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Catherine Tesnière[1], Martine Pradal[1] , Jean-Luc Legras[1,2]

[1] SPO, Univ Montpellier, INRAE, Institut Agro, Montpellier, France

[2] CIRM-Levures, SPO, Univ Montpellier, INRAE, Institut Agro, Montpellier, France

Address correspondence to Jean-Luc Legras, jean-luc.legras@inrae.fr

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Abstract

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

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

Introduction

Sterols are essential lipids that determine membrane properties such as permeability, transport, resistance to stress. In *Saccharomyces cerevisiae*, a higher sterol content can change membrane fluidity and thus resistance to ethanol stress (Tesnière, 2019). *S. cerevisiae* cells are able to synthesize sterols as ergosterol only in aerobic conditions, this ergosterol being a typical, main sterol of the membranes (Weete *et al.*, 2010). Indeed, oxygen is required for the epoxidation of squalene by squalene epoxidase *Erg1p,* which is a limiting step for the biosynthesis of ergosterol. Under strict anaerobic conditions, the growth of *S. cerevisiae* strains is extremely reduced, but some residual growth is maintained (Da Costa *et al.*, 2019). However, when available, *S. cerevisiae* is able to incorporate a variety of exogenous sterols (Lorenz & Parks, 1987), such as cholesterol, ergosterol or phytosterols (Luparia *et al.*, 2004; Zavrel *et al.*, 2013). While, cholesterol is the major sterol of the animals, plants produce specific sterols called phytosterols; in grape skins, four such sterols have been identified, i.e.β-

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

Because wine alcoholic fermentations are performed under anaerobiosis, lipid compounds represent important nutrients in winemaking that are necessary for cells to complete fermentation (Rosenfeld *et al.*, 2003; Tesnière, 2019). The high amount of carbon dioxide produced (2 CO_2 molecules per molecule of glucose) during wine fermentation, together with oxygen consumption through yeast growth, provoke a reduction in dissolved oxygen concentration (Salmon et al., 2004; Saa *et al.*, 2012; Cerda-Drago *et al.*, 2016), which may lead to a change in membrane lipid composition (Ochando *et al.*, 2017). However, an increased ergosterol content protects yeast cells against the membrane thickness reduction effect caused by high ethanol concentration (Vanegas *et al.*, 2012).

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

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

synthesis by *S. cerevisiae* of a peptide may explain its dominance over these two species (Pérez Nevado *et al.*, 2006, Branco *et al.*, 2014); for *Torulaspora delbrueckii* and *Lachancea thermotolerans* the reduction of oxygen availability participates to the reduction of fitness in comparison to *S. cerevisiae* (Nissen *et al.*, 2003). In contrast to *S. cerevisiae*, for which sterol import plays a key role under anaerobiosis, the lack of sterol uptake transporters has been noticed in some yeast species such as *Yarrowia lipolytica* and *Kluyveromyces lactis*; it has been hypothesized to be one of the factors explaining the absence of growth of *K lactis* under anaerobiosis (Bolotin-Fukuhara *et al.*, 2006; Snoek & de Steensma, 2006). While some authors have focused on nitrogen consumption in non-*Saccharomyces* species (Su *et al.*, 2020), sterol uptake has not been investigated for other yeast species of the wine microbial community, such as *Torulaspora delbrueckii* or *Hanseniaspora uvarum* that are well known for their ability to grow and ferment in grape must. Indeed, yeast species from genera other than *Saccharomyces* are increasingly investigated for their ability to interact with *S. cerevisiae*, and to modulate the flavor and taste of wines (Masneuf-Pomarede *et al.*, 2016), indicating that this ecological specialization has technological consequences. Thus, studying sterol import in *Saccharomyces* and non-*Saccharomyces* wine strains will help understanding the behavior of these fungi during fermentation and eventually help to design optimal practices to favor the selective growth of specific species.

In this paper, we investigate sterol import under wine fermentation conditions in *S. cerevisiae* from different origins as well as in several other yeast species (non-*cerevisiae Saccharomyces* and non-*Saccharomyces*). In a first step aimed at assessing whether sterols uptake can promote growth under fermentation, the uptake of a fluorescent derivative of cholesterol (25-NBD Cholesterol) was measured using flow cytometry. Complementary spot test assays performed in anaerobic jars on a medium containing phytosterols alone or combined with fluconazole provide additional clues on sterol uptake ability to restore growth under anaerobiosis for several species. In a second step, the presence of genes homologues to genes coding for ABC transporters involved in sterol transport in *S. cerevisiae* and other closely related genes (*AUS1, PDR11, PDR18* and *SNQ2*) and their regulators (*UPC2* and *ECM22*) were searched among these non-*cerevisiae* S. and non-*Saccharomyces* strains. The analysis of their expression with that of *ERG6 (as* a gene marker of the ergosterol biosynthetic pathway) by quantitative RT-PCR for strains of the different

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

Materials and methods

Yeast strain constructions

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

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

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

Strains and growth conditions

The 39 strains selected in the present study (Table S1), consisting of 13 *S. cerevisiae* (among which 5 mutants as controls: 59A*amn1; 59Aamn1,aus1; 59Aamn1, pdr11; 59Aamn1,aus1,pdr11 and 59Aamn1,dan1*), 3 non-*cerevisiae Saccharomyces* and 18 non-*Saccharomyces,* were used to evaluate cell sterol uptake, CO_2 production, population and viability. To that end, strains were grown at least 22h in 20ml flasks containing 15 ml fermentation medium, closed by a stopper in which a 200ml filtered cone was inserted in order to release CO_2 excess. The medium was the MS425 synthetic fermentation medium (Bely *et al.*, 1990) containing 0.05% Tween 80 and 20µg/mL of a cholesterol mixture (cholesterol/ 25-NBD Cholesterol, 1:1, w/w).

