosterol. Under strict
39anaerobic conditions, the growth of *S. cerevisiae* strains is extremely reduced, but
40some residual growth is maintained (Da Costa *et al.*, 2019). However, when available,
41*S. cerevisiae* is able to incorporate a variety of exogenous sterols (Lorenz & Parks,
421987), such as cholesterol, ergosterol or phytosterols (Luparia *et al.*, 2004; Zavrel *et*
43*al.*, 2013). While, cholesterol is the major sterol of the animals, plants produce specific
44sterols called phytosterols; in grape skins, four such sterols have been identified, i.e. β -

45sitosterol, campesterol, stigmasterol and lanosterol, β -sitosterol representing more
46than 85% of the total detected phytosterols (Le Fur *et al.*, 1994).

47Because wine alcoholic fermentations are performed under anaerobiosis, lipid
48compounds represent important nutrients in winemaking that are necessary for cells to
49complete fermentation (Rosenfeld *et al.*, 2003; Tesnière, 2019). The high amount of
50carbon dioxide produced (2 CO₂ molecules per molecule of glucose) during wine
51fermentation, together with oxygen consumption through yeast growth, provoke a
52reduction in dissolved oxygen concentration (Salmon *et al.*, 2004; Saa *et al.*, 2012;
53Cerdeira-Drago *et al.*, 2016), which may lead to a change in membrane lipid composition
54(Ochando *et al.*, 2017). However, an increased ergosterol content protects yeast cells
55against the membrane thickness reduction effect caused by high ethanol concentration
56(Vanegas *et al.*, 2012).

57Yeast anaerobic sterol uptake varies according to the concentration of sterol present
58in their cells. For instance, cells cease to import exogenous sterols when they are
59saturated in free internal sterols (Lorenz *et al.*, 1986). Under anaerobiosis, i.e. when
60sterol biosynthesis is compromised, sterol uptake is required for cell viability (Wilcox *et*
61*al.*, 2002) and for complete fermentation as already mentioned. In *S. cerevisiae*
62laboratory strains such as S288C, two paralog ABC transporters (*AUS1* and *PDR11*)
63located in the plasma membrane are involved in the anaerobic sterol uptake (Wilcox
64*et al.*, 2002; Kohut *et al.*, 2011). Either transporter alone can take up enough sterol to
65restore growth under anaerobiosis (Wilcox *et al.*, 2002); however *AUS1* has been
66shown to be the major importer of non-yeast sterols such as cholesterol and plant
67sterols (Papay *et al.*, 2020). Growth recovery afforded by exogenous sterols may
68impair cells functions such as weak acid export by *Pdr12* or G-protein-coupled receptor
69*Ste2* (Souza *et al.*, 2011; Morioka *et al.*, 2013). During wine fermentation, phytosterol
70uptake has been reported to impair fermentation completion (Luparia *et al.*, 2004);
71however, several publications report its use without noticeable effect on fermentation
72kinetics (Rollero *et al.*, 2015; Deroite *et al.*, 2018).

73In non-inoculated wine fermentations, *S. cerevisiae* dominates progressively in the
74fermentation medium whereas it initially represents a very minor fraction among the
75dozen of yeast species present in grape must (Fleet & Heard, 1993; Holm Hansen *et*
76*al.*, 2001; Williams *et al.*, 2015; Albergaria & Arneborg, 2016). Several explanations
77have been proposed for the unrivaled ability of *S. cerevisiae* to outperform its
78competitors. While for *Hanseniaspora uvarum* and *Hanseniaspora guilliermondii*, the

79synthesis by *S. cerevisiae* of a peptide may explain its dominance over these two
80species (Pérez Nevado *et al.*, 2006, Branco *et al.*, 2014); for *Torulaspora delbrueckii*
81and *Lachancea thermotolerans* the reduction of oxygen availability participates to the
82reduction of fitness in comparison to *S. cerevisiae* (Nissen *et al.*, 2003). In contrast to
83*S. cerevisiae*, for which sterol import plays a key role under anaerobiosis, the lack of
84sterol uptake transporters has been noticed in some yeast species such as *Yarrowia*
85*lipolytica* and *Kluyveromyces lactis*; it has been hypothesized to be one of the factors
86explaining the absence of growth of *K lactis* under anaerobiosis (Bolotin-Fukuhara *et*
87*al.*, 2006; Snoek & de Steensma, 2006). While some authors have focused on nitrogen
88consumption in non-*Saccharomyces* species (Su *et al.*, 2020), sterol uptake has not
89been investigated for other yeast species of the wine microbial community, such as
90*Torulaspora delbrueckii* or *Hanseniaspora uvarum* that are well known for their ability
91to grow and ferment in grape must. Indeed, yeast species from genera other than
92*Saccharomyces* are increasingly investigated for their ability to interact with *S.*
93*cerevisiae*, and to modulate the flavor and taste of wines (Masneuf-Pomarede *et al.*,
942016), indicating that this ecological specialization has technological consequences.
95Thus, studying sterol import in *Saccharomyces* and non-*Saccharomyces* wine strains
96will help understanding the behavior of these fungi during fermentation and eventually
97help to design optimal practices to favor the selective growth of specific species.
98In this paper, we investigate sterol import under wine fermentation conditions in *S.*
99*cerevisiae* from different origins as well as in several other yeast species (non-
100*cerevisiae Saccharomyces* and non-*Saccharomyces*). In a first step aimed at
101assessing whether sterols uptake can promote growth under fermentation, the uptake
102of a fluorescent derivative of cholesterol (25-NBD Cholesterol) was measured using
103flow cytometry. Complementary spot test assays performed in anaerobic jars on a
104medium containing phytosterols alone or combined with fluconazole provide additional
105clues on sterol uptake ability to restore growth under anaerobiosis for several species.
106In a second step, the presence of genes homologues to genes coding for ABC
107transporters involved in sterol transport in *S. cerevisiae* and other closely related genes
108(*AUS1*, *PDR11*, *PDR18* and *SNQ2*) and their regulators (*UPC2* and *ECM22*) were
109searched among these non-*cerevisiae S.* and non-*Saccharomyces* strains. The
110analysis of their expression with that of *ERG6* (as a gene marker of the ergosterol
111biosynthetic pathway) by quantitative RT-PCR for strains of the different

112 *Saccharomyces* species provided further insight into the functioning of these
113 mechanisms. These results will have direct implications for winemakers.

114 **Materials and methods**

115 **Yeast strain constructions**

116 *S. cerevisiae* 59A*amn1* is derived from strain 59A, a meiotic haploid spore isolated
117 from the industrial starter Lalvin EC1118 and selected for its similar fermentation
118 performance and metabolite production (Marsit *et al.*, 2015). This strain was deleted
119 for the *AMN1* gene to limit aggregation (Wloch-Salamon *et al.*, 2013), which eases
120 flow-cytometry counting.

121 To obtain 59A*amn1* deleted strains, ORFs of the *AUS1*, *PDR11* and *DAN1* genes were
122 replaced by the loxP–kanMX–loxP disruption cassette that confers resistance to
123 geneticin (G-418) (Güldener *et al.*, 1996). To that end, PCR runs using respective
124 primers (B and C in Table 1) designed with homologous bases upstream and
125 downstream regions of the genes to be deleted in 59A*amn1* strain were performed
126 using DNA from *aus1*-, *pdr11*- and *dan1*- deleted BY4742 strains (Euroscarf). Gene
127 disruptions and constructs were confirmed by PCR (primers A, and D and KanB, KanC,
128 for 59A *aus1*::KanMX, 59A *pdr11*::KanMX and 59A *dan1*::KanMX respectively, Table
129).

