Title: Analysis of lipid composition reveals mechanisms of ethanol tolerance in the model yeast Saccharomyces *cerevisiae*

Lairón-Peris M,[a*] Routledge SJ,[b*] Linney JA,[b] Alonso-del-Real J,[a] Spickett, C.M.[b] Pitt AR,[b,c] Guillamon JM,[a] Barrio E,[a,d] Goddard AD[#,b] Querol A[#a]

[a]Food Biotechnology Department, Institute of Agrochemistry and Food Technology, CSIC, Valencia, Spain
[b]College of Health and Life Sciences, Aston University, Birmingham, UK
[c]Manchester Institute of Biotechnology and Department of Chemistry, University of Manchester, Manchester, UK
[d]Genetics Department, University of Valencia, Valencia, Spain

Running head: Membrane lipid composition in *S. cerevisiae* strains

# These authors contributed equally. Address correspondence to Alan Goddard, a.goddard@aston.ac.uk Amparo Querol, aquerol@iata.csic.es

* María Lairón-Peris and Sarah J. Routledge contributed equally to this work. Author order was determined on the basis of alphabetical order.

Abstract

Saccharomyces cerevisiae is an important unicellular yeast species within the biotechnological and food and beverage industries. A significant application of this species is the production of ethanol, where concentrations are limited by cellular toxicity, often at the level of the cell membrane. Here, we characterize 61 *S. cerevisiae* strains for ethanol tolerance and further analyse five representatives with varying ethanol tolerances. The most tolerant strain, AJ4, was dominant in co-culture at 0% and 10% ethanol. Unexpectedly, although it does not have the highest NIC or MIC, MY29 was the dominant strain in co-culture at 6% ethanol, which may be linked to differences in its basal lipidome. Whilst relatively few lipidomic differences were observed between strains, a significantly higher PE concentration was observed in the least tolerant strain, MY26, at 0% and 6% ethanol compared to the other strains that became more similar at 10%, indicating potential involvement of this lipid with ethanol sensitivity. Our findings reveal that AJ4 is best able to adapt its membrane to become more fluid in the presence of ethanol and lipid extracts from AJ4 also form the most permeable membranes. Furthermore, MY26 is least able to modulate fluidity in response to ethanol and membranes formed from extracted lipids are least leaky at physiological ethanol concentrations. Overall, these results

reveal a potential mechanism of ethanol tolerance and suggests a limited set of membrane compositions that diverse yeast species use to achieve this.

Importance

Many microbial processes are not implemented at the industrial level because the product yield is poorer and more expensive than can be achieved by chemical synthesis. It is well established that microbes show stress responses during bioprocessing, and one reason for poor product output from cell factories is production conditions that are ultimately toxic to the cells. During fermentative processes, yeast cells encounter culture media with high sugar content, which is later transformed into high ethanol concentrations. Thus, ethanol toxicity is one of the major stresses in traditional and more recent biotechnological processes. We have performed a multilayer phenotypic and lipidomic characterization of a large number of industrial and environmental strains of *Saccharomyces* to identify key resistant and non-resistant isolates for future applications.

Keywords: ethanol, *S. cerevisiae*, membrane properties

Introduction

Saccharomyces cerevisiae is a unicellular eukaryotic microorganism that has been employed as a model organism to study diverse relevant phenomena in biology at molecular level (1). Due to its high fermentative capability, it is also widely used in the biotechnology field for the performance of industrial fermentations of products such as wine, beer or bread (2) or traditional Latin American beverages like pulque, masato, chicha, tequila, or cachaça (3–7). *S. cerevisiae* also has a relevant role in bioethanol production (8). *S. cerevisiae* has been isolated from different sources and environments all over the world, including fruits, soils, cactus, insects, oak, and cork tree barks (9, 10). The physiological and genetic diversity among the *Saccharomyces* genus is high, due to their colonization of different environments; the most studied species are those associated with industrial processes of economic importance as wine production (11–17), cider (18) and beer (11). *Saccharomyces* yeasts that have been selected to carry out these fermentations in a controlled manner show particular characteristics, as selective pressures imposed by the fermentative environment, such as low pH and the high ethanol levels in the media, favor yeasts with the most efficient fermentative catabolism, particularly *S. cerevisiae* strains, but there are species in the *Saccharomyces* genus which are also found spontaneously in these fermentation products including *S. uvarum*. Depending on the fermentation process, other factors apart from alcohol concentration, as temperature, can be considered stress factors (19–21).

Ethanol (CH_3CH_2OH) is a small molecule containing a methyl group and a hydroxyl group and consequently it is soluble in both aqueous and lipidic phases. Because of these properties, it can penetrate inside cells, which generates important stresses; incorporation into the cell membrane can increase fluidity, which is a fundamental driver of membrane properties (22, 23).

This fluidity change induces a loss of membrane integrity, becoming more permeable (24). Ethanol causes other detrimental effects to the cells, including alterations on mitochondrial structure, reducing ATP levels and respiratory frequency and favoring acetaldehyde and reactive oxygen species (ROS) generation, which can cause lipid peroxidation, DNA damage and oxidative stress (25, 26). As a consequence, a notable reduction in cellular viability occurs. Cell membranes are composed of lipids (mainly phospholipids and sterols, but also sphingolipids and glycolipids) and proteins. Membrane lipids are amphipathic, possessing hydrophobic (apolar) and hydrophilic (polar) regions. Embedded membrane proteins are strongly associated with the apolar core of the bilayer and peripheral proteins are more loosely associated with the membrane via several mechanisms. A key factor contributing to membrane fluidity is the fatty acids and sterol composition of the membrane (27).

The molecular structure of ethanol allows passive diffusion across the membrane and likely incorporation into the bilayer structure (28). When this happens, van der Waals attractive forces decrease, increasing membrane fluidity (29). Using fluorescence anisotropy studies a direct relationship between plasma membrane fluidity and ethanol concentration has been reported (30, 31). This increase in fluidity, together with the loss of structural integrity previously mentioned, result in loss of various intracellular components including amino acids and ions (24), producing alterations in a cellular homeostasis.

The alterations in membrane properties are fundamental in the mechanism of ethanol toxicity but the physical changes that the membrane structure undergoes as a result of ethanol presence in the media have not been completely described. It is widely accepted that ethanol is intercalated in lipidic heads of the membrane, with the OH group of the ethanol associated with the phosphate group of the lipidic heads and the hydrophobic tails aligned with the hydrophobic core of the membrane. When this interaction takes place, ethanol molecules substitute interfacial water molecules**,** generating lateral spaces between polar heads, and, as a consequence, spaces in the hydrophobic core (32). These gaps result in unfavorable energy, so the system tries to minimize it by creating an interdigitated phase. This modification in the membrane causes a decrease in its thickness of at least 25% (33, 34) and as a consequence of this thinning, alterations in membrane protein structure and function can occur, leading to cellular inactivation during the fermentation process (35).