Experiments were generally performed twice. Anaerobiosis was induced by the CO_2 flush produced by alcoholic fermentation.

Among these strains, 13 were selected (Table 2) to evaluate the ability of exogenous sterols to restore growth under anaerobiosis. They were mainly isolated from fruits except strains CBS 432 and IFO 1802. They consisted in 4 *S. cerevisiae* (K1_28-1A, LALVIN 2056, Lava32.15 and 59A*amn1* that could be considered as a positive control), 3 *Saccharomyces* (*S. paradoxus*, *S. kudriavzevii* and *S. uvarum*), and 5 non-*Saccharomyces* (*Hanseniaspora guillermondii*, *Hanseniaspora uvarum*, *Starmerella bacillaris*, *Lachancea thermotolerans*, and *Torulaspora delbrueckii*). In addition, the 59A*amn1* strain deleted for the two *AUS1* and *PDR11* sterol transporter genes was studied in parallel. This last strain was considered as a negative control.

Spot tests in anaerobic jars

These 13 strains were propagated on YPD agar plates after an overnight preculture in YPD medium. Aliquots were grown at 28°C in 5ml complete liquid medium up to 1 OD_{660}. 1 OD (10^7 cells) were collected (700g, 5min 20°C) and the resulting pellet was washed two times with water. Cells were resuspended in 1ml water and 4 successive 1/10 dilutions were prepared. 10µL of each dilution was spotted on SM425 gelled with 25g/L agar containing Tween 80 (0.05%). According to the tested modalities, phytosterols (20µg/mL) and Fluconazole (FLC) (256µg/mL) were added to the media to evaluate their effect. Agar plates were incubated at 28°C in anaerobic conditions. To that end, plates were introduced in anaerobic jars (2.5L), and anaerobiosis was provoked using the Anaerocult™ A reagent (Merck, Millipore) according to the manufacturer's instructions. Cell growth was evaluated after 6 days. Experiments were repeated twice.

Assays of sterol uptake by flow cytometry

Before flow cytometry analysis cells were harvested from 0.5mL liquid medium and washed twice with cold PBS (PBS : 130mM NaCl, 2.6mM KCl, 7mM Na_2HPO_4, 1.2mM KH_2PO_4, pH 7.4) containing 0.5% (w/v) Nonidet P-40) and once with PBS at room temperature, before resuspension in 0.5mL PBS. 5µL of propidium iodide (0.1mg/mL) was added to cells just before cytometry analysis on an C6 Accuri apparatus (BD Biosciences). The histogram for red fluorescence (propidium iodide) was used for gating, allowing to exclude dead cells from the analysis. The green fluorescence corresponding to the 25-NBD Cholesterol present in live cells was registered (OD_{ex} 488nm; OD_{em} 500-530nm). For each species, measures were taken on at least two

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

RNA isolation

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

Search of orthologues of and gene phylogeny

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

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

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

Quantitative RT-PCR analysis

For quantitative RT-PCR analysis, specific primers were designed (Table 3). Real-time quantitative PCR was conducted with SYBRGreen I PCR Master Mix using the gene-specific primers. Each PCR reaction (25µL final volume) contained 5 µL of template cDNA, 250nM of each primer and 1µL 2 x Power SYBR Green I PCR Master Mix (Applied Biosystems, Warrington, UK). Thermocycling conditions were as follows: an initial enzyme activation of 10 min at 95 °C, followed by 40 cycles of denaturation for 15s at 95°C, annealing and extension for 1 min at 60°C, with a final melt gradient starting from 60°C and heating to 95°C at a rate of 0.03°Cs^{-1}. The real-time PCR reactions were carried out in a 7300 Fast Real Time PCR System (Applied Biosystems, Warrington, UK). Fluorescence was measured at the 497nm (excitation) and 521nm (detection) wavelengths at the end of each extension step and at each 1°C increment of the melt profile. Primer specificity was confirmed by analyzing dissociation curves of the PCR amplification products. All cDNA samples to be compared for transcript levels were analyzed in triplicate for each gene in a single batch for each primer pair. To ascribe a relative transcript copy number to each cDNA sample, a purified PCR fragment of each gene sequence was serially diluted 10-fold to obtain template standards. The most concentrated standard was assigned an arbitrary transcript copy number and subsequent n-fold dilutions were accordingly assigned as relative copy numbers. Standards from 10^5 to 10^9 were included in the real-time PCR assay of cDNA samples. In each case, a dilution series of standards showed a linear change in the

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

Statistical analyses

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

Results

Strain variability of fluorescent sterol uptake in fermentation-induced anaerobiosis

To determine sterol uptake in 39 yeast strains from different origins, we measured viable cells fluorescence after 25h growth in synthetic fermentation medium (SM) supplemented with a 25-NBD fluorescent Cholesterol probe. Results are presented according to the strain categories (non-Saccharomyces, *S. cerevisiae* and. non-*cerevisiae Saccharomyces*) and their fluorescence level (Fig. 1, Table 4). Data showed that the fluorescent signal could vary widely. The strains in the non-*Saccharomyces* category displayed reproducibly low fluorescence signals (300 to 4800 AU depending on the species), while strains from the *Saccharomyces* genus (*cerevisiae* or not) presented higher fluorescence signals (868 to 73000 AU). *Saccharomyces* strains had a significantly higher ability to take up sterols from the medium than non-*Saccharomyces* species under fermentation conditions (Kruskal Wallis test, p-value = $1.6e^{-5}$). In comparison, the ability to import sterol in *S. cerevisiae* and non-*cerevisiae* strains was not found significantly different; however, this might be linked to the fact that we only tested one strain for *S. paradoxus*, *S. kudriavzevii* and *S. uvarum*.