130 The 59A*amn1,aus1, pdr11* mutant was obtained by PCR-mediated gene disruption
131 from the 59A*amn1, pdr11* strain using the pAgTEF1-hphMX-tAgTEF1 cassette of
132 pAG32 amplified with primers F-AUS1hygro and R-AUS1hygro (Table 1) to confer
133 hygromycin B resistance. Gene disruption and construct were confirmed by PCR
134 (primers A-AUS1, D-AUS1, Hygro1, Hygro2, Table 1).

135 **Strains and growth conditions**

136 The 39 strains selected in the present study (Table S1), consisting of 13 *S. cerevisiae*
137 (among which 5 mutants as controls: 59A*amn1*; 59A*amn1,aus1*; 59A*amn1, pdr11*;
138 59A*amn1,aus1,pdr11* and 59A*amn1,dan1*), 3 non-*cerevisiae* *Saccharomyces* and 18
139 non-*Saccharomyces*, were used to evaluate cell sterol uptake, CO₂ production,
140 population and viability. To that end, strains were grown at least 22h in 20ml flasks
141 containing 15 ml fermentation medium, closed by a stopper in which a 200ml filtered
142 cone was inserted in order to release CO₂ excess. The medium was the MS425
143 synthetic fermentation medium (Bely *et al.*, 1990) containing 0.05% Tween 80 and
144 20µg/mL of a cholesterol mixture (cholesterol/ 25-NBD Cholesterol, 1:1, w/w).

145Experiments were generally performed twice. Anaerobiosis was induced by the CO₂
146flush produced by alcoholic fermentation.

147Among these strains, 13 were selected (Table 2) to evaluate the ability of exogenous
148sterols to restore growth under anaerobiosis. They were mainly isolated from fruits
149except strains CBS 432 and IFO 1802. They consisted in 4 *S. cerevisiae* (K1_28-1A,
150LALVIN 2056, Lava32.15 and 59Aamn1 that could be considered as a positive control),
1513 *Saccharomyces* (*S. paradoxus*, *S. kudriavzevii* and *S. uvarum*), and 5 non-
152*Saccharomyces* (*Hanseniaspora guilliermondii*, *Hanseniaspora uvarum*, *Starmerella*
153*bacillaris*, *Lachancea thermotolerans*, and *Torulaspora delbrueckii*). In addition, the
15459Aamn1 strain deleted for the two *AUS1* and *PDR11* sterol transporter genes was
155studied in parallel. This last strain was considered as a negative control.

156Spot tests in anaerobic jars

157These 13 strains were propagated on YPD agar plates after an overnight preculture in
158YPD medium. Aliquots were grown at 28°C in 5ml complete liquid medium up to 1
159OD₆₆₀. 1 OD (10⁷ cells) were collected (700g, 5min 20°C) and the resulting pellet was
160washed two times with water. Cells were resuspended in 1ml water and 4 successive
1611/10 dilutions were prepared. 10µL of each dilution was spotted on SM425 gelled with
16225g/L agar containing Tween 80 (0.05%). According to the tested modalities,
163phytosterols (20µg/mL) and Fluconazole (FLC) (256µg/mL) were added to the media
164to evaluate their effect. Agar plates were incubated at 28°C in anaerobic conditions.
165To that end, plates were introduced in anaerobic jars (2.5L), and anaerobiosis was
166provoked using the Anaerocult™ A reagent (Merck, Millipore) according to the
167manufacturer's instructions. Cell growth was evaluated after 6 days. Experiments were
168repeated twice.

169Assays of sterol uptake by flow cytometry

170Before flow cytometry analysis cells were harvested from 0.5mL liquid medium and
171washed twice with cold PBS (PBS : 130mM NaCl, 2.6mM KCl, 7mM Na₂HPO₄, 1.2mM
172KH₂PO₄, pH 7.4) containing 0.5% (w/v) Nonidet P-40) and once with PBS at room
173temperature, before resuspension in 0.5mL PBS. 5µL of propidium iodide (0.1mg/mL)
174was added to cells just before cytometry analysis on an C6 Accuri apparatus (BD
175Biosciences). The histogram for red fluorescence (propidium iodide) was used for
176gating, allowing to exclude dead cells from the analysis. The green fluorescence
177corresponding to the 25-NBD Cholesterol present in live cells was registered (OD_{ex}
178488nm; OD_{em} 500-530nm). For each species, measures were taken on at least two

179independent cultures. For *S. cerevisiae*, measures were not duplicated for most strains
180but analyses were interpreted per group (i.e. wine strains or Oak strains). When
181displayed, errors bars correspond to one standard deviation.

182

183**RNA isolation**

184Total RNAs were isolated from yeast cells after 20h under induced fermentation
185anaerobiosis at 28°C in a MS425 medium containing phytosterols (20µg/mL). The
186TRIzol method was used according to Chomczynski and Sacchi (1987). Aliquots of 10⁹
187cells were harvested and quickly washed with 750mL cooled (4°C) DEPC-treated
188water. Cells were pelleted and frozen in a -80°C methanol bath. Frozen cells were
189mechanically lysed by vortexing with glass beads (d = 0.3mm) in 400mL TRIzol
190(GIBCO BRL) at 4°C for 15min. The liquid phase was collected and TRIzol added to a
191final volume of 4mL. The samples were then mixed and incubated for 5min at room
192temperature, and 800mL chloroform was added. The mixture was vortexed and then
193incubated for 3min and centrifuged (9,000g for 15min). The supernatant was
194centrifuged again (2,000g for 2min) in swing buckets. The aqueous phase was
195recovered, RNAs were precipitated with the the same volume of cooled isopropanol (-
19620°C) and further incubated for 10min. The samples were centrifuged (9,000g for
19710min) and the resulting nucleic acid pellet was washed twice with 750mL 75%
198ethanol/DEPC-treated water and then dissolved in 150mL of nuclease-free water
199(Qiagen). Total RNA from 100mg aliquots of these preparations was purified with a
200RNeasy mini kit (Qiagen) following the RNA cleanup protocol, including membrane
201DNase digestion. RNAs were eluted with 30µL (2 x) of the provided RNase-free water.
202RNA quality was verified through capillary electrophoresis with an RNA 6000 Nano
203LabChip Kit (Agilent Technologies).

204**Search of orthologues of and gene phylogeny**

205In order to obtain the sequence of *ACT1*, *AUS1*, *ECM22*, *ERG6*, *PDR11*, *PDR18*,
206*SNQ2* and *UPC2*, known orthologues were searched in the available databases
207(<http://sss.genetics.wisc.edu/cgi-bin/s3.cgi> , <http://gryc.inra.fr/>,
208<https://www.ncbi.nlm.nih.gov/>, http://phylomedb.org/phylome_3). When these
209orthologues were not known, (i.e. in the case of non-annotated genomes), we
210searched for them using a bidirectional blast approach. For each of these proteins in
211S288C, we first searched the protein with the highest identity and the highest coverage
212in the considered yeast species using the tblastn tool. This target protein was then

213 compared back to S288C and when the target protein was identical to the protein
214 searched for, we concluded to orthologue proteins. When no orthologues genes were
215 found, we included the nearest proteins in phylogenetic trees, using a specific name
216 containing the name of the second-best matching protein and the text “closest_to” with
217 the name of the initial protein (i.e. *PDR12*closest_to_*AUS1-PDR11* HANGU means
218 that, when searching for an ortholog to *AUS1*, and *PDR11*, in *Hanseniaspora*
219 *guilliermondii* genome, a protein was found, and the reciprocal blast in *S. cerevisiae*
220 with this protein indicated *PDR12* as the best hit).