It has been demonstrated that membrane thickness affects membrane protein functionality, in which maximum activity takes place with a defined thickness (36, 37). If this thickness changes, exposure of hydrophobic amino acid residues in integral membrane proteins can take place, resulting in a

104 phenomenon known as hydrophobic maladjustment (35), that can lead to aggregation of membrane
105 proteins to minimize the exposition of their hydrophobic parts in the aqueous media (38). Studies that
106 use membrane models formed by phosphatidylcholine and ergosterol that are exposed to different
107 ethanol concentrations have demonstrated that lipid composition protects the membrane because
108 interdigitated phase formation is delayed (39).
109 In Arroyo-López et al. (40) different S*accharomyces* species were characterized for their ethanol
110 tolerance, identifying *S. cerevisiae* as the most ethanol tolerant. In the present work, we have selected
111 61 *S. cerevisiae* strains, from different origins and isolation sources. The purpose of this study was to
112 establish differences in the behavior of strains that represent the different *S. cerevisiae* groups, to
113 determine the most resistant ones, so they are better to perform industrial fermentations. With this
114 aim, we both monitored the growth in a liquid medium with different ethanol concentrations, using
115 absorbance measurements, and in a solid media, carrying out drop test analysis on ethanol plates.
116 Growth data were statistically analyzed for each of the *S. cerevisiae* strains and strains showing a
117 different behavior under ethanol stress were selected to conduct membrane studies that allow
118 correlations of lipid composition in yeast populations with responses to environmental stress such as
119 ethanol.
120
121 **Results**
122 **Ethanol tolerance of the strains in solid media.**
123 A total of 61 yeast strains belonging to *S. cerevisiae* were selected to assess ethanol tolerance. The
124 strains have been identified by sequencing of the D1/D2 26S, sequencing of the D1/D2 26S rRNA
125 gene was deposited in GenBank with the accession numbers MW559910-MW559970. All the strains
126 have been identified as *S. cerevisiae* with the exception of MY62 that is a *S. cerevisiae* strain
127 containing a limited amount of *S. kudriavzevii* genome. 21 are industrial strains and were selected for
128 their use in winemaking and 40 of them belong to the IATA-CSIC collection. The sources from which
129 these 40 strains were retrieved are diverse: agave, beer, bioethanol, chicha, cider, cocoa, honey water,
130 masato, sake, sugar cane, wine, natural wild strains, etc. *S. cerevisiae* yeast strains' ethanol tolerance
131 was first assessed in plates with GPY + different ethanol percentages. To observe the influence of
132 ethanol on these strains we performed four biological replicates of each strain growth in 6 different
133 media. One biological replicate for each of the strains and media can be seen in Fig. S1. With the
134 growth data of each of the strains and taking into account, the 4 replicates values of growth for each
135 strain, a heatmap with the growth data in ethanol was constructed (Fig. 1). This heatmap is
136 hierarchically clustered into two big clusters with different subclusters. The first cluster is made up
137 of the strains which are more tolerant of ethanol (a total number of 22 of the 61 strains) and another
138 one with the rest of the strains which show intermediate and low growth with this compound (39

strains). Among the first cluster, with the most tolerant strains, it is interesting that 19 of the 22 strains belong to commercial wine strains. The other 3 strains which are included in this heatmap are AJ4, a Lallemand commercial strain, which is also one of the most tolerant strains of all the screened ones, MY48, a cachaça strain and MY43, a cider yeast strain.

The other cluster, with the 39 intermediate-low tolerant strains, appears to be divided into two subclusters too. One of the subclusters is composed of MY33 and MY34, which are the less ethanol tolerant strains, and belong to the sake group. It is interesting to note that in the other subcluster, there are strains with different behaviors. As an example, strains MY46 (cachaça) and MY44 (cider) growth in ethanol media are affected by low ethanol concentrations (ethanol percentage of 6%), but they can grow (at a low rate) until 16% of ethanol is present in solid media. On the other hand, there are other strains, such as MY37 (Masato) and MY22 (natural), whose growth is not affected until 10% of ethanol is present in GPY solid media but in the next ethanol step (14%) they do not grow at all.

Ethanol tolerance of the strains in liquid media.

Ethanol tolerance of the set of *S. cerevisiae* strains was evaluated in minimal YNB liquid media at 28ºC. Yeast growth was evaluated by OD_{600} determination in microtiter plates containing this media with different ethanol concentrations and for each strain, the area under the curve during these growths was calculated. With the area under the curve reduction due to the addition of ethanol, NIC (non-inhibitory concentration) and MIC (minimum inhibitory concentration) parameters were calculated for 57 of the 61 strains. Not all of the 61 strains could be evaluated following this method: the data obtained with flor strains MY28 and MY31 could not be used because these strains flocculate and the data obtained with them are not reproducible. The data obtained with the strains MY55 and MY56 were not used as they have problems growing in minimal media YNB. The complete list with the NIC and MIC values for each one of the selected strains can be found in Table S1. Fig. 2 depicts a graph representing these values for each one of the strains.

Strain selection

After performing the phenotypic characterization in ethanol of our collection of 61 strains, to further characterize some representatives of the different behaviors we decided to select 5 of them as they showed a range of tolerances: AJ4, MY3, MY14, MY26, and MY29. Fig. 3A shows the results of the drop test in GPY+ethanol media of these 5 strains and Fig. 3B the NIC and MIC parameters of growth in YNB liquid media+ethanol.

AJ4 shows high NIC and MIC values during YNB growth in liquid media, and in solid media in GPY + ethanol it clusters amongst the most tolerant *S. cerevisiae* strains too. This strain is a Lallemand commercial strain that has been reported as a highly tolerant ethanol strain (41). It has a high NIC

value 11.62% ± (0.33%), which means that a high concentration of ethanol is needed to affect its growth.

MY29, which is a flor strain isolated from sherry wine, is classified within the second cluster with the strains that show an intermediate growth in GPY+ethanol in solid media. It grows well until 14% ethanol; however, viability is reduced in 16% ethanol, and it is unable to grow at 18% ethanol. Regarding the liquid assay in YNB+ethanol, its MIC value is amongst the highest MIC values of all the strains (15.41% ± 2.93%), but its NIC value (7.5% ± 1.48%) can be classified as a medium-low value. This result shows that MY29 is a *S. cerevisiae* strain whose behaviour can be classified as intermediate in ethanol conditions. Moreover, MY29 is the most tolerant sherry wine strain of the five strains analyzed.

MY26, which is an agave strain, is among the least tolerant strains in solid media and is also the strain that shows the lowest growth among the three agave strains that we selected for our study. In liquid media, its NIC value is also low, being affected by an ethanol concentration of 7.24% ±0.77 (%) but its MIC value is high (15.34% ± 0.4%). This strain shows similar behavior in liquid media as MY29, but in solid media, it proved to be less tolerant as it was not able to grow in 14% ethanol plates, and MY26 could grow in this condition too.

MY3 and MY14 are commercial wine strains, which are classified in the cluster of the most tolerant strains regarding their growth on ethanol plates. Nevertheless, MY14 appears to be affected by the ethanol at low concentrations (NIC value of 6.787% ± 0.337% and MIC value of 13.93% ± 0.91%) and MY3 seems to start being affected by ethanol at higher concentrations but has a low range, as it has a low MIC value (NIC 8.89% ± 1.26% and MIC 12.97% ± 0.13%).