Interestingly, *S. cerevisiae* wine strains presented a higher cell population (p-value = 0.004) but not a higher capacity to take up sterols when considering all groups. This results very likely from the dispersion of values observed for flor and rum strains combined with a small size of the sample. Indeed, the comparison of the sole wine and oak strains indicate that wine strains significantly take up more sterol than oak isolates during fermentation (p-values =0.043) (Fig. S1). As expected, the analysis of mutants of 59A amn1 strains suggests that the deletion of *AUS1* or *PDR11* reduces 25-NBD cholesterol import, and that the import was minimal when both genes were deleted.

If sterol can be taken up from the medium, and contribute to yeast growth, then an increase in 25-NBDCholesterol should lead to an increase in cell population and thus improve fermentation. In order to evaluate these hypotheses, we tested the correlation between 25-NBD Cholesterol cell content and cell populations and between 25NBD-Cholesterol and CO_2 production after 25h of fermentation (Fig S2). The correlation between the cell content in 25-NBD Cholesterol and cell population was 0.48 and highly significant (adj.p-value=3.4.e-03), and the correlation between the cell content in 25-NBD Cholesterol and CO2 release was 0.65 and highly significant (adj.p-value=2.2.e-05). While the correlation between cell population and CO2 production was highly significant (adj.p-value=4.1.e-11), as well as the correlation of cell viability with either cell population or CO2 production (adj.p-value=5.e-3), the correlation between 25-NBD Cholesterol content and cell viability was not significant (Fig. S3).

Effect of exogeneous phytosterols on cell growth in microaerophilic conditions

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

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

59A*amn1,aus1*, *pdr11* that did not grow. The deletion of either *AUS1* or *PDR11* was not sufficient to cause growth inhibition (data not shown), indicating that the presence of one of these two genes was sufficient to provide enough sterol to maintain cell growth under these conditions. The same trend observed for *S. cerevisiae* strains was found for the 3 non-*cerevisiae Saccharomyces* studied (Fig. 2B), which indicates that, under anaerobiosis, *Saccharomyces* yeasts can import phytosterols from the medium in order to sustain their growth.

For the other 5 non-*Saccharomyces* strains (Fig. 2C), various patterns were found: *Hanseniaspora uvarum V4_11* grew in media containing Tw with or without sterols, but did not grow in presence of FLC without sterols. Growth was only partially restored when phytosterols were added in the presence of FLC, in a similar manner to what was observed for *Saccharomyces* strains. *Starmerella bacillaris*, *Lachancea thermotolerans* and *Torulaspora delbrueckii* strains grew on agar media containing Tw with or without phytosterols, but did not grow when FLC was added. This pattern is similar to that observed with the 59A*amn1*,*aus1*,*pdr11* strain, suggesting in this case that phytosterols were not taken up. For the last yeast strain, *Hanseniaspora guillermondii,* growth was partially inhibited by FLC in contrast with other yeast species, but the presence of sterol did not mitigate FLC inhibition. Given that 25-NBD Cholesterol provided similar results for non-Saccharomyces strains, these results suggest that, for all these species, sterols are not taken up from the medium.

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

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

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

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

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

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

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

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

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

AUS1, *PDR11*, *UPC2* and *ECM22* gene expression analyses were performed for *S. cerevisiae* and non *cerevisae Saccharomyces* strains (Fig. 4). Results showed that their expression fluctuated between strains, without major differences, but more variations could be seen for *PDR11* and *ECM22*. As expected, *AUS1* and *PDR11* expression levels were almost undetectable (residual detection) for the 59A*amn1*,*aus1, pdr11* strain. Interestingly, for this last strain, *UPC2* expression level was, on the contrary, significantly increased compared to the non-deleted 59A*amn1* strain. However, this was not the case for *ECM22* gene expression. Relative *ERG6* gene expression was analyzed in all *Saccharomyces* (Fig. 4 E). For *S. cerevisiae* and non-*cerevisiae* strains, levels varied between 0.18 and 0.38 AU. Interestingly, the analysis of the correlation between the expression of these 5 genes revealed a significant correlation of the expression of *ECM22* with 25-NBD cholesterol uptake (adj.p-value= 0.036) (Fig. 4 F). It is noteworthy that the correlation of 25-NBD Cholesterol uptake with *UPC2* expression was just below the threshold (adj.p-value= 0.079), and that the third highest correlation could be observed between the expressions of *AUS1* and *UPC2*, (adj.p-value= 0.108).

Discussion

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

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

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

One reason for such discrepancies in sterol uptake could be due to differences in the genetic makeup of the strains. In *S. cerevisiae,* two sterol uptake transporters (*AUS1* and *PDR11* genes) have been characterized in lab strain W303-A (Wilcox *et al.*, 2002). However, we lack data for strains from other strains or species (other *S. cerevisiae*, *S.* non-*cerevisiae* and non-*Saccharomyces*). Our results of the spot tests performed under microaerophilic conditions indicated that, for all the selected *Saccharomyces* strains, sterol biosynthetic pathway functioning was necessary for growth, this growth being inhibited by FLC, albeit recovering when sterols were added. This confirms the importance of sterol uptake in anaerobic conditions for non-*cerevisiae Saccharomyces* strains. Interestingly, the presence of either *AUS1* or *PDR11* genes was sufficient to promote growth in the conditions tested, suggesting that these two paralog genes are rather substituable. In addition, the analysis of the expression of genes involved in the transport of sterols, revealed a high correlation between the expression of *ECM22* with 25-NBD Cholesterol uptake, and a slightly lower correlation for *UPC2*, which indeed

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

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

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

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

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

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

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

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

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

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The authors declare no conflict of interest.