221 Gene sequence alignment and phylogeny were performed using the muscle module of
222 Genious V9.1.6 after 10 iterations. From the alignments, the best tree was inferred
223 using IQ-TREE 1.5.5 (Nguyen *et al.*, 2015) with the MFP option in order to find the best
224 model, and the topology of the tree was tested with 1000 bootstraps.

225

226 **Quantitative RT-PCR analysis**

227 For quantitative RT-PCR analysis, specific primers were designed (Table 3). Real-time
228 quantitative PCR was conducted with SYBRGreen I PCR Master Mix using the gene-
229 specific primers. Each PCR reaction (25µL final volume) contained 5 µL of template
230 cDNA, 250nM of each primer and 1µL 2 x Power SYBR Green I PCR Master Mix
231 (Applied Biosystems, Warrington, UK). Thermocycling conditions were as follows: an
232 initial enzyme activation of 10 min at 95 °C, followed by 40 cycles of denaturation for
233 15s at 95°C, annealing and extension for 1 min at 60°C, with a final melt gradient
234 starting from 60°C and heating to 95°C at a rate of 0.03°Cs⁻¹. The real-time PCR
235 reactions were carried out in a 7300 Fast Real Time PCR System (Applied Biosystems,
236 Warrington, UK). Fluorescence was measured at the 497nm (excitation) and 521nm
237 (detection) wavelengths at the end of each extension step and at each 1°C increment
238 of the melt profile. Primer specificity was confirmed by analyzing dissociation curves of
239 the PCR amplification products. All cDNA samples to be compared for transcript levels
240 were analyzed in triplicate for each gene in a single batch for each primer pair. To
241 ascribe a relative transcript copy number to each cDNA sample, a purified PCR
242 fragment of each gene sequence was serially diluted 10-fold to obtain template
243 standards. The most concentrated standard was assigned an arbitrary transcript copy
244 number and subsequent n-fold dilutions were accordingly assigned as relative copy
245 numbers. Standards from 10⁵ to 10⁹ were included in the real-time PCR assay of cDNA
246 samples. In each case, a dilution series of standards showed a linear change in the

247cycle threshold values and cDNA templates were thus ascribed a relative transcript
248copy number by comparing their cycle threshold values with the standards. All
249templates and standards were run in triplicate and expressed as the average standard
250deviation. Sample values were normalized using the corresponding expression level
251of *ACT1*, an isogene constitutively expressed. Results correspond to the relative
252expression change under fermentation.

253**Statistical analyses**

254Significance tests were adjusted to the data after check of normality (Shapiro test) ,
255and homoscedasticity (Barlett tests. Kruskal Wallis test, Welch's Heteroscedastic F
256Test, and ANOVA)were performed under R environment v3.63 using the package and
257onewaytests v2.4 or lme4 v1.1-26 for sterol uptake and cell population measurements.
258Correlation between 25-NDB Cholesterol content and cell population and CO₂
259production was tested after log transformation, and check of residues normality.
260Correlation between these variables and between the different genes expression levels
261were performed with the R package ggstatsplot v0.6.8. (Patil, 2018).

262

263**Results**

264**Strain variability of fluorescent sterol uptake in fermentation-induced** 265**anaerobiosis**

266To determine sterol uptake in 39 yeast strains from different origins, we measured
267viable cells fluorescence after 25h growth in synthetic fermentation medium (SM)
268supplemented with a 25-NBD fluorescent Cholesterol probe. Results are presented
269according to the strain categories (non-Saccharomyces, *S. cerevisiae* and. non-
270*cerevisiae* *Saccharomyces*) and their fluorescence level (Fig. 1, Table 4). Data showed
271that the fluorescent signal could vary widely. The strains in the non-*Saccharomyces*
272category displayed reproducibly low fluorescence signals (300 to 4800 AU depending
273on the species), while strains from the *Saccharomyces* genus (*cerevisiae* or not)
274presented higher fluorescence signals (868 to 73000 AU). *Saccharomyces* strains had
275a significantly higher ability to take up sterols from the medium than non-
276*Saccharomyces* species under fermentation conditions (Kruskal Wallis test, p-value =
2771.6e⁻⁵). In comparison, the ability to import sterol in *S. cerevisiae* and non-*cerevisiae*
278strains was not found significantly different; however, this might be linked to the fact
279that we only tested one strain for *S. paradoxus*, *S. kudriavzevii* and *S. uvarum*.

280 Interestingly, *S. cerevisiae* wine strains presented a higher cell population (p-value =
281 0.004) but not a higher capacity to take up sterols when considering all groups. This
282 results very likely from the dispersion of values observed for flor and rum strains
283 combined with a small size of the sample. Indeed, the comparison of the sole wine and
284 oak strains indicate that wine strains significantly take up more sterol than oak isolates
285 during fermentation (p-values = 0.043) (Fig. S1). As expected, the analysis of mutants
286 of 59A *amn1* strains suggests that the deletion of *AUS1* or *PDR11* reduces 25-NBD
287 cholesterol import, and that the import was minimal when both genes were deleted.
288 If sterol can be taken up from the medium, and contribute to yeast growth, then an
289 increase in 25-NBD Cholesterol should lead to an increase in cell population and thus
290 improve fermentation. In order to evaluate these hypotheses, we tested the correlation
291 between 25-NBD Cholesterol cell content and cell populations and between 25-NBD-
292 Cholesterol and CO₂ production after 25h of fermentation (Fig S2). The correlation
293 between the cell content in 25-NBD Cholesterol and cell population was 0.48 and
294 highly significant (adj.p-value=3.4.e-03), and the correlation between the cell content
295 in 25-NBD Cholesterol and CO₂ release was 0.65 and highly significant (adj.p-
296 value=2.2.e-05). While the correlation between cell population and CO₂ production
297 was highly significant (adj.p-value=4.1.e-11), as well as the correlation of cell viability
298 with either cell population or CO₂ production (adj.p-value=5.e-3), the correlation
299 between 25-NBD Cholesterol content and cell viability was not significant (Fig. S3).

300 **Effect of exogenous phytosterols on cell growth in microaerophilic conditions**

301 To gain further insight into the understanding of the discrepancies between these
302 differences in sterol uptake, 13 strains isolated from grapes or other fruits (except CBS
303 432 and IFO 1802 chosen for their reference status) (Table 2) were selected. These
304 were used to perform drop tests in anaerobic jars. Cell growth was evaluated on a
305 synthetic fermentation medium with agar and Tween 80 (Tw), supplemented or not
306 with phytosterols. The impact of fluconazole (FLC), which inhibits lanosterol 14- α -
307 demethylase, the enzyme converting lanosterol to ergosterol, was also evaluated in
308 the absence or presence of phytosterols.

309 Detectable cell growth was observed on media containing Tween (Tw) only or Tw and
310 sterols for the first 4 *Saccharomyces cerevisiae* strains, with generally a slightly higher
311 growth when sterols were present (Fig. 2A). When FLC was added to the medium ,
312 sterol synthesis was blocked and cell growth was largely inhibited. However, growth
313 was restored when phytosterols were included in the media, except for strains

31459Aamn1,aus1, pdr11 that did not grow. The deletion of either *AUS1* or *PDR11* was
315not sufficient to cause growth inhibition (data not shown), indicating that the presence
316of one of these two genes was sufficient to provide enough sterol to maintain cell
317growth under these conditions. The same trend observed for *S. cerevisiae* strains was
318found for the 3 non-*cerevisiae* *Saccharomyces* studied (Fig. 2B), which indicates that,
319under anaerobiosis, *Saccharomyces* yeasts can import phytosterols from the medium
320in order to sustain their growth.