Competition fermentations

These five strains, AJ4, MY3, MY14, MY26, and MY29 were selected for their different behavior regarding ethanol susceptibility. They were inoculated into mixed culture fermentations to assess the correlation between ethanol tolerance and competition capacity under different ethanol concentrations (0%, 6%, and 10%). As one GPY fermentation would be insufficient for observing domination of the culture by one single strain, we followed a method in which we inoculated a sample of the culture after sugar depletion into new fresh media with the corresponding ethanol concentration.

After the tenth pass, AJ4 completely dominated the 0% and the 10% fermentations. However, in 6% fermentations, MY29 strain completely dominated one of the three replicate fermentations and clearly dominated the other two. The other 2 strains which are present in this 6 % fermentation when sugar is depleted are AJ4 and MY14, although in low proportion. Neither MY3 nor MY26 colonies were found in any of the fermentation (Fig. 4). AJ4 dominating high ethanol concentration cultures was

quite an expected result regarding its ethanol tolerance determined in the present work. However, it does not seem clear why MY29 dominates 6% ethanol cultures, given its moderate tolerance compared to other strains such as AJ4, MY3 or even MY14. Here, probably, complex interaction among strains play an important role in domination, which has been studied previously for another set of strains (42), and demonstrated to be of importance together with growth capacity under the studied media conditions (43).

Lipid composition and membrane properties

Several studies have demonstrated that yeasts can adapt their membrane composition in response to ethanol stress (44–46). To better understand the effects of ethanol upon the yeast strains, we investigated the properties of the membranes in the presence and absence of ethanol. We determined the total lipid composition of each of the strains by mass spectrometry (Table S2 and S3). The number of species identified for major lipid classes for strains grown in media containing 0% or 6% ethanol is shown in Fig. 5. For the strains grown in the absence of ethanol, for ceramide 1-phosphates (CerP), there were significantly fewer species observed in MY29 (109.6 ± 6.61) compared to AJ4 and MY3 (128.2 ± 1.49 and 130 ± 0.55), where $P < 0.01$ (two-way anova and Tukey's multiple comparisons test) and MY14 (126.6 ± 1.86) where $P < 0.05$. For cardiolipin species (CL), there were significantly fewer observed in AJ4 and MY3 (3.0 ± 0.45 and 3.0 ± 0.31); ($P < 0.01$), and MY14 and MY26 (4.2 ± 1.3 and 4.0 ± 0.55); ($P< 0.05$) when compared to MY29 (9.67 ± 1.8). There were fewer diacylglycerols observed in MY29 compared to MY3 (180.2 ± 1.93 and 193.0 ± 1.41); ($P < 0.05$). For glycerophosphatidicacid (GPA) species, there were significantly fewer species identified for MY29 (126.4 ± 15.17) compared to AJ4 (178.0 ± 2.28; $P < 0.0001$), MY3 (175.0 ± 1.05; $P < 0.001$), MY14 (170.4 ± 5.30; $P < 0.001$), and MY26 (167.8 ± 6.67; $P < 0.01$). There were also fewer glycerophosphatidylethanolamine GPEth species identified for MY29 compared to each of the strains ($P < 0.01$ in each case) (259.6 ± 3.2 AJ4; 258.4 ± 1.36 MY3; 254.8 ± 2.85 MY14; 252.4 ± 3.26 MY26 and 186.2 ± 35.034 for MY29). For glycerophosphoserine species (GPSer), there were fewer species in MY29 (120.0 ± 12.99) compared to AJ4 and MY3 (157.6 ± 2.50 and 159 ± 1.41; $P < 0.001$), MY14 (151.6 ± 3.41; $P < 0.01$) and MY26 (147.4 ± 3.94; $P < 0.05$). Lastly, there were less monoacylglycerols (MG) species observed in MY29 (19.0 ± 0.84) than for MY3 (24.6 ± 0.51; $P < 0.01$).

There were no significant differences observed between the species grown in the presence of 6% ethanol; however, significant changes were seen between the 0% and 6% ethanol samples. For CL, there were significantly fewer species observed for MY29 grown in 6% compared to 0% ethanol (3.0 ± 0.44 and 9.66 ± 1.80; $P < 0.01$). For DG, there were more species in 0% MY3 than 6% (193.0 ± 1.41 and 178.4 ± 2.13; $P < 0.05$), for GPA there were significantly fewer species in MY29 at 0%

compared to 6% (126.4 ± 15.17 and 157.0 ± 4.03; $P < 0.05$), and for GPEth there were also significantly fewer species in MY29 at 0% compared to 6% ethanol (186.2 ± 35.04 and 241.2 ± 1.82; $P < 0.05$). There were significantly more MG species in MY3 at 0% (24.6 ± 0.51 and 20 ± 1.22; $P < 0.05$) and more TG species in MY3 at 0% compared to 6% ethanol (73.2 ± 1.39 and 66.6 ± 1.03; $P < 0.01$). Strikingly, MY29 seems to have the most different total lipid composition at 0% ethanol and to remodel this most dramatically, in terms of species diversity, at 6%. However, at 6% ethanol, species diversity in MY29 is similar to the other strains, perhaps indicating an optimal membrane composition for ethanol tolerance.

Acyl chain length and saturation have been shown to be important factors in regulating membrane fluidity and ethanol tolerance in yeast (44–46). We therefore investigated this for AJ4, MY3, MY14, MY26 and MY29 strains in both 0% and 6% ethanol. While there were no significant changes in average carbon length of the acyl chains for each of the strains grown in 0% compared to 6% ethanol (Fig. S2), there were significant differences in saturation (Fig. 6). For the strains grown in 0% ethanol (Fig. 6A), DG species contained a significantly lower percentage saturated acyl chains in MY29 compared to AJ4 (37.95 ± 0.35 and 40.22 ± 0.30; $P < 0.01$). There was a significantly higher percentage of monounsaturated CL species in MY29 (30 ± 7.83) compared to AJ4 and MY3 (0 ± 0.0 in both cases; $P < 0.01$), and MY26 (3.33 ± 3.33; $P < 0.05$). For GPA, there was a significantly higher percentage saturated chains in MY29 (34.51 ± 1.07) compared to MY14 (31.30 ± 0.88); $P < 0.05$. For GPEth, there were more saturated chains in MY29 compared to AJ4, MY3, MY14, and MY26 (31.21 ± 3.79; 25.30 ± 0.24; 24.92 ± 0.16; 24.96 ± 0.26; 24.38 ± 0.26; $P < 0.05$ in each case). There was a significantly greater number of saturated GPSer species in MY29 compared to MY26 (32.44 ± 1.70 and 29.24 ± 0.22; $P < 0.05$) and a lower number of monounsaturated species in MY29 (40.07 ± 2.20) compared to MY3 and MY14 (45.11 ± 0.62 and 44.7 ± 0.59; $P < 0.05$). Lastly, there was a significantly higher percentage of MG species containing two unsaturations in MY29 (10.59 ± 0.40) compared to MY3 (8.14 ± 0.17) ($P < 0.05$). Once again, MY29 is the most different in terms of saturated species at 0% ethanol and remodels its membrane to be more similar to the other strains at 6%.