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659 conditions in three fungal species, Candida albicans, Candida glabrata, and Saccharomyces
660 cerevisiae. *Eukaryot Cell* **12**: 725–738.

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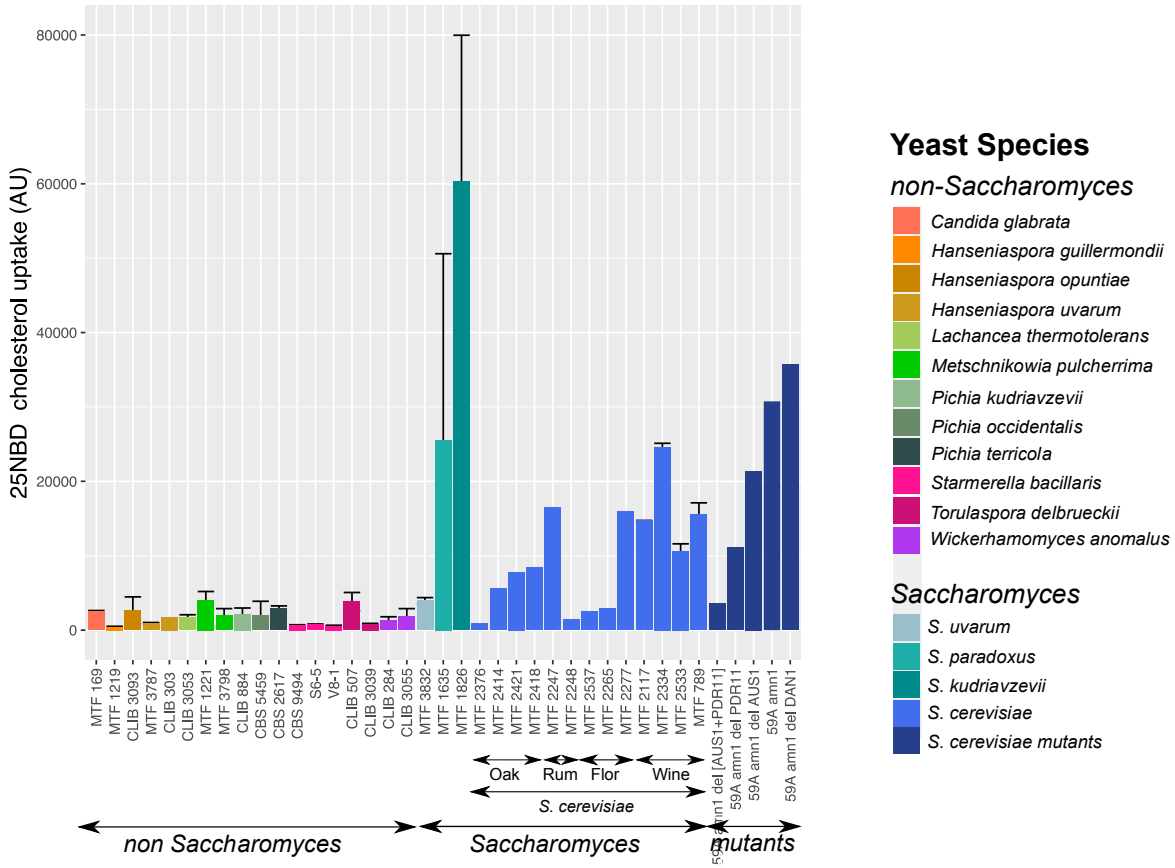
Fig. 1. 25-NBD Cholesterol fluorescence uptake (arbitrary units, AU) detected by flow cytometry in alive cells from 39 *Saccharomyces cerevisiae*, *Saccharomyces* non-*cerevisiae* and non-*Saccharomyces* strains grown 25h after inoculation at 28°C. Measures were performed twice for strains with error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

A- 25NBD-Cholesterol uptake



B- Cell populations

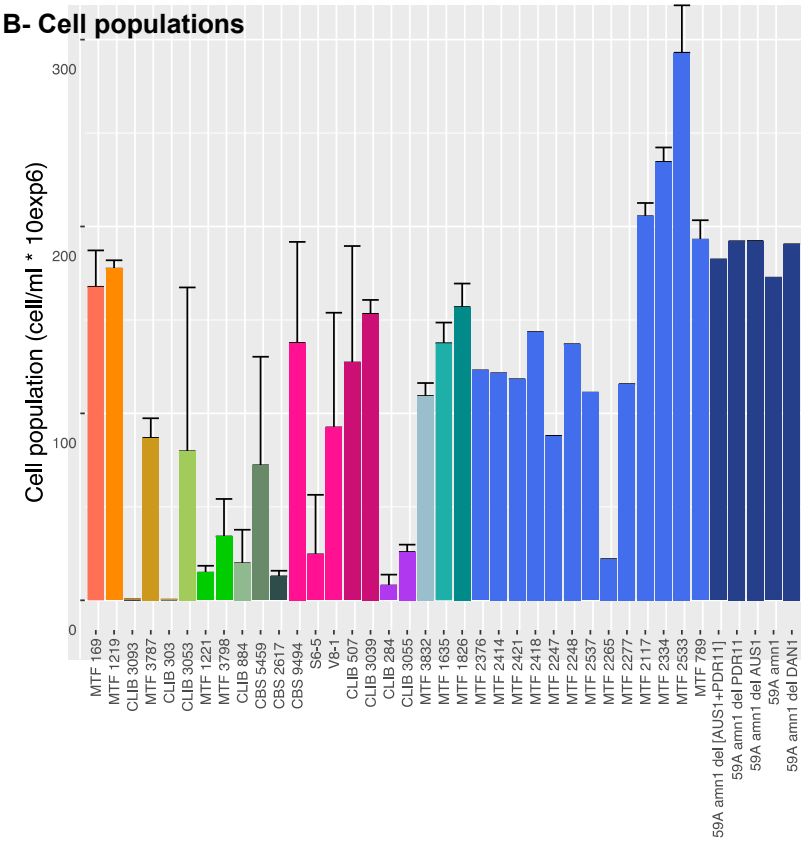


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Fig. 2. Analysis of phytosterol uptake under microaerophilic environment by droptest. Four serial tenfold dilutions of *S. cerevisiae* (A), S. non-*cerevisiae* (B) and *non-Saccharomyces* (C) strains spotted onto various synthetic standard agar media (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.