321For the other 5 non-*Saccharomyces* strains (Fig. 2C), various patterns were found:
322*Hanseniaspora uvarum* V4_11 grew in media containing Tw with or without sterols, but
323did not grow in presence of FLC without sterols. Growth was only partially restored
324when phytosterols were added in the presence of FLC, in a similar manner to what
325was observed for *Saccharomyces* strains. *Starmerella bacillaris*, *Lachancea*
326*thermotolerans* and *Torulaspora delbrueckii* strains grew on agar media containing Tw
327with or without phytosterols, but did not grow when FLC was added. This pattern is
328similar to that observed with the 59Aamn1,aus1,pdr11 strain, suggesting in this case
329that phytosterols were not taken up. For the last yeast strain, *Hanseniaspora*
330*guilliermondii*, growth was partially inhibited by FLC in contrast with other yeast species,
331but the presence of sterol did not mitigate FLC inhibition. Given that 25-NBD
332Cholesterol provided similar results for non-*Saccharomyces* strains, these results
333suggest that, for all these species, sterols are not taken up from the medium.

334

335**Sequence comparison and phylogeny of *AUS1*, *PDR11*, *UPC2*, *ECM22*, *ERG6*, 336*PDR18* and *SNQ2* genes of 9 selected strains from various origins**

337For the 8 species corresponding to the 13 previous studied strains, and *Metschnikowia*
338*pulcherrima*, we tried to retrieve the sequences of these 7 genes involved in sterol
339uptake from the draft genome assemblies available. When the orthologues of these
340genes were known, they were taken into account, otherwise the best candidate was
341searched for.

342The phylogeny of species in the *Saccharomycetaceae* separates genera that derived
343after an event of genome duplication (*Saccharomyces*, *Kazachstannia*,
344*Nakaseomyces*, *Naumovia*, *Tetrapisispora*, *Vanderwaltozyma*) from others that
345differentiated before this event (*Zygossaccharomyces*, *Zygotorulaspora*, *Torulaspora*,
346*Lachancea*, *Kluyveromyces*, *Eremothecium*, *Hansenispora*, *Pichia*...). As the strains
347analyzed here arose in the *Saccharomycetaceae* family either before and after genome

348duplication, two paralogues in *S. cerevisiae* will have two orthologues in post-
349duplication species, and only one in pre-duplication species. This is the case for the two
350paralogues *ECM22* and *UPC2*, for which all pre-duplication species contain one
351ancestral gene, with the exception of *Lachancea thermotolerans* that contains a
352supplementary gene highly similar to *ECM22*.

353A phylogeny tree drawn for these two genes suggested that *UPC2* presented the most
354ancestral version of the gene, whereas *ECM22* appeared to diverge after genome
355duplication occurred (Fig. 3 A). The *Lachancea thermotolerans* gene homologous to
356*ECM22*, branched in an unexpected position inside the *Saccharomyces* group,
357suggesting a potential horizontal transfer in *Lachancea thermotolerans*.

358When considering ABC transporters, it has recently been shown that *PDR18* derives
359from the duplication of *SNQ2*, which occurred just before the *Saccharomyces* genus
360speciation (Godinho *et al.* 2018). This limits the presence of *PDR18* to the
361*Saccharomyces* genus. The basal position of the gene with the highest identity to
362*SNQ2-AUS1-PDR11* genes in *Torulaspora delbrueckii*, *Lachancea thermotolerans*
363and *Metschnikowia pulcherima*, is in agreement with the differentiation of *PDR18* from
364*SNQ2* (Fig. 3 B). For the pre-duplication species, it was highly difficult to identify
365homologue genes to the ABC transporters *AUS1*, and *PDR11*. In the three species
366*Lachancea thermotolerans*, *Torulaspora delbrueckii*, and *Metschnikowia pulcherima*,
367one gene only presented a high similarity with *PDR11* and *AUS1*, but the reverse
368search in *S. cerevisiae* indicated *SNQ2*. In the two *Hanseniaspora* species, two genes
369presented a high similarity with *SNQ2* and *PDR12* or with *PDR11* and *AUS1*. (Fig. 3 B).
370Last, for *Starmerella bacillaris*, the gene with the highest similarity to *SNQ2*, *AUS1* and
371*PDR11* was more similar to *PDR5* and was also basal to the *SNQ2/PDR18* and
372*AUS1/PDR11* genes clusters (Fig. 3 B). Strikingly, the branch leading to the *AUS1* and
373*PDR11* genes clusters in the *Saccharomyces* species was also highly divergent with
374*SNQ2*. In contrast with these transporters, we could easily identify orthologues to
375*ERG6* gene in these species.

376As expected, a high level of sequence identity (Table 5) was observed between *S.*
377*cerevisiae* and other *Saccharomyces* species alleles for all proteins (ranging from 83.3
378to 96.9 %). The sequence identity of *Erg6* was also significant between the most distant
379species and *S. cerevisiae* (67% for *Starmerella bacillaris*). However, for the
380transporters *Aus1*, *Pdr11*, and *Snq2*, or the regulator *Upc2/Ecm22*, identity dropped
381sharply between *S. cerevisiae* and *Hanseniaspora* or *Starmerella* species.

383 ***AUS1*, *PDR11*, *UPC2*, *ECM22*, *ERG6*, *PDR18* and *SNQ2* gene expression analyses** 384 **in *Saccharomyces* yeast strains after 20h of wine fermentation**

385 Using these data, primers were designed for genes associated with sterol uptake
 386 (*AUS1* and *PDR11*), their transcription control (*ECM22* and *UPC2*) or the sterol
 387 biosynthesis pathway (*ERG6*) and used for assays of expression patterns by
 388 quantitative RT-PCR.

389 *AUS1*, *PDR11*, *UPC2* and *ECM22* gene expression analyses were performed for *S.*
 390 *cerevisiae* and non *cerevisiae* *Saccharomyces* strains (Fig. 4). Results showed that
 391 their expression fluctuated between strains, without major differences, but more
 392 variations could be seen for *PDR11* and *ECM22*. As expected, *AUS1* and *PDR11*
 393 expression levels were almost undetectable (residual detection) for the
 394 59Aamn1, *aus1*, *pdr11* strain. Interestingly, for this last strain, *UPC2* expression level
 395 was, on the contrary, significantly increased compared to the non-deleted 59Aamn1
 396 strain. However, this was not the case for *ECM22* gene expression. Relative *ERG6*
 397 gene expression was analyzed in all *Saccharomyces* (Fig. 4 E). For *S. cerevisiae* and
 398 non-*cerevisiae* strains, levels varied between 0.18 and 0.38 AU. Interestingly, the
 399 analysis of the correlation between the expression of these 5 genes revealed a
 400 significant correlation of the expression of *ECM22* with 25-NBD cholesterol uptake
 401 (adj.p-value= 0.036) (Fig. 4 F). It is noteworthy that the correlation of 25-NBD
 402 Cholesterol uptake with *UPC2* expression was just below the threshold (adj.p-value=
 403 0.079), and that the third highest correlation could be observed between the
 404 expressions of *AUS1* and *UPC2*, (adj.p-value= 0.108).