There were no significant differences observed between strains for 6% ethanol samples (Fig. 6B), but there were between strains grown in 0% compared to 6% ethanol. There was a significantly higher percentage of saturated DG species for AJ4 at 0% than 6% ethanol (40.22 ± 0.30 and 38.08 ± 0.44), and a lower percentage of monounsaturated species for AJ4 (32.80 ± 0.09 and 34.75 ± 0.38; $P < 0.001$) and MY3 (33.06 ± 0.21 and 34.54 ± 0.25; $P < 0.05$) at 0% compared to 6% ethanol. For saturated GPEth species, there was a significantly higher percentage in 0% MY29 than 6% MY29

(31.21 ± 3.79 and 24.65 ± 0.26; $P < 0.05$), and significantly fewer monounsaturated species in 0% MY29 compared to 6% (40.23 ± 0.55 and 41.94 ± 0.42; $P < 0.05$). There were significantly more monounsaturated GPGro species in MY29 at 0% compared to 6% ethanol (19.12 ± 4.95 and 12.37± 1.05). In addition, there were significantly fewer monounsaturated GPSer species in 0% MY29 than in 6% (40.07 ± 2.20 and 44.77 ± 0.23). Lastly, for TG species, there were significantly more saturated species in MY14 at 0% ethanol than in MY14 at 6% (35.94 ± 0.58 and 30.86 ± 1.16; $P < 0.001$), more monounsaturated species in AJ4 6% (26.33 ± 0.503; $P < 0.01$), MY14 6% (6.24 ± 0.55; $P < 0.01$), and MY26 6% (25.73 ± 0.26; $P < 0.05$) compared to the 0% samples (23.40 ± 0.64; 23.60 ± 0.40 and 23.55 ± 0.25 respectively), and fewer species containing two unsaturations in MY3 (26.50 ± 0.47; $P < 0.01$) and MY14 at 0% (26.98 ± 0.55; $P < 0.05$) compared to 6% (29.43 ± 0.68 and 29.39 ± 0.48) samples.

To assess variation in overall lipid unsaturation the unsaturation index (UI) was calculated at the lipid level by lipid class for species identified in each strain at 0% and 6% ethanol (Table 2) using the percentage of lipids weighted by the number of unsaturated bonds: UI = % with one unsaturation + (2 x % with two unsaturations) + (3 x % with three unsaturations) + (4 x % with four unsaturations). The UI for DG was significantly lower for AJ4 compared to MY29 at 0% ethanol (86.76 ± 0.64 and 90.03 ± 0.61, $P < 0.01$) and higher for GPEth species in the 0% AJ4, MY14, MY26 strains compared to MY29 (108.72 ± 0.35, 108.72 ± 0.28, 109.36 ± 0.60 and 97.36 ± 7.13 respectively, where $P < 0.05$ in each case). The UI for MY29 at 0% was also significantly lower than at 6% ethanol (108.73 ± 0.92, $P < 0.05$). Lastly, the UI for MG species at 0% ethanol was significantly lower for MY3 compared to MY29 (73.30 ± 16.58 and 83.27 ± 18.95, $P < 0.05$), and the UI for MY29 at 0% ethanol was significantly higher compared to 6% MY29 (83.27 ± 18.95 and 78.74 ± 1.52, $P < 0.05$).

Due to changes observed in phosphatidylethanolamine (PE) and phosphatidylserine (PS) species diversity in Fig. 5, we undertook quantitative TLC analysis of these lipids. This showed significant differences in the abundance of PE in MY26 grown in 0% ethanol (0.41 ± 0.02), where the abundance was higher compared to AJ4 (0.03 ± 0.01; $P < 0.0001$), MY3 (0.08 ± 0.01; $P < 0.0001$), MY14 (0.17 ± 0.01; $P < 0.0001$) and MY29 (0.18 ± 0.04; $P < 0.0001$) grown in 0% ethanol as illustrated by Fig. 7. There was also a significantly greater abundance of PE in 6% MY26 (0.41 ± 0.05) compared to 6% AJ4 (0.08 ± 0.03; $P < 0.05$), MY3 (0.07 ± 0.02; $P < 0.0001$), MY14 (0.09 ± 0.01; $P < 0.0001$) and MY29 (0.13 ± 0.01; $P < 0.0001$). In addition, there was a lower abundance of PE in MY26 at 10% ethanol (0.20 ± 0.06) compared to MY26 at both 0% (0.41 ± 0.02) and 6% ethanol (0.41 ± 0.051); $P < 0.001$). There was a significantly lower abundance of PS in AJ4 at 0% ethanol (0.06 ± 0.01) compared to MY14 and MY29 (0.36 ± 0.06 and 0.30 ± 0.09; $P < 0.01$ and $P < 0.05$, respectively).

There was also a significantly lower abundance of PS in MY3 compared to MY14 at 0% ethanol (0.09 ± 0.01 and 0.36 ± 0.06; $P < 0.05$). It is notable that MY26, the least tolerant strain, is the most different at 0% and 6% ethanol, but has a similar composition to the other strains at 10%.

We next examined the effect of ethanol upon the fluidity of the yeast membranes as they grew in cultures with and without ethanol. We utilized the fluorescent dye, Laurdan, which has been used to study phase properties of membranes as it is sensitive to the polarity of the membrane environment (47). GP (Generalized Polarization) values, which correlate inversely with fluidity, were calculated at six timepoints during the growth of AJ4, MY3, MY14, MY26 and MY29 strains in GPY, GPY containing 6% ethanol and GPY containing 10% ethanol. The assay suggests that the fluidity of the yeast membranes decreases with culture time as shown by the increase in GP (Fig. 8). AJ4 and MY14 strains demonstrated large changes in fluidity when treated with 10% ethanol (AJ4 showed a GP value change of -0.0002 ± 0.0009 at 10% and a GP value change of 0.0233 ± 0.0025 at 0% and MY14 showed a GP value change of -0.0101 ± 0.002 at 10% and a GP value change of 0.009 ± 0.002 at 0%) ($P < 0.001$ and $P < 0.01$, respectively). MY29 also became significantly more fluid at 10% ethanol (GP value change of -0.0016 ± 0.0011 at 10% and a GP value change of 0.0084 ± 0.0019 at 0%) ($P < 0.05$). However, these strains did not show any increases in fluidity with 6% ethanol. The other strains showed no significant differences to fluidity with ethanol treatment. It is notable that the most tolerant strains show the largest increases in membrane fluidity in response to ethanol exposure.

To examine membrane permeability, we investigated the integrity of liposomes composed of lipids extracted from each of the strains and loaded with carboxyfluorescein (CF) dye. The liposomes were challenged with increasing concentrations of exogenous ethanol, and the fluorescence increase from CF dye release was measured. The data in Fig. 9 shows that the liposomes containing lipids extracted from AJ4 demonstrated a significantly greater increase in fluorescence at high ethanol concentrations than those composed of lipids from the other strains (ANOVA and Tukey's multiple comparisons test (90.98 ± 4.29 fluorescence increase; $P < 0.001$). MY3 and MY26 liposomes were less "leaky" overall (46.38 ± 2.97 and 47.41 ± 7.84 of fluorescence increase). This increase in fluorescence indicates increased "leakiness" of the membranes.