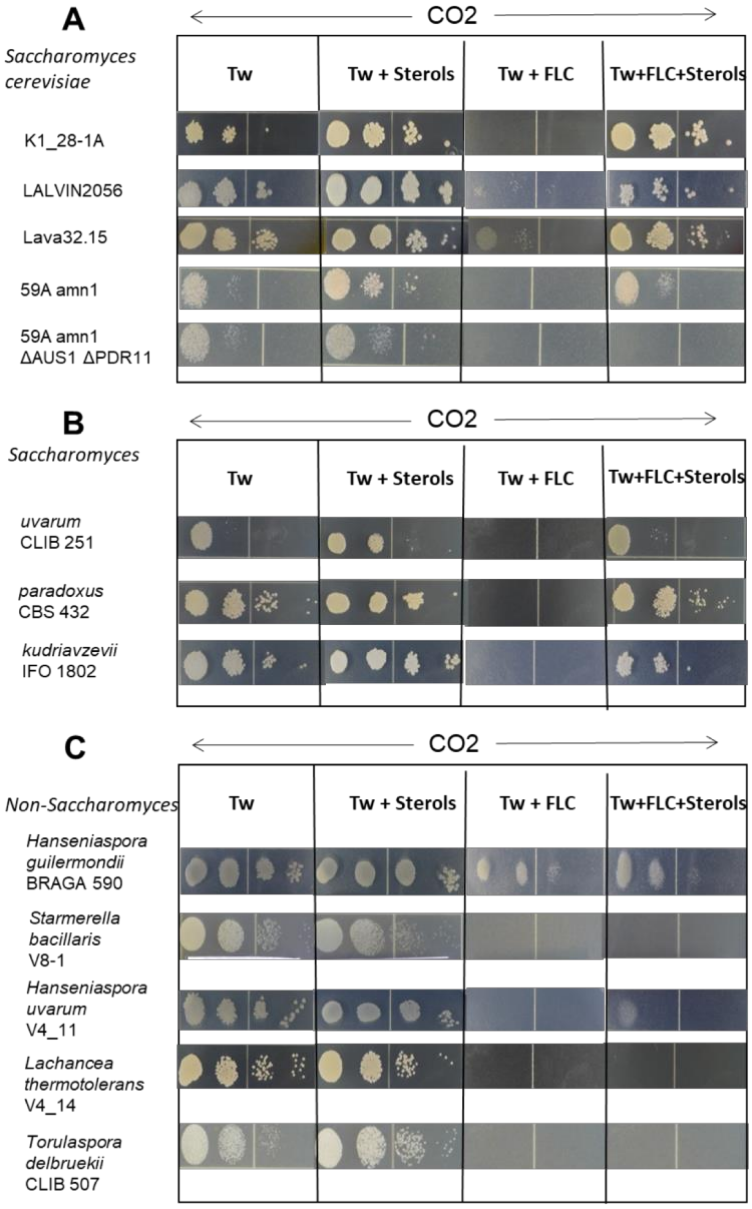

Figure 2

Fig. 3. Maximum likelihood phylogenic 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*).

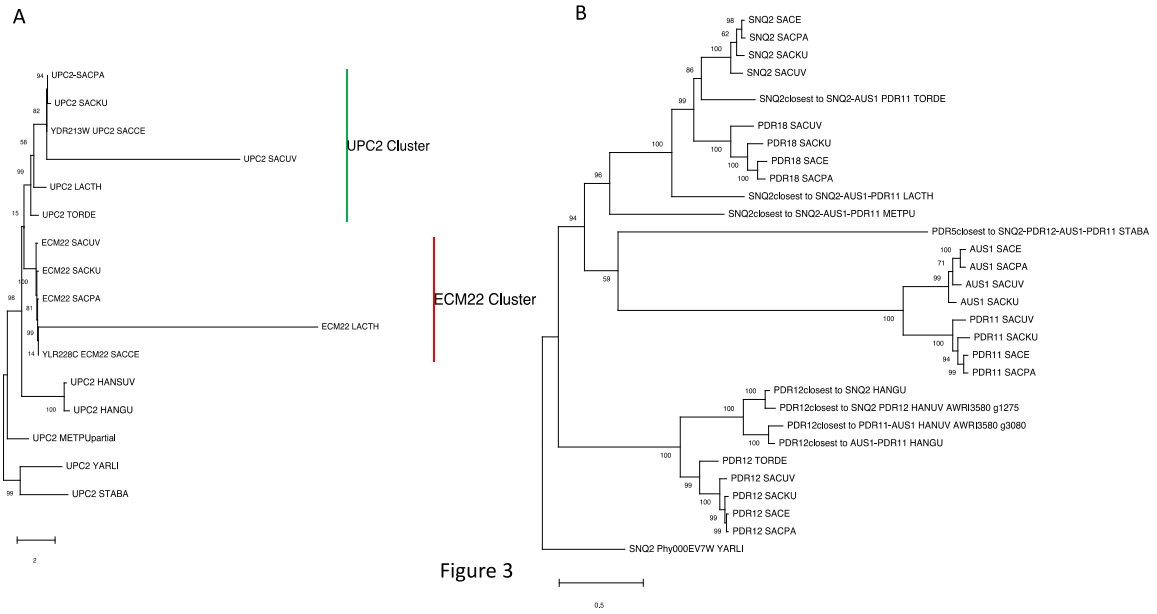

Figure 3

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.