405 **Discussion**

406 A large panel of yeast strains from various origins and species- many of them having
 407 been isolated from a winemaking environment-, from the *Saccharomyces* genus or
 408 from other genera were selected to obtain a general overview of the ability to import
 409 sterol from the medium during fermentation. To evaluate the sterol uptake process, we
 410 used a fluorescent probe, 25-NBD Cholesterol, that enabled us to quantify by flow
 411 cytometry sterol import during wine fermentation. 25-NBD Cholesterol is a fluorescent
 412 sterol analog that possesses a large reporter group attached to the aliphatic side chain.
 413 Some studies have reported that its localization could differ from that of natural sterol

414(Mukherjee *et al.*, 1998), but it is nevertheless widely used to study sterol transport
415(Reiner *et al.*, 2005, 2006).

416In this paper, we present for the first time evidence of discrepancies in sterol uptake
417between yeast strains depending on their genetic background. We observed that non-
418*Saccharomyces* strains imported less sterol than *Saccharomyces* strains whereas we
419did not observe significant differences between *S. cerevisiae* strains and strains from
420other *Saccharomyces* species. In addition, some variation could be noticed among *S.*
421*cerevisiae* strains: under fermentation, wine isolates displayed a significantly higher
422ability to import 25NDB cholesterol than wild oak isolates, but wide variations in 25-
423NBD Cholesterol uptake could be observed for some rum and flor strains. Interestingly,
424this is in line with our recent results on the adaptation of *S. cerevisiae* from different
425niches, in which we highlighted that the genomic region of the sterol importer *AUS1*
426was under selection for wine yeast (Legras *et al.*, 2018). These differences in sterol
427uptake for the different species are correlated to differences in cell populations and
428fermentation progress at the beginning of fermentation. This is quite in agreement with
429the ability of yeast species to complete alcoholic fermentation, but it cannot be
430excluded that some additional differences could be observed considering the data at
431the end of the fermentation. In fact, it is known that sterols participate to resistance to
432ethanol stress and thus to cell viability when ethanol accumulate in the medium (Ding
433*et al.*, 2009).

434One reason for such discrepancies in sterol uptake could be due to differences in the
435genetic makeup of the strains. In *S. cerevisiae*, two sterol uptake transporters (*AUS1*
436and *PDR11* genes) have been characterized in lab strain W303-A (Wilcox *et al.*, 2002).
437However, we lack data for strains from other strains or species (other *S. cerevisiae*, *S.*
438non-*cerevisiae* and non-*Saccharomyces*). Our results of the spot tests performed
439under microaerophilic conditions indicated that, for all the selected *Saccharomyces*
440strains, sterol biosynthetic pathway functioning was necessary for growth, this growth
441being inhibited by FLC, albeit recovering when sterols were added. This confirms the
442importance of sterol uptake in anaerobic conditions for non-*cerevisiae* *Saccharomyces*
443strains. Interestingly, the presence of either *AUS1* or *PDR11* genes was sufficient to
444promote growth in the conditions tested, suggesting that these two paralog genes are
445rather substitutable. In addition, the analysis of the expression of genes involved in the
446transport of sterols, revealed a high correlation between the expression of *ECM22* with
44725-NBD Cholesterol uptake, and a slightly lower correlation for *UPC2*, which indeed

448 suggests that the differential regulation of regulators, and thus of transporters, explains
449 the differences in sterol transport among *Saccharomyces*.

450 The behavior of non-*Saccharomyces* strains - except for *Hanseniaspora guilliermondii*
451 that displayed a lower FLC sensitivity (Kuo *et al.*, 2010) in spot test experiments- was
452 different, which suggests that growth depends solely on the synthesis of ergosterol by
453 cells, sterol uptake being very low or undetectable. These physiological tests are
454 coherent with the apparent absence of orthologues for sterol uptake transporters in the
455 genome of the non-*Saccharomyces* strains analyzed here: *Hanseniaspora uvarum* and
456 *Hanseniaspora guilliermondii*, *Lachancea thermotolerans*, *Torulaspora delbrueckii*,
457 *Metschnikowia pulcherima* and *Starmarella bacillaris*. As the proteins in these species
458 with the highest identities with *Aus1* and *Pdr11* have a higher similarity with *Snq2* or
459 *Pdr12*, this suggests there is no specialization for sterol import function among the pre-
460 duplicated genome species. Interestingly, *Candida glabrata*, a post-duplicated genome
461 species, presents orthologues of sterol transporters to *Aus1* and *Pdr11* and of their
462 regulator *UPC2* and has been shown to take up cholesterol in a microaerophilic
463 environment (Zavrel *et al.*, 2013). However, in presence of Fluconazole, *C. glabrata*
464 growth was inhibited even in the presence of ergosterol (Kuo *et al.*, 2010). This yeast
465 species has also been reported to be able to compete efficiently with *S. cerevisiae*
466 during fermentation (Williams *et al.*, 2015). The strain that was included in our
467 screening for 25-NBD Cholesterol uptake did not show any ability to take up sterols
468 under our conditions, suggesting that sterol uptake may not have been active in *C.*
469 *glabrata* in our conditions.

470 The ability of lineages that underwent whole-genome duplication to take up sterol from
471 their environment are in line with the results of Merico *et al.* (2007) who showed that
472 post-duplication yeast species exhibit a good ability to grow under anaerobiosis on a
473 rich media containing ergosterol, whereas pre-duplication species presented no or
474 delayed growth on such medium. In addition, in *Kluyveromyces lactis*, there were
475 identified 20 genes essential for growth under anaerobiosis in *S. cerevisiae*, but that
476 are missing in *K. lactis* genome which may explain its inability to grow under anaerobic
477 conditions (Snoek & de Steensma, 2006). Among these genes, 7 (*ARV1*, *UPC2*, *AUS1*,
478 *PDR11*, *SUT1*, *SUT2* and *UPC2*) are related to or involved in sterol uptake. In a similar
479 manner, Bolotin-Fukuhara *et al.* (2006) compared of the genome of *Yarrowia lipolytica*,
480 *K. lactis* and *S. cerevisiae* and emitted the hypothesis that the ability to uptake sterol
481 might have been a key function for the adaptation to anaerobiosis.

482In order to test if the sterol content in the cell is the limiting factor for cell growth of non-
483*Saccharomyces* yeast under anaerobic environment, it would be interesting to
484overcome the import of sterol barrier through the heterolog expression of the *S.*
485*cerevisiae* gene *AUS1* in non-*Saccharomyces* species. Kuo *et al.* (2010) have reported
486that the overexpression of *AUS1* in *K. lactis* relieved some of the Fluconazole inhibition
487when ergosterol is added in the media. The comparison of the fermentation
488performances of these strains might provide some insight about the importance of
489sterol import for wine fermentation. However, given the low ability of *K. lactis* to grow
490under anaerobiosis (Merico *et al.*, 2007), an enhanced uptake of exogenous sterol
491might not be sufficient to achieve complete alcoholic fermentation. By contrast, species
492of the *Torulaspora* clade that are able to grow anaerobically (Merico *et al.*, 2007) and
493are moderately short- and long-term Crabtree-positive (Hagman *et al.*, 2014) might be
494good candidates for such tests. Interestingly, *T. delbrueckii* displays good fermenting
495ability, albeit lower than *S. cerevisiae*.