Principal component analysis

With the aim of grouping the 5 selected strains based on their lipid composition and their ethanol tolerance, the data obtained in the previous sections was used to perform a PCA (Fig. 10). The data from the variables NIC, MIC, and the drop test growth value at 14% and 16% of ethanol in the plates, related to the ethanol tolerance were used. For the lipid composition, the data of the

carboxyfluorescein release at the last time point; the data from the Laurdan experiments of the differential GP value at 10% of ethanol and when no ethanol is present in the last time point, and the PE abundance at 0% and 6% of ethanol in the media was used. The two commercial wine strains MY3 and MY14 group together, and MY26 (the most sensitive to ethanol) and AJ4 (the most tolerant) are the two strains that demonstrate the most differences among them. It is interesting to note that MY26 is associated in the PCA with an accumulation of PE in the membrane at low ethanol concentration and a higher membrane rigidity, and the most tolerant stain, AJ4, associated with a high membrane fluidity in the presence of ethanol.

Discussion

In this study, we investigated the membrane properties of the selected yeast strains to try to understand their different levels of ethanol tolerance. The mass spectrometry analysis of the lipid composition of each strain in the absence of ethanol highlighted differences, in particular between MY29 and the other strains, not only in the variety of species observed for the lipid classes but also in their saturation. MY29 is a flor yeast. These yeasts constitute a separate phylogenetic group within *S. cerevisiae* species. They are characterized by forming a layer on top of wine known as flor, which allows them to access the oxygen during the fermentation of sherry wines, so they show different behavior and thus physiological characteristics to wine yeast. Moreover, they have been reported to survive under extreme conditions (ethanol content over 15%) (48, 49), which could relate to their membrane structure.

Upon treatment with 6% ethanol, the lipid composition of MY29 underwent significant changes; the composition was then found to be more similar to that of the other strains, suggesting that the membrane of MY29 underwent more drastic changes than the other strains in response to ethanol. The lack of significant differences at 6% ethanol suggests that each of the strains move towards a more common lipid composition in response to ethanol. However, despite the fewer differences to lipid composition at 6% ethanol between the strains, MY29 dominated the fermentation at this concentration. In addition, the lipid composition of AJ4 was not significantly different from the other strains at 6% ethanol, although it is the most tolerant to ethanol. It is possible that there may be further adaptation of the membrane at higher ethanol concentrations than were investigated in this study, but it is likely that other factors contribute to the ethanol tolerance of these strains.

Indeed, this has been suggested by other studies, where the relationship between H^+-ATPase activity and ergosterol content as well as the sterol to phospholipid and protein to phospholipid ratios are important (45, 50, 51). Ethanol tolerance is a complex phenotype, and different mechanisms may lead to improved tolerance. Fluidisation of the yeast membranes by ethanol is also known to activate the unfolded protein response (UPR), and it is speculated that a better response could lead to greater

tolerance (52). Moreover, yeast cells can increase their tolerance to ethanol by other mechanisms, such as the increase the biosynthesis of some amino acids, as tryptophan (53) and trehalose accumulation (54).

Nevertheless, it is striking that yeast strains with different membrane compositions in the absence of ethanol become more similar upon exposure, suggesting a common, or limited number, of membrane compositions that maximize tolerance to ethanol.

Incorporation of longer acyl chains and a decrease in shorter chains has previously been shown to occur in yeast in response to ethanol (46, 55); however, we did not observe any significant changes in chain length. Our study does suggest that there were significant differences in saturation between the species upon ethanol treatment. These changes occurred in GPGro and GPEth in MY29, and occurred predominantly in DG and TG for the other strains, with shifts towards increased saturation for AJ4 and increased unsaturation for MY3 and MY14. These changes appear to be complex and specific to each strain. Documented changes to the membrane of yeast upon ethanol challenge are conflicting (56); while some studies have shown that increased levels of unsaturated fatty acids are linked to improved ethanol tolerance (46), changes to the unsaturation index may not necessarily be associated with improved tolerance, or lead to the expected changes in membrane fluidity, and it is rather the potential of the cell to alter its composition (45, 57). The lipid membrane is a highly complex environment and multiple factors can influence membrane fluidity and permeability. Further study of these strains is required to determine if their different compositions have similar biophysical properties.

We investigated the fluidity of the membranes and the Laurdan assay demonstrated that the fluidity of the membranes for each strain decreased over the duration of the fermentation, which has been observed previously (58), and may be linked to nutrient depletion and changes in the growth rate of the cells. In our study, the most tolerant strain, AJ4, underwent the largest changes in fluidity, where the membranes were significantly more fluid at 10% ethanol than in the other conditions. AJ4 lipid-containing liposomes were also the “leakiest” when compared to the other strains. This strain may therefore be better able to tolerate the fluidizing effects of ethanol upon the membrane or to modulate its membrane composition to lead to an increase in fluidity; this more fluid composition may allow more efficient movement of ethanol across the membrane. The membranes of one of the least tolerant strains, MY26, did not alter in fluidity in any of the conditions and liposomes comprised of MY26 lipids were less leaky when challenged with ethanol. In addition, our analysis of PE abundance shows that MY26 contained significantly more PE than the other strains in both 0% and 6% ethanol, while the most tolerant strain, AJ4, contained less PE in general than other strains. PE has a small headgroup and can form hydrogen bonds with adjacent PE molecules (59). It influences lipid packing and therefore membrane fluidity, where increased PE content results in less fluid membranes (60, 61),

consistent with our hypothesis. Lower PE content in relation to PC has been correlated with more tolerant strains (46, 62). These findings suggest that more tolerant strains are more fluid and permeable, while less tolerant strains are more rigid and less permeable. Several studies have correlated membrane fluidity and ethanol tolerance, and many of these point to increased fluidity being associated with more tolerant strains (45, 57), although another study suggests that less fluid membranes are associated with more tolerant strains (58). In this study, we provide further support for the concept that low PE content is beneficial for ethanol tolerance. This result can guide engineering to improve ethanol tolerance towards the reduction of PE synthesis. This compound is produced by four separate pathways, but the Psd pathway, which utilizes PS as a substrate is predominant in *S. cerevisiae* (63,64), so future works can be addressed in this direction.

In summary, the lipid composition of most of the yeast strains in this study were comparable but there were significant differences between these and the MY29 strain. Upon ethanol treatment, this composition changed significantly and a more similar composition was reached, suggesting an adaptation mechanism in common with the other strains. Changes in saturation were observed for each of the strains upon ethanol treatment, but it is not clear if these changes have a direct impact upon fluidity and tolerance, and it is likely that other factors beyond the scope of this study play a critical role and further investigation is needed. The PE abundance of the least tolerant strain, MY26, was significantly higher than in the other strains. Our investigation therefore suggests that the membranes of more tolerant strains are more fluid and contain less PE. Overall, our results point to a reduced set of desirable membrane compositions and features that promote ethanol tolerance with increased fluidity and permeability appearing to be key.