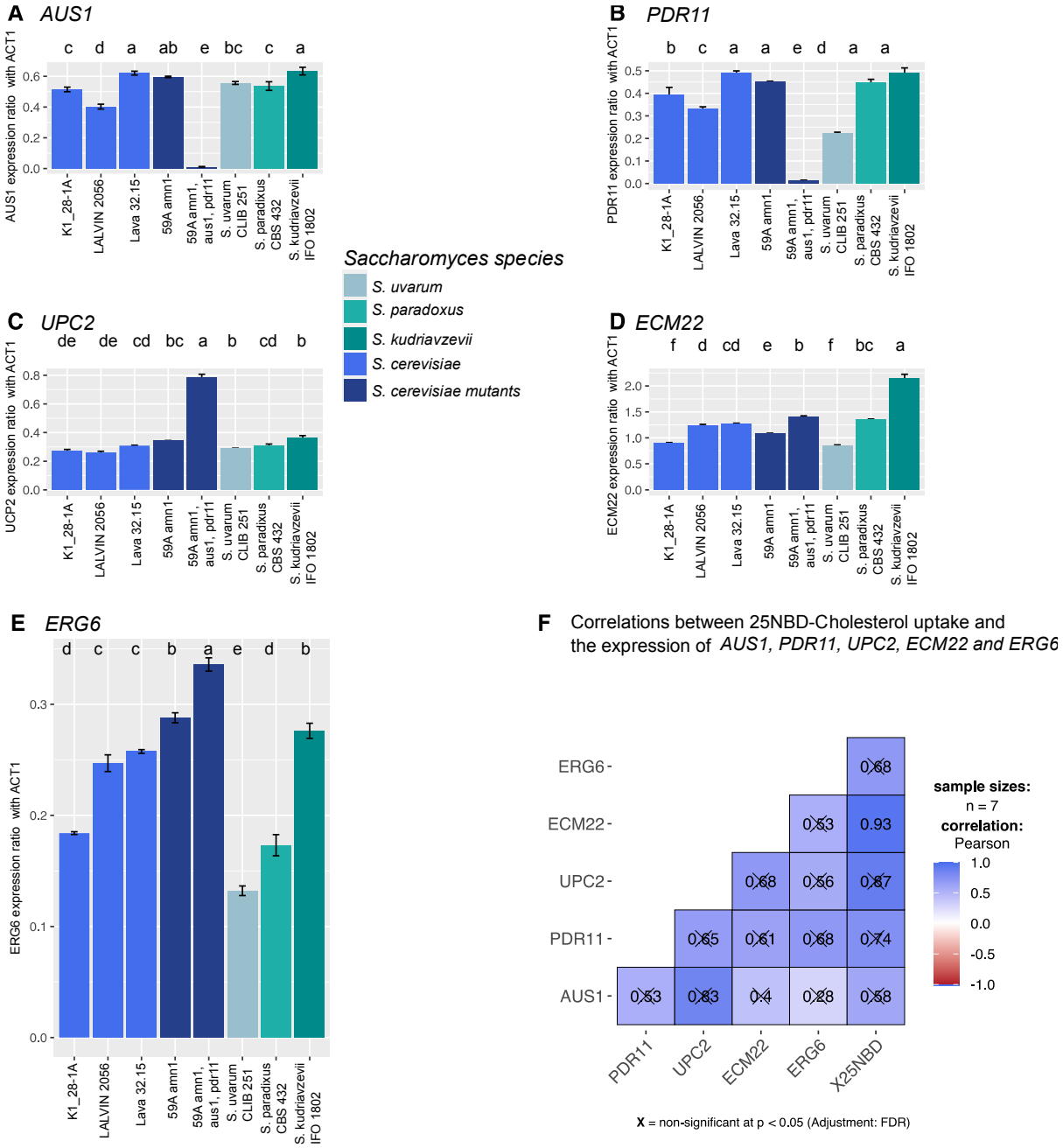

Table1. Oligonucleotides used for generation of deletion mutants

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

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

Species	Yeast strains	Origin	Isolation year	Collection , person who isolated
Hanseniaspora guillermondii	Braga 590 / MTF 1219	Wine (Braga, Portugal)	2004	Universidade do Minho, D. Schuler
Hanseniaspora uvarum	V4_11 / MTF 3787	Viognier grape must (Montpellier France)	2015	SPO collection, JL Legras
Lachancea thermotolerans	CLIB 3053/ V4_14	Viognier grape must (Montpellier France)	2015	CIRM Levures, INRAE, Montpellier JL Legras
Saccharomyces cerevisiae	K1_28-1A_Mata a / MTF 2534	Wine (haploid derivative from K1 starter)	2010	SPO collection, S. Dequin (1)
	LALVIN 2056	Wine (Rhone Valley, France)	<1990	Lallemand collection
	Lava32.15	Wine (Açores, Protugal)	2004	U. Minho (D. Schuler
	59A*amn1*	haploid derived from EC1118	2009	SPO collection, S. Dequin
	59A*amn1,aus1, pdr11*	from 59A*amn1*	2020	SPO collection, this work
Saccharomyces kudriavzevii	IFO 1802	oak liter, Japan	2009	NBRC collection
Saccharomyces paradoxus	CBS 432/CLIB 228	Unknown, The Netherlands	<1994	CIRM Levures , INRAE, Montpellier
Saccharomyces uvarum	CLIB 251 / CBS 395	Black current juice, The Netherlands	1898	CIRM Levures, INRAE, Montpellier
Starmerella bacillaris	V8-1	Viognier grape must, Sommières, France	2016	SPO collection, JL Legras
Torulaspora delbrueckii	CLIB 507	Grapes, Bordeaux, France	<1965	CIRM Levures, INRAE, Montpellier