496In contrast to transporters for sterol uptake, we could identify orthologues of the *ERG6*
497gene that codes for the delta(24)-sterol C-methyltransferase of the ergosterol
498biosynthetic pathway, whose sequences were conserved between *Saccharomyces*
499and non-*Saccharomyces* wine yeast strains. We could observe, in the non-
500*Saccharomyces* strains tested in drop test assays, a still active ergosterol biosynthesis
501pathway under our anaerobiosis conditions. This is interesting as it has been reported
502that sterol biosynthesis in the absence of oxygen did not occur in *Saccharomyces*
503*cerevisiae* strains (Wilcox *et al.*, 2002), suggesting a different behavior for non-
504*Saccharomyces* strains. This may reflect a better ability to use traces of oxygen present
505in our experimental setup for sterol biosynthesis.

506In conclusion, we have shown a good conservation of the sterol uptake mechanisms
507among *Saccharomyces* strains (*cerevisiae* and non-*cerevisiae*), but a different
508behavior of the non-*Saccharomyces* belonging to the pre-duplicated genome species.
509Wine strains of *S. cerevisiae* displayed a higher ability than oak strains to take up
510sterols from the medium during wine fermentation, suggesting a possible adaptation to
511anaerobic fermentation. By contrast, the non-*Saccharomyces* species in pre-
512duplication genera tested here did not develop such sterol uptake function permitting
513growth in an environment with limiting oxygen availability. This suggests that, in
514addition to the apparition of the Crabtree effect, one key step in the evolution of the
515*Saccharomycetaceae* (Dashko *et al.*, 2014) may have been the acquisition of sterol

516uptake. As this innovation contributed to an improved growth in anaerobic/fermentative
517environments, it should have quickly favored the expansion of these fungal species in
518this environment, especially for the *Saccharomyces* genus.

519These different abilities to take up sterols from the medium might also offer
520opportunities to wine makers. Those who wish to replace yeast starters by preparing a
521“pied de cuve” from grapes (Börlin *et al.*, 2020) should prepare it under anaerobiosis
522from unsettled grape juice. The higher cell population and higher phytosterols content
523in unsettled must should mainly favor strains from the *Saccharomyces* genus, while
524limiting the growth of non-*Saccharomyces*. By contrast, the growth of non-
525*Saccharomyces* yeast can only be favored through the addition of some oxygen to the
526grape must.

527As a consequence, more work has to be performed in order to decipher the cross
528relations between oxygen, the regulation of sterol uptake and ergosterol biosynthesis,
529in *S. cerevisiae* and the other yeast species in the wine making environment.

530

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Fig. 1. 25-NBD Cholesterol fluorescence uptake (arbitrary units, AU) detected by flow cytometry in alive cells from 39 *Saccharomyces cerevisiae*, *Saccharomyces non-cerevisiae* and non-*Saccharomyces* strains grown 25h after inoculation at 28°C. Measures were performed twice for strains with error bars.

A- 25NBD-Cholesterol uptake

B- Cell populations

Fig. 2. Analysis of phytosterol uptake under microaerophilic environment by droptest. Four serial tenfold dilutions of *S. cerevisiae* (A), *S. non-cerevisiae* (B) and *non-Saccharomyces* (C) strains spotted onto various synthetic standard agar media (SM425, 425 mg/L assimilable nitrogen) with Tween 80 (0.05%), supplemented with either phytosterols (sterols), in the presence or not of fluconazole (FLC, 256 µg/mL). Plates were incubated at 28°C for six days under anaerobiosis in an anaerobic jar (Anaerocult™ A, Merck, Millipore) with an anaerobic indicator.

Figure 2

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Fig. 3. Maximum likelihood phylogenetic tree of the genes with the highest identity with *UPC2* and *ECM22* (A), *AUS1*, *PDR11*, *PDR18* and *SNQ2* (B) and *ERG6* (C) for the 9 species used here (*HANGU* : *Hanseniaspora guilliermondii*, *HANUVI* : *Hanseniaspora uvarum*, *LACTH*: *Lachancea thermotolerans*, *STABA* : *Starmerella bacillaris*, *SACE* : *Saccharomyces cerevisiae*, *SACKU* : *Saccharomyces kudriavzevii*, *SACPA* : *Saccharomyces paradoxus*, *SACUV* : *Saccharomyces uvarum*, *TORDE* : *Torulaspora delbrueckii*).

Fig. 4. Analysis of the expression of *AUS1* (A), *PDR11* (B), *UPC2* (C), *ECM22* (D), and *ERG6* (E) genes by RT-PCR in *Saccharomyces cerevisiae* and non-*cerevisiae* strains from different origins, and correlation between the expression of the different genes. Analyses were performed after 20h of fermentation at 28°C. Expressions were normalized to *ACT1* gene. Graphs represent the average of three technical replicates with the standard errors.

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695 **Table1. Oligonucleotides used for generation of deletion mutants**

Primer name	Sequence
A-AUS1	CTGAAGATCACTCCATATACAGGCT
B-AUS1	GGCAAATCAACTAAATGACATAACC
C-AUS1	CTTTCATAGAAGAATACGGTGGCTA
D-AUS1	ACTCTGAGAAACAACAAAATAACGG
A-PDR11	CACTTTTGTTTCCTACAACCTCCAC
B-PDR11	GTGAAGTCGTCTTAGTACTGGGAAA
C-PDR11	TGAGTTATCATCACAGATGGAGAAA
D-PDR11	ATTAGAACATTCCTTGATTGTCATC
A-DAN1	AAAATTGATGAATGTATCCTCCGTA
B-DAN1	TTATGATGAAAGGGTCAATTTGATT
C-DAN1	CCAAAGCTCAATCTACAGCTACTTC
D-DAN1	TACGATCCAATAGCGACGTATATAT
KanB	CTGCAGCGAGGAGCCGTAAT
KanC	ATTACGCTCGTCATCAAAATCA
F-AUS1hygro	CTCTCAGTCCTTGCAGTCTGCTTTTTCTGGAATTAATATGTTTCGTACGCTGCAGGTGAC
R-AUS1hygro	TATTAAGTAGAAAAGTAGAAATATATTTAAAAATGGTATTAGCATAGGCCACTAGTGGATCTG
Hygro1	GCAGCTATTTACCCGCAGGA
Hygro2	TTCTGGAGGCCGTGGTTGGC

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698**Table 2. List of the 13 selected strains to study the origin of sterol uptake**
699**discrepancies by drop test experiments.**

Species	Yeast strains	Origin	Isolation year	Collection , person who isolated
<i>Hanseniaspora guillermondii</i>	Braga 590 / MTF 1219	Wine (Braga, Portugal)	2004	Universidade do Minho, D. Schuler
<i>Hanseniaspora uvarum</i>	V4_11 / MTF 3787	Viognier grape must (Montpellier France)	2015	SPO collection, JL Legras
<i>Lachancea thermotolerans</i>	CLIB 3053/ V4_14	Viognier grape must (Montpellier France)	2015	CIRM Levures, INRAE, Montpellier JL Legras
<i>Saccharomyces cerevisiae</i>	K1_28-1A_Mata a / MTF 2534	Wine (haploid derivative from K1 starter)	2010	SPO collection, S. Dequin (1)
	LALVIN 2056	Wine (Rhône Valley, France)	<1990	Lallemand collection
	Lava32.15	Wine (Açores, Portugal)	2004	U. Minho (D. Schuler)
	59Aamn1	haploid derived from EC1118	2009	SPO collection, S. Dequin
	59Aamn1,aus1, pdr11	from 59Aamn1	2020	SPO collection, this work
<i>Saccharomyces kudriavzevii</i>	IFO 1802	oak liter, Japan	2009	NBRC collection
<i>Saccharomyces paradoxus</i>	CBS 432/CLIB 228	Unknown, The Netherlands	<1994	CIRM Levures , INRAE, Montpellier
<i>Saccharomyces uvarum</i>	CLIB 251 / CBS 395	Black current juice, The Netherlands	1898	CIRM Levures, INRAE, Montpellier
<i>Starmerella bacillaris</i>	V8-1	Viognier grape must, Sommières, France	2016	SPO collection, JL Legras
<i>Torulaspora delbrueckii</i>	CLIB 507	Grapes, Bordeaux, France	<1965	CIRM Levures, INRAE, Montpellier