Material and methods

Strains and media conditions.

The *Saccharomyces cerevisiae* yeast strains used in this study are listed in Table 1. A total number of 61 strains from different isolation sources were selected. These strains were maintained in GPY-agar medium (%w/v: yeast extract 0.5, peptone, 0.5, glucose 2, agar 2). Yeast identity was confirmed by sequencing the D1/D2 domain of the 26S rRNA gene (65).

Drop test experiments. Assay in ethanol plates

To assess yeast strains' ethanol tolerance, drop test experiments were carried out. Rectangular GPY plates supplemented with different ethanol percentages (0, 6, 10, 14, 16 and 18%) were prepared. Yeast cells were grown overnight at 28ºC on GPY media and diluted to an OD_{600}= 0.1 in sterile water. Then, serial dilutions of cells (10^{-1} to 10^{-3}) were transferred on the plates with replicates and incubated

at 28ºC for ten days with the plates wrapped in parafilm to avoid ethanol evaporation. Each strain was inoculated twice on the same plate but at different positions, and an exact replicate of the plate was made. With this method, four biological replicates of each strain were performed. Growth values were assigned to each of the replicates: 0 no growth, 1 weak growth, 2 intermediate growth and 3 remarked growth. Median growth values were assigned for each ethanol concentration. Hierarchical clustering used in heatmap plot was elaborated using www.heatmapper.ca tool, (66) with Euclidean distance measurement method and group clustering was based on growth in different ethanol media averages (average linkage).

Assay in liquid media.

Optical density measurements.

GPY precultures of each strain were prepared and incubated at 28ºC overnight. These cultures were washed with sterile water and adjusted to an OD_{600}= 0.1 in each one of the culture media (YNB liquid media supplemented with different ethanol percentages (0, 1, 6, 8, 10, 13, 16 and 18 %)). YNB is composed of 6.7 g/L of aminoacids and ammonium sulfate (YNB, Difco) and supplemented with 20 g/L of D-glucose as carbon source. Growth was monitored in a SPECTROstar Omega instrument (BMG Labtech, Offenburg, Germany) at 28ºC. NuncTM MicroWellTM 96 well plates (ThermoFisher Scientific) wrapped in parafilm and with water in each of its 4 repositories were employed. Measurements were taken at 600 nm every 30min, with 10 seconds of preshaking before each measurement until 64 hours of growth monitoring. All the experiments were carried out in triplicate.

Estimation of the NIC and MIC parameters.

The basis of the technique, used as in (40), is the comparison of the area under the OD–time curve of positive control (absence of ethanol, optimal conditions) with the areas of the tested condition (presence of ethanol, increasing inhibitory conditions). As the amount of inhibitor in the well increases, the effect on the growth of the organism also increases. This effect on the growth is manifested by a reduction in the area under the OD–time curve relative to the positive control at any specified time.

Briefly, the areas under the OD–time curves were calculated by integration using GCAT software (http://gcat-pub.glbrc.org/). Then, for each ethanol condition and strain replicate, the fractional area (fa) was obtained by dividing the tested area between the positive control area (f a = (test area) / (positive control area). The plot of the fa vs log10 ethanol concentration produced a sigmoid-shape curve that could be well fitted with the modified Gompertz function for decay (67) $fa = A+C\times\exp[-\exp(B(x-M)])$. After this modelling, the NIC (non-*inhibitory* concentration) and MIC (Minimum Inhibitory Concentration) parameters could be estimated as in (66).

NIC = 10[M−(1.718/B)] MIC = 10[M+(1/B)]

To check for significant differences among yeast species for NIC and MIC parameters, an analysis of variance was performed using the one-way ANOVA module of Statistica 7.0 software. Tukey's test was employed for mean comparison. ggplot2 package (68) implemented in R software, version 3.2.2 (RDevelopment Core Team 2011) was employed for graphic representation of these NIC and MIC values.

Strains selection and competition fermentation

Competition fermentations were carried out in 30 mL GPY, GPY+6% ethanol and GPY+10% ethanol in triplicate. 0.1 OD of each of the 5 strains (AJ4, MY3, MY14, MY26, MY29) were inoculated in every initial culture. Every 3/5 days 1 mL of the culture was transferred into the corresponding fresh media. After 5 and 10 rounds, culture plates of samples from every tube were obtained. 20 colonies from every plate were randomly picked for their identification. This was carried out by means of mitochondrial digestion profile identification (69), which allowed differentiation of all the strains except for MY14 and MY29, which shared the same exact profile. As an alternative, as we had available the genome sequences of MY14 and MY29 (70), we identified a divergent region among these two strains which encomprises gene *MMS1*. We amplified a region of gene *MMS1* with primers *f1* (AACGGATCCTTTTTCCCAAC) and *r1* (CGGTCGCAAAAATTAACG) and used *RsaI* digestion to differentiate specially these two strains. Theoretical results for digestion bands sizes in an agarose gel were calculated based on Sanger sequencing of the amplicon for the strains of interest (Figure S3).

Lipid composition and membrane studies.

Lipid extraction and quantification by ammonium ferrothiocyanate assay

Yeast precultures of each one of the five selected strains (AJ4, MY3, MY12, MY26 and MY29) were first propagated in 25 mL of GPY media at 200 rpm and 28°C. The cultures were harvested after 24 h and total lipids were extracted using a modified Bligh and Dyer protocol (71). To quantify the lipids, 10 µL sample was taken from the above 100 µL reconstituted lipids in chloroform and added to 2 mL chloroform with 1 mL of assay reagent (0.1M $FeCl_3.6H_2O$, 0.4 M ammonium thiocyanate) in a 15 mL glass tube. Samples were vortexed for 1 min and centrifuged at 14,500 g for 5 mins. The lower layer was collected into quartz cuvettes. The absorbance was measured at 488 nm, and the concentration of lipid was determined by comparison with a standard curve of a mixture of phospholipid standards (POPC, POPE and POPG) (Sigma).

Mass spectrometry of lipids present in the strains

The lipids from each of the five yeast strains extracted as previously described were reconstituted in 100 µL chloroform to contain 5 µg/µL lipid as determined by ammonium ferrothiocyanate assay, and then diluted 1 in 50 in solvent A (50:50 acetonitrile:H_2O, 5 mM ammonium formate and 0.1% v/v formic acid). Analysis of 10 µL samples was performed by LCMS. LC was performed on a U3000 UPLC system (Thermo scientific, Hemel Hempstead) using a Kinetex C18 reversed phase column (Phenomenex, 2.6 µm particle size, 2.1 mm x 150 mm), at a flow rate of 200 µL/min with a gradient from 10% solvent B to 100% solvent B (85:10:5 isopropanol: acetonitrile: H_2O, 5 mM ammonium formate and 0.1% v/v formic acid) with the following profile: t=0 10% A, t=20 86%A, t=22 96%A, t=26 95%A. MS analysis was carried out in positive and negative ionization mode on a Sciex 5600 Triple TOF. Source parameters were optimized on infused standards. Survey scans were collected in the mass range 250-1250 Da for 250 ms. MSMS data was collected using top 5 information dependent acquisition and dynamic exclusion for 5 s, using a fixed collision energy of 35V and a collision energy spread of 10V for 200 ms per scan. ProgenesisQI® was used for quantification and LipidBlast (https://fiehnlab.ucdavis.edu/projects/LipidBlast) for identification. All data were manually verified and curated. Data were analysed by two-way ANOVA and Tukey's multiple comparisons test, where n = 5. Data sets were uploaded to: https://doi.org/10.17036/researchdata.aston.ac.uk.00000495

TLC analysis.

