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Wine Yeast Strains Under Ethanol-Induced Stress: Morphological and Physiological Responses

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Abstract: During alcoholic fermentation, ethanol accumulation significantly impacts yeast cells by disrupting membrane integrity, increasing permeability, and reducing cell viability. This study evaluated the effects of ethanol stress on the growth, membrane fluidity, and cell surface morphology of *Saccharomyces cerevisiae* and non-*Saccharomyces* yeast strains, specifically *Torulaspora delbrueckii* and *Metschnikowia pulcherrima*. These strains, commercialized by AEB SpA and preserved at the Unimore Microbial Culture Collection (UMCC), were tested for fermentative performance in grape must and grown under varying ethanol concentrations. Membrane fluidity was measured using Laurdan generalized polarization (GP), while Atomic Force Microscopy (AFM) assessed cell surface morphology. Results indicated that at 10% ethanol, membrane fluidity increased, particularly in strains able to tolerate up to 16% ethanol, which also demonstrated superior fermentative performance. Less tolerant strains, such as *T. delbrueckii* UMCC 5 and *M. pulcherrima* UMCC 15, showed smaller increases in fluidity. At 18% ethanol, these strains exhibited severely altered surface morphology and larger surface roughness values, suggesting increased instability under high ethanol stress, while more tolerant strains displayed better-preserved surface morphology and lower roughness values, reflecting enhanced adaptability. These findings offer insights into yeast responses to ethanol stress, supporting the development of more resilient strains for improved fermentation.

Keywords: ethanol stress; alcoholic fermentation; yeast strains; membrane fluidity; *Saccharomyces cerevisiae*; non-*Saccharomyces* yeasts; fermentative performance

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1. Introduction

Yeasts play a crucial role in various biotechnological processes, particularly in producing alcoholic beverages. During alcoholic fermentation, yeast cells encounter multiple stress factors, including osmotic stress due to high sugar concentrations in the initial stages, temperature fluctuations, nutrient depletion, and the accumulation of metabolites, especially ethanol [1–4]. Yeasts of the *Saccharomyces cerevisiae* species have a native capability to withstand high levels of ethanol that would be lethal to or severely impair the physiology of other organisms. However, ethanol is one of the most significant stressors because of its ability to penetrate cell membranes due to its small size and amphiphilic nature—possessing both a hydroxyl group and a methyl group. This dual solubility allows ethanol to integrate into both the aqueous and lipid phases of the cell, facilitating its penetration of the cell membrane, which is primarily composed of phospholipids, sterols, and proteins. Once

ethanol permeates the membrane, it disrupts membrane integrity by altering its fluidity and permeability, leading to the leakage of intracellular components such as amino acids and ions, thereby compromising cellular homeostasis [5–7]. Furthermore, ethanol impacts mitochondrial structure, reducing ATP levels and respiration rates while promoting the production of acetaldehyde and reactive oxygen species. These events can result in lipid peroxidation, DNA damage, and oxidative stress, ultimately decreasing cell viability [8,9]. The alteration of membrane properties is central to the mechanism underlying ethanol toxicity. The composition of membrane fatty acids and sterols, such as ergosterol, plays a key role in determining membrane fluidity, which is essential for maintaining the proper structure and function of the membrane [10]. Membrane fluidity is influenced by the chain length and degree of saturation of fatty acids: a higher proportion of saturated fatty acids (SFAs) results in increased membrane rigidity, which can lead to lipid bilayer stress and trigger the unfolded protein response (UPR) [11,12]. On the other hand, the failure to adequately regulate unsaturated fatty acids (UFAs) can disrupt organelle organization and, in severe cases, lead to cell death. In *S. cerevisiae*, the production of unsaturated fatty acids largely depends on the Δ9 acyl-CoA desaturase, Ole1p. This enzyme's activity is tightly controlled through the OLE pathway, which plays a critical role in preserving membrane fluidity, especially under ethanol-induced stress [13]. The OLE pathway is responsible for the regulated activation of the transcription factors Mga2 and Spt23 from the endoplasmic reticulum (ER) [14,15]. These factors then migrate to the nucleus to promote the expression of the *OLE1 gene*, which encodes the essential Δ9-fatty acid desaturase. This enzyme is responsible for the de novo biosynthesis of UFAs, which serve as fundamental lipid building blocks for maintaining membrane integrity and cellular function [16]. Although *S. cerevisiae* yeasts have traditionally been regarded as the primary agents in alcoholic fermentation, the role of non-*Saccharomyces* yeasts should not be overlooked. A diverse range of yeast species is known to inhabit freshly crushed grape juice, primarily from the genera *Hanseniaspora* (anamorph *