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Table 3. Oligonucleotides used for quantitative RT-PCR (F : forward, R : reverse, Hg : *Hanseniaspora guilliermondii*, Hu : *Hanseniaspora uvarum*, Lt : *Lachancea thermotolerans*, nS : non *Saccharomyces*, Sb : *Starmerella bacillaris*, Sc : *Saccharomyces cerevisiae*, Sk : *Saccharomyces kudriavzevii*, Snc : *S. non cerevisiae*, Sp : *Saccharomyces paradoxus*, Su : *Saccharomyces uvarum*, Td : *Torulaspora delbrueckii*).

Primer name	Primer sequence
ACT1_F_Sc_Snc	GGACTTCGAACAAGAAATGC
ACT1_F_Sb	AAAGCCGGTTTCGGTGGTGAGGA
ACT1_F_Hu_Hg	AAAGCCGGTTTTGCTGGTGATGA
ACT1_F_Lt	AAAGCAGGGTTTAGCGGTGAAGA
ACT1_F_Td	AAAGCAGGCTTTTGTGGTGAAGA
ACT1_R_Sc_Snc	GCTCTGAATCTTTCGTTACC
ACT1_R_Sb	CCAGATCTTCTCCATATCATC
AUS1_F_Sc_Snc	AGAGCTTTTCAA(C/A)GAAGTTTGGGTGATA
AUS1_R_Sc_Snc	AGGT(C/T)AA(G/A)GAACCTCTTGAGTATGAACC
ECM22_F_Sc_Snc	AC(A/T/C)ATAAC(T/C)GAAGCAGG(T/C)ATTTCTGG
ECM22_R_Sc_Snc	TC(C/T)CTTAA(C/T)AG(T/C)CT(T/C)AA(C/T)GCTTCTAAACG
ERG6_F_Sc_Snc	AAGATT(T/C)AC(T/C)GG(T/C)TGTAACGTCATCGG
ERG6_F_nS	AC(A/T/G/C)GA(C/T)TT(C/T)TA(T/C)GA(A/G)TA(C/T)GG(A/C/T)TGGGG
ERG6_R_Sc_Snc	CA(T/C)TCGTAAAC(A/G)GCAAAGGTACCACC
ERG6_R_nS	C(T/G)(A/T/G/C)GC(T/A)GG(A/T/G/C)CC(A/T/G/C)CC(A/T/G/C)ACACC
PDR11_F_Sc_Snc	TCC(C/T)GCTATGTTTGCCATGGAAGC
PDR11_R_Sc_Snc	C(G/A)CCTTGCCA(G/A)GCACAAGCTTTATG
UPC2_F_Sc_Snc	GC(G/A)(TC)TA(C/T)TGATGACAGGTGA(C/T)TTAGG(C/T)GC
UPC2_R_Sc_Snc	AGGAAATC(T/C)AGCATCATATGCATACCACC

1 Table 4. Species summary of the uptake of 25-NBD Cholesterol and of cell populations.

2 Measures were performed after 25 hours of growth during fermentation in a synthetic must (induced anaerobiosis). Standard
3 variations have been estimated from two independent cultures (1 strain) or from the mean per strain (2 or more).

Genus	Species	Number of strains tested	25-NBD Cholesterol absorption under anaerobiosis (AU)	Cell population (x10 ⁶ /ml) under anaerobiosis
<i>Saccharomyces</i>				
<i>Saccharomyces</i>	<i>cerevisiae</i>	13	9804 ± 7266	193 ± 69.5
<i>Saccharomyces</i>	<i>paradoxus</i>	1	25579 ± 25021	138 ± 11.1
<i>Saccharomyces</i>	<i>kudriavzevii</i>	1	60299 ± 19676	157 ± 12.6
<i>Saccharomyces</i>	<i>uvarum</i>	1	3938 ± 425	109 ± 6.9
<i>Non-Saccharomyces</i>				
<i>Candida</i>	<i>glabrata</i>	1	2477 ± 150	181 ± 19.4
<i>Hanseniaspora</i>	<i>uvarum</i>	2	1335 ± 631	59 ± 61
<i>Hanseniaspora</i>	<i>guillermundii</i>	1	407 ± 105	177 ± 4.5
<i>Hanseniaspora</i>	<i>opuntiae</i>	1	2690 ± 1784	0.38 ± 0.04
<i>Lachancea</i>	<i>thermotolerans</i>	1	1720 ± 350	141.7 ± 87
<i>Metschnikowia</i>	<i>pulcherrima</i>	2	3000 ± 1473	30 ± 13.6
<i>Pichia</i>	<i>kudriavzevii</i>	1	2170 ± 793	18.0 ± 1
<i>Pichia</i>	<i>occidentalis</i>	1	2000 ± 1879	72.2 ± 58.1
<i>Pichia</i>	<i>terricola</i>	1	2904.5 ± 350	12.8 ± 3
<i>Starmerella</i>	<i>bacillaris</i>	3	660 ± 84	84.9 ± 57.0
<i>Torulaspora</i>	<i>delbrueckii</i>	2	2334 ± 2115	120 ± 18.4
<i>Wickerhamomyces</i>	<i>anomalus</i>	2	1568 ± 429	16.8 ± 11.7
Significance tests			p-value	p-value
<i>Saccharomyces</i> /	<i>Non-Saccharomyces</i>	(Kruskal-Wallis test)	1.6 * 10 ⁻⁰⁵	0.011
<i>S. cerevisiae</i>	<i>All origins</i>	ANOVA	0.163	0.004
<i>S. cerevisiae</i> wine /	<i>S. cerevisiae</i> oak	ANOVA	0.015	0.003

Table 5. Comparison of the similarities of the homologues of Aus1p, Pdr11p, Upc2p, Ecm22p, Erg6p, Pdr18p and Snq2p of non-*Saccharomyces* and *S. non-cerevisiae* strains with *S. cerevisiae* S288C (sequence length in aa is indicated in parenthesis).