Yeast lipids extracted as above after 24 h growth were analysed by TLC. Briefly, 20 µg of lipid sample and 10 µg phospholipid lipid standards (POPE and POPS) (Sigma) were loaded onto silica gel TLC plates (Sigma) and separated using chloroform/methanol/acetic acid/water 25:15:4:2. The plates were air dried and sprayed with ninhydrin reagent (0.2% ninhydrin in ethanol) (Sigma) and charred at 100°C for 5 mins. Images of plates were captured with a digital camera ad spot intensity was determined using ImageJ software.

Laurdan membrane fluidity assay.

Yeast cultures were set up in GPY and incubated at 200 rpm and 28°C overnight. Then, 25 mL of GPY media containing 0% ethanol, 6% ethanol or 10% ethanol was inoculated to an OD_{595} of 0.5. Samples were taken at different time points during the fermentation, and live yeast were diluted to an OD_{595} of 0.4 in GPY and incubated with 5 µM Laurdan (6-dodecanoyl-2-dimethylaminonaphthalene) for 1 h. Fluorescence emission of these cells stained with Laurdan was taken using a microplate reader (Mithras, Berthold) with the following filters; λex=460 λem=535. Generalized Polarization (GP), derived from fluorescence intensities at critical wavelengths, can be considered as an index of membrane fluidity and is calculated as $GP = (I_{460}-I_{535})/(I_{460}+I_{535})$. Data were analyzed by one-way ANOVA and Tukey's multiple comparisons test, where n = 3.

Carboxyfluorescein dye leakage assay

Lipids for each of the five selected yeast strains extracted as described previously were used to generate 400 nm liposomes loaded with 100 mM Carboxyfluorescein (CF) in protein buffer (50 mM tris, 50 mM NaCl, pH 7.4). Dye leakage assays were performed with at 0.125 mg/mL liposomes and increasing concentrations of ethanol in protein buffer at room temperature, and the fluorescence emission measured (λex= 492 nm, λem=512 nm). Liposomes were treated with 5% Triton X-100 to fully disrupt them, and fluorescence measurements were normalized to the maximum reading for each liposome composition. Data were analyzed by one-way ANOVA and Tukey's multiple comparisons test, where n = 3.

PCA analysis

To visualize the relationships among different ethanol tolerance parameters and lipid composition of the selected *S. cerevisiae* strains, a principal component analysis (PCA) was performed using the prcomp function and ggbiplot (0.55 version) and ggplot (3.2.1 version) implemented in R.

Data availability

The sequencing of the D1/D2 26S rRNA gene of the strains was deposited in GenBank with the accession numbers MW559910-MW559970 (73).

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Figure legends.

FIG 1. Heatmap representation of growth values (from 0 to 3) of the analysed strains at plates with increasing ethanol concentrations. Each line corresponds to a strain (AJ4, MY1-MY63) and each column to a particular ethanol concentration (0%, 6%, 10%, 14%, 16% and 18%). The color key bar at the top indicates growth values, from yellow (low growth value) to pink (high growth value). Hierarchical clustering is showed on the left. Color dots on the right of the Figure indicate the source/origin of each one of the strains and shapes their classification. In Fig. S1 can be seen one of the four replicates from which these heatmap was constructed.

FIG 2. Representation of each strain NIC (yellow) and MIC (red) parameters of the selected strains in relation with its ethanol tolerance (%). Values are averages from triplicate experiments and standard deviation is represented too. Color dots on the right of the Figure indicate the source/origin of each one of the strains and shapes their classification. Strains are ordered by MIC value.

FIG 3. Photograph of the drop tests in ethanol plates (A) and the NIC and MIC parameters (B) for each one of the 5 selected strains.

FIG 4. Percentage of strains present in GPY+ethanol media determined by molecular identification after 10 rounds of fermentations. Every biological replicate is indicated by letters *A*, *B* and *C* and the ethanol concentration present in the media in the *X* axis.

FIG 5. Number of species identified by lipid class for AJ4, MY3, MY14, MY26 and MY29 strains in the presence of 0% ethanol and 6% of ethanol. Lipids were extracted and analysed by LC-MS in positive and negative ion mode (n = 5).

FIG 6. Percentage of saturated, monounsaturated and polyunsaturated chains by lipid class showing significant changes for A) AJ4, MY3, MY14, MY26 and MY29 strains in the presence of 0% ethanol, and B) AJ4, MY3, MY14, MY26 and MY29 strains in the presence of 6% ethanol. Lipids were extracted and analysed by LC-MS in positive and negative ion mode (n = 5).

FIG 7. TLC analysis of phosphatidylethanolamine (PE) and phosphatidylserine (PS) abundance for AJ4, MY3, MY14, MY26 and MY29 strains in the presence of 0%, 6% and 10% of ethanol.

Samples were loaded in triplicate and spot intensity was analyzed using ImageJ. Spot intensity is plotted relative to phospholipid standards loaded onto each plate.

FIG 8. The effects of ethanol upon the fluidity of live yeast throughout the fermentation, measured by changes to Laurdan generalized polarization (GP).

FIG 9. The effects of ethanol upon liposomes composed of lipids extracted from AJ4, MY3, MY14, MY26 and MY29 strains normalized to the maximum amount of dye released upon treatment with 5% Triton X-100.

FIG 10. Plot of the first two factors of a Principal Component Analysis (PCA) of the five *S. cerevisiae* strains regarding their lipid composition and their ethanol tolerance.

825 **TABLE 1.** List of the 61 *Saccharomyces* strains selected used in this work.

Strain name	Strain repository / Collection	Isolation source and origin	Strain properties / Description
Wine comercial fermentation strains			
MY1	Lallemand	Wine	White and rosé wines
MY2	Lallemand	Wine	White wines
MY3	Lallemand	Wine	Rosé and red wines
MY4	Lallemand	Wine	White and rosé wines
MY6	Lallemand	Wine	White, rosé and red wines
MY7	Lallemand	Wine	Red wines
MY8	Lallemand	Wine	Red wines
MY11	Lallemand	Wine	White wines
MY12	Lallemand	Wine	Red wines
MY13	Lallemand	Wine	White, rosé and red wines
MY14	Lallemand	Wine	Sparkling wines, fruit wines and ciders
MY15	Lallemand	Wine	White wines
MY16	Lallemand	Wine	White, rosé and red wines
MY17	Lallemand	Wine	White wines
MY18	Lallemand	Wine	Stuck fermentations
MY19	Lallemand	Wine	Red wines
MY20	Lallemand	Wine	Red wines
MY21	Lallemand	Wine	Red wines
MY51	Lallemand / AQ29	Wine	Red wines
MY62	Lallemand	Wine	Red wines[a]
MY63	Lallemand	Wine	White and rosé wines

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832 **TABLE 1. Continuation**
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Strain name	Strain repository / Collection	Isolation source and origin	Strain properties / Description
Wine non-commercial fermentation strains			
MY52	AQ1336	Wine, South Africa	-
MY53	AQ923	Wine, Spain	-
MY54	AQ924	Wine, Spain	-
MY55	AQ2371	Bili wine, West Africa	-
MY56	AQ2375	Bili wine, West Africa	-
MY61	I.CF 14[b]	Wine, Hungary	High Temperature
MY28	AQ2492	Flor wine, Spain	-
MY29	AQ2356	Flor wine, Spain	-
MY30	AQ94	Flor wine, Spain	-
MY31	AQ636	Flor wine, Spain	-
Other commercial fermentation strains			
AJ4	Lallemand	Fermentations	
MY50	Lallemand	Fermenting cacao	-
MY60	Fermentis	Bioethanol	Ethanol Red
Other non-commercial fermentation strains			
MY25	AQ2579	*Agave salmiana,* Peru	-
MY26	AQ2493	*Agave salmiana,* México	-
MY27	AQ2591	Chicha de jora, Perú	-
MY32	AQ594	Sake, Japan	-
MY33	AQ1312	Sakeye, Japan	-
MY34	AQ1314	Sakeye, Japan	-
MY35	AQ2332	Chicha de jora, Perú	-
MY36	AQ2469	Chicha de jora, Perú	-
MY37	AQ2363	Masato, Perú	-
MY38	AQ2473	Masato, Perú	-
MY43	AQ1180	Cider, Ireland	-
MY44	AQ1182	Cider, Ireland	-
MY45	AQ1184	Cider, Ireland	-
MY46	AQ2851	Sugar cane, Brazil	-
MY47	AQ2543	Sugar cane, Brazil	-
MY48	AQ2506	Sugar cane, Brazil	-
MY57	AQ843	Beer, Belgium	-
MY58	AQ1323	Sorghum beer, Burkina Faso	-
MY49	AQ1085	Fermenting cacao, Indonesia	-
MY59	UFLA	Bioethanol	-

TABLE 1. Continuation

Strain name	Strain repository / Collection	Isolation source and origin	Strain properties / Description
Natural Environmental strains			
MY22	AQ2458	*Agelaia vicina,* Peru	-
MY23	AQ2163	*Quercus faginea,* Spain	-
MY24	AQ997	*Prunus armeniaca,* Hungary	-
Clinical strains			
MY39	AQ2587	Dietetic product, Spain	-
MY40	AQ2654	Faeces, Spain	-
MY41	AQ435	Vagina, Spain	-
MY42	AQ2717	Lung, Spain	-

AQ (Amparo Querol collection),

[a] MY62 is a *S. cerevisiae* strain containing a limited amount of *S. kudriavzevii* genome (72, 73)

[b] kindly provided by Professor Sipiczki

Table 2. Unsaturation index (UI) for lipids identified in each strain was calculated using the percentage of lipids with each number of unsaturated bonds: one unsaturation + (2 x two unsaturations) + (3 x three unsaturations) + (4 x four unsaturations). Statistically significant differences between strains and ethanol conditions are highlighted in bold (two-way anova and Tukey's multiple comparisons test). Errors (SD) are shown in brackets, n = 5.

	0% ethanol					6% ethanol				
Lipid species	**AJ4**	**MY3**	**MY14**	**MY26**	**MY29**	**AJ4**	**MY3**	**MY14**	**MY26**	**MY29**
CerP	41.38 (± 1.09)	42.50 (± 1.01)	41.86 (± 0.54)	41.90 (± 0.50)	40.23 (± 4.34)	83.25 (± 0.58)	82.93 (± 0.41)	84.39 (± 1.17)	83.93 (± 0.43)	83.25 (± 0.58)
CL	33.33 (± 13.92)	70.00 (± 8.15)	62.22 (± 23.36)	90.00 (± 31.83)	88.00 (± 35.91)	107.24 (± 34.56)	60.00 (± 4.08)	111.79 (± 32.31)	30.00 (± 19.96)	84.33 (± 32.53)
DG	**86.76 (± 0.64)**	89.02 (± 0.29)	88.99 (± 0.62)	89.25 (± 0.48)	**90.03 (± 0.61)**	89.10 (± 4.08)	89.91 (± 0.31)	88.31 (± 0.63)	87.65 (± 0.64)	89.71 (± 0.69)
GPA	104.16 (± 0.98)	103.65 (± 0.34)	107.60 (± 1.63)	104.22 (± 1.01)	103.12 (± 0.87)	103.96 (± 23.31)	104.26 (± 1.00)	105.53 (± 0.34)	104.08 (± 1.02)	105.09 (± 1.13)
GPCho	50.20 (± 0.57)	50.29 (± 0.17)	50.03 (± 0.43)	50.72 (± 0.32)	50.36 (± 0.35)	96.64 (± 19.96)	96.66 (± 0.49)	96.47 (± 0.59)	96.35 (± 0.50)	95.77 (± 0.71)
GPEth	**108.72 (± 0.35)**	108.20 (± 0.35)	**108.72 (± 0.28)**	**109.36 (± 0.60)**	**97.36 (± 7.13)**	109.69 (± 32.53)	110.43 (± 0.50)	110.04 (± 1.06)	109.81 (± 0.36)	**108.73 (± 0.92)**
GPGro	124.20 (± 0.97)	123.76 (± 1.02)	119.56 (± 1.01)	124.69 (± 2.03)	125.84 (± 5.31)	121.53 (± 3.14)	120.00 (± 0.31)	120.03 (± 2.85)	120.87 (± 2.67)	127.20 (± 2.24)
GPIns	82.24 (± 1.21)	88.80 (± 3.42)	80.55 (± 2.65)	88.05 (± 3.25)	92.06 (± 2.02)	81.06 (± 2.40)	84.26 (± 1.81)	85.24 (± 4.14)	90.56 (± 2.16)	86.07 (± 2.39)
GPSer	96.68 (± 0.59)	95.85 (± 0.33)	95.92 (± 0.62)	97.30 (± 0.43)	95.06 (± 1.70)	97.83 (± 22.32)	96.80 (± 22.09)	96.52 (± 22.01)	97.00 (± 22.13)	96.68 (± 22.05)
MG	77.42 (± 17.68)	**73.30 (± 16.58)**	78.09 (± 17.71)	78.43 (± 17.77)	**83.27 (± 18.95)**	79.90 (± 1.88)	83.21 (± 1.26)	80.59 (± 2.78)	78.74 (± 0.90)	**78.74 (± 1.52)**
TG	116.88 (± 1.35)	118.43 (± 2.36)	118.00 (1.16)	114.93 (± 0.62)	118.19 (± 0.54)	118.75 (± 1.18)	120.76 (± 1.65)	125.55 (± 2.90)	118.22 (± 0.91)	122.29 (± 1.91)