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

Table 4. Species summary of the uptake of 25-NBD Cholesterol and of cell populations.
Measures were performed after 25 hours of growth during fermentation in a synthetic must (induced anaerobiosis). Standard 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
Saccharomyces				
Saccharomyces	*cerevisiae*	13	9804 ± 7266	193 ± 69.5
Saccharomyces	*paradoxus*	1	25579 ± 25021	138 ± 11.1
Saccharomyces	*kudriavzevii*	1	60299 ± 19676	157 ± 12.6
Saccharomyces	*uvarum*	1	3938 ± 425	109 ± 6.9
Non-Saccharomyces				
Candida	*glabrata*	1	2477± 150	181 ± 19.4
Hanseniaspora	*uvarum*	2	1335± 631	59 + 61
Hanseniaspora	*guillermondii*	1	407 ± 105	177 ± 4.5
Hanseniaspora	*opuntiae*	1	2690 ± 1784	0.38 ± 0.04
Lachancea	*thermotolerans*	1	1720 ± 350	141.7 ± 87
Metschnikowia	*pulcherrima*	2	3000 ± 1473	30 ± 13.6
Pichia	*kudriavzevii*	1	2170 ± 793	18.0 ± 1
Pichia	*occidentalis*	1	2000 ± 1879	72.2 ± 58.1
Pichia	*terricola*	1	2904.5 ± 350	12.8 ± 3
Starmerella	*bacillaris*	3	660 ± 84	84.9± 57.0
Torulaspora	*delbrueckii*	2	2334 ± 2115	120 ± 18.4
Wickerhamomyces	*anomalus*	2	1568 ± 429	16.8 ± 11.7
Significance tests			p-value	p-value
Saccharomyces /	*Non-Saccharomyces*	*(Kruskal-Wallis test)*	$1.6 * 10^{-05}$	0.011
S. cerevisiae	*All origins*	ANOVA	0.163	0.004
S. cerevisiae wine /	*S. cerevisiae 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).

Species \ Gene	***Aus1***	***Pdr11***	***Pdr18***	***Snq2***	***Upc2***	***Ecm22***	***Erg6***
Saccharomyces cerevisiae	100 (1394)	100 (1411)	100 (1333)	100 (1501)	100 (913)	100 (814)	100 (383)
Saccharomyces paradoxus	95.3 (1394)	95.5 (1412)	93.1 (1333)	96.9 (1501)	94.1 (916)	92.6 (810)	96.9 (384)
Saccharomyces uvarum	90.9 (1394)	88.7 (1413)	81.6 (1431)	90.2 (366)	87.0 (486)	83.3 (813)	92.7 (384)
Saccharomyces kudriavzevii	90.3 (1393)	89.9 (1407)	87.1 (1332)	93.7 (1502)	87.9 (512)	83.8 (590)	94.5 (384)
Lachancea thermotolerans	36.2* (1499)	35.3* (1499)		65.8 (1499)	60.3 (788)	45.8 (722)	83.3 (384)
Torulaspora delbrueckii		35.6* (1500)		73.8 (1500)	64.2 (810)		82 (374)
Hanseniaspora uvarum		31.7* (1514)		63.4 (1514)	56.5 (1106)		71.9 (401)
Hanseniaspora guillermondii		32.2* (1369)		64.5 (1523)	43.4 (1090)		72.2 (400)
Metchnikowia pulcherima		35.0* (1352)		54.0 (1352)	51.7 (498)		65.9 (376)
Starmerella bacillaris				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
Candida glabrata	ICV D222 / MTF169	grapes, France	<1990	SPO internal collection, INRAE, Montpellier
Hanseniaspora guillermondii	Braga 590 / MTF 1219	grapes, Portugal	2004	Universidade do Minho, D. Schuler
Hanseniaspora opuntiae	CLIB 3093 / S1-2	grape must, Puylacher, France	2015	CIRM Levures, SPO INRAE Montpellier JL Legras
Hanseniaspora uvarum	CLIB 303 T	Grapes, Ukraine	<1996	CIRM Levures, SPO INRAE Montpellier
Hanseniaspora uvarum	V4_11 / MTF 3787	grape must, France	2015	SPO internal collection, INRAE Montpellier, JL Legras
Lachancea thermotolerans	CLIB 3053 / V4_14	grape must, France	2015	CIRM Levures, SPO Montpellier JL Legras
Metschnikowia pulcherrima	Braga 604 / MTF 1221	unknown	<2004	Universidade do Minho, D. Schuler
Metschnikowia pulcherrima	V4_22 /MTF3798	grape must, France	2015	CIRM Levures, SPO Montpellier JL Legras
Pichia kudriavzevii	CLIB 884 T	fruit juice,	<2002	CIRM-Levures, INRAE, Montpellier,
Pichia occidentalis	CBS 5459 T	spoiled figs ? – USA.	unknown	Westerdijk Institute, The Netherlands
Pichia terricola	CBS 2617 T	Soil, South Africa	unknown	Westerdijk Institute, The Netherlands
Saccharomyces cerevisiae	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 (Rhone 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)
	59A*amn1 /MTF 2521*	haploid derived from wine strain EC1118	2015	SPO internal collection S. Dequin (3)
	59Aamn1,aus1,pdr11	from 59A*amn1*	2020	SPO internal collection, this work,
	59Aamn1,aus1	from 59A*amn1*	2020	SPO internal collection, this work
	59Aamn1,pdr11	from 59A*amn1*	2020	SPO internal collection, this work
	59Aamn1,dan1	from 59A*amn1*	2020	SPO internal collection, this work
Saccharomyces kudriavzevii	IFO 1802 T	oak liter , Japan	2009	SPO internal collection
Saccharomyces paradoxus	CBS 432 T	unknown origin	<1994	CIRM-Levures, SPO, INRAE, Montpellier
Saccharomyces uvarum	CLIB 251 T	beverage, The Netherlands	1989	CIRM-Levures, SPO INRAE Montpellier
Starmerella bacillaris	CBS 9494 T	Wine, Tokaj, Hungary	2000	Westerdijk Institute, The Netherlands, M. Sipiczki
Starmerella bacillaris	S6-5	Sauvignon grape must, Gruissan, France	2016	SPO internal collection, INRAE Montpellier, JL Legras
Starmerella bacillaris	V8-1	Viognier grape must, Sommières, France	2016	SPO internal collection, INRAE Montpellier, JL Legras
Torulaspora delbrueckii	CLIB 507	grapes, Bordeaux, France	<1965	CIRM Levures, INRAE, Montpellier
Torulaspora delbrueckii	S1_D / CLIB 3039	Sauvignon grape must, Puylacher, France	2015	CIRM-Levures, INRAE Montpellier, JL Legras
Wickerhamomyces anomalus	CLIB 284	unknown origin, USA	<1994	CIRM-Levures, INRAE Montpellier
Wickerhamomyces anomalus	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.