<i>Species \ Gene</i>	<i>Aus1</i>	<i>Pdr11</i>	<i>Pdr18</i>	<i>Snq2</i>	<i>Upc2</i>	<i>Ecm22</i>	<i>Erg6</i>
<i>Saccharomyces cerevisiae</i>	100 (1394)	100 (1411)	100 (1333)	100 (1501)	100 (913)	100 (814)	100 (383)
<i>Saccharomyces paradoxus</i>	95.3 (1394)	95.5 (1412)	93.1 (1333)	96.9 (1501)	94.1 (916)	92.6 (810)	96.9 (384)
<i>Saccharomyces uvarum</i>	90.9 (1394)	88.7 (1413)	81.6 (1431)	90.2 (366)	87.0 (486)	83.3 (813)	92.7 (384)
<i>Saccharomyces kudriavzevii</i>	90.3 (1393)	89.9 (1407)	87.1 (1332)	93.7 (1502)	87.9 (512)	83.8 (590)	94.5 (384)
<i>Lachancea thermotolerans</i>	36.2* (1499)	35.3* (1499)		65.8 (1499)	60.3 (788)	45.8 (722)	83.3 (384)
<i>Torulaspora delbrueckii</i>		35.6* (1500)		73.8 (1500)	64.2 (810)		82 (374)
<i>Hanseniaspora uvarum</i>		31.7* (1514)		63.4 (1514)	56.5 (1106)		71.9 (401)
<i>Hanseniaspora guilliermondii</i>		32.2* (1369)		64.5 (1523)	43.4 (1090)		72.2 (400)
<i>Metchnikowia pulcherima</i>		35.0* (1352)		54.0 (1352)	51.7 (498)		65.9 (376)
<i>Starmerella bacillaris</i>				37.4* (1502)	46.8 (829)		66.7 (392)

* : When the orthologue was not found, the % of similarity obtained for the gene with the highest homology is given.

Table S1. List of the 39 strains used in this study

Species	Yeast strains	Origin	Year of isolation	Provider
<i>Candida glabrata</i>	ICV D222 / MTF169	grapes, France	<1990	SPO internal collection, INRAE, Montpellier
<i>Hanseniaspora guillermondii</i>	Braga 590 / MTF 1219	grapes, Portugal	2004	Universidade do Minho, D. Schuler
<i>Hanseniaspora opuntiae</i>	CLIB 3093 / S1-2	grape must, Puylacher, France	2015	CIRM Levures, SPO INRAE Montpellier JL Legras
<i>Hanseniaspora uvarum</i>	CLIB 303 T	Grapes, Ukraine	<1996	CIRM Levures, SPO INRAE Montpellier
<i>Hanseniaspora uvarum</i>	V4_11 / MTF 3787	grape must, France	2015	SPO internal collection, INRAE Montpellier, JL Legras
<i>Lachancea thermotolerans</i>	CLIB 3053 / V4_14	grape must, France	2015	CIRM Levures, SPO Montpellier JL Legras
<i>Metschnikowia pulcherrima</i>	Braga 604 / MTF 1221	unknown	<2004	Universidade do Minho, D. Schuler
<i>Metschnikowia pulcherrima</i>	V4_22 /MTF3798	grape must, France	2015	CIRM Levures, SPO Montpellier JL Legras
<i>Pichia kudriavzevii</i>	CLIB 884 T	fruit juice,	<2002	CIRM-Levures, INRAE, Montpellier,
<i>Pichia occidentalis</i>	CBS 5459 T	spoiled figs ? – USA.	unknown	Westerdijk Institute, The Netherlands
<i>Pichia terricola</i>	CBS 2617 T	Soil, South Africa	unknown	Westerdijk Institute, The Netherlands
<i>Saccharomyces cerevisiae</i>	Bdx 21-1/MTF2376	Oak liter, France	2011	SPO internal collection, INRAE Montpellier, JL Legras (2)
	Rom 3-2/MTF2414	Oak liter, Romania	2011	SPO internal collection, INRAE Montpellier, JL Legras (2)
	ZP 611 /MTF 2421	Oak bark, Canada	2012	Universidade Nova de Lisboa, Portugal, JP Sampaio (4)
	ZP 851 / MTF 2418	oak bark, Spain	2012	Universidade Nova de Lisboa, Portugal, JP Sampaio (4)
	245 Antilles / MTF2248	rum, Guadeloupe, France	<2004	SPO internal collection, INRAE Montpellier, S. Dequin (2)
	309 Antilles / MTF 2247	rum, Guadeloupe, France	<2004	SPO internal collection, INRAE Montpellier, S. Dequin (2)
	CLIB 1769 /P3-D5-Mata	flor, France	<2004	SPO internal collection, INRAE Montpellier, S. Dequin (1)
	FINO : 7.7 / MTF 2265	flor, Spain	<2009	SPO internal collection, INRAE Montpellier, S. Dequin (1)
	TS12-A7 / MTF 2277	flor, Hungary	<2009	SPO internal collection, INRAE Montpellier, S. Dequin (1)
	K1_28-1A_Mata / MTF 2533	Wine (haploid derivative from K1 starter)	2011	SPO internal collection, INRAE Montpellier, S. Dequin (1)
	LALVIN2056 / MTF 2117	Wine (Rhône Valley, France)	1980	Lallemand collection

	MC_N 1500 3C / MTF 789	wine, Montpellier, France	2003	SPO internal collection, INRAE Montpellier, S. Dequin (2)
	Lava 32.15 /MF 2334	wine, Portugal	2004	U. Minho, Braga, D. Schuler (2)
	59Aamn1 /MTF 2521	haploid derived from wine strain EC1118	2015	SPO internal collection S. Dequin (3)
	59Aamn1,aus1,pdr11	from 59Aamn1	2020	SPO internal collection, this work,
	59Aamn1,aus1	from 59Aamn1	2020	SPO internal collection, this work
	59Aamn1,pdr11	from 59Aamn1	2020	SPO internal collection, this work
	59Aamn1,dan1	from 59Aamn1	2020	SPO internal collection, this work
<i>Saccharomyces kudriavzevii</i>	IFO 1802 T	oak liter , Japan	2009	SPO internal collection
<i>Saccharomyces paradoxus</i>	CBS 432 T	unknown origin	<1994	CIRM-Levures, SPO, INRAE, Montpellier
<i>Saccharomyces uvarum</i>	CLIB 251 T	beverage, The Netherlands	1989	CIRM-Levures, SPO INRAE Montpellier
<i>Starmerella bacillaris</i>	CBS 9494 T	Wine, Tokaj, Hungary	2000	Westerdijk Institute, The Netherlands, M. Sipiczki
<i>Starmerella bacillaris</i>	S6-5	Sauvignon grape must, Gruissan, France	2016	SPO internal collection, INRAE Montpellier, JL Legras
<i>Starmerella bacillaris</i>	V8-1	Viognier grape must, Sommières, France	2016	SPO internal collection, INRAE Montpellier, JL Legras
<i>Torulaspora delbrueckii</i>	CLIB 507	grapes, Bordeaux, France	<1965	CIRM Levures, INRAE, Montpellier
<i>Torulaspora delbrueckii</i>	S1_D / CLIB 3039	Sauvignon grape must, Puylacher, France	2015	CIRM-Levures, INRAE Montpellier, JL Legras
<i>Wickerhamomyces anomalus</i>	CLIB 284	unknown origin, USA	<1994	CIRM-Levures, INRAE Montpellier
<i>Wickerhamomyces anomalus</i>	V4_45 /CLIB 3055	Viognier grape must, Fabrezan, France	2015	CIRM-Levures, INRAE Montpellier, JL Legras

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Fig. S1. Bar plot comparing sterol uptake (A) and cell population (B) of *Saccharomyces cerevisiae* isolated from oak, flor, rum and wine origins after 25h fermentation.

24**Fig. S2.** CO₂ production by the 39 strains after 25h of growth during fermentation at 25 conditions at 28°C. This parameter reflect sugar consumption

CO2 production after 25h of fermentation

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28**Fig. S3.** Cell viability measured in the 39 strains after 25h of growth under fermentation
29at 28°C. Experiments were performed twice for all strains in error bars.

Cell viability after 25h of fermentation

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