Fig. S2. CO_2 production by the 39 strains after 25h of growth during fermentation at conditions at 28°C. This parameter reflect sugar consumption

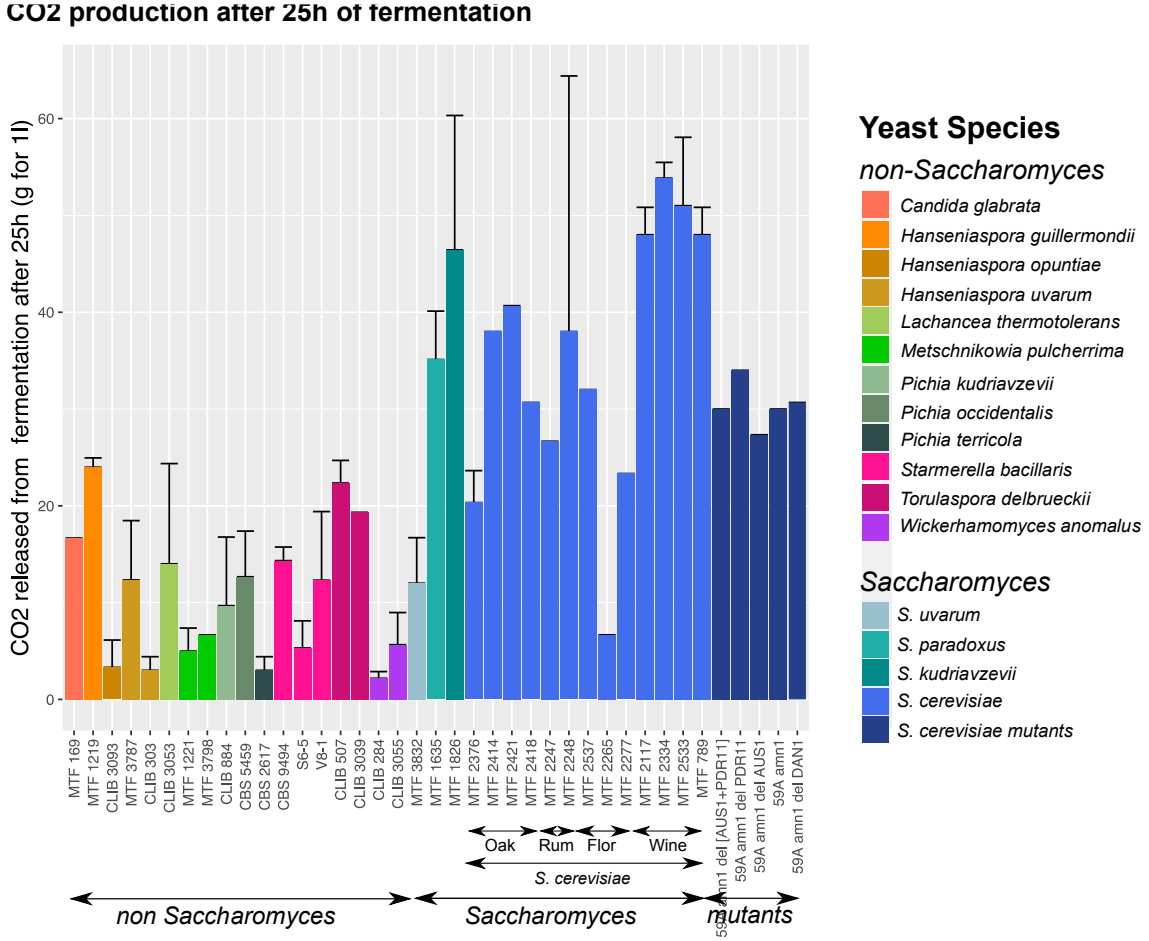

Fig. S3. Cell viability measured in the 39 strains after 25h of growth under fermentation at 28°C. Experiments were performed twice for all strains in error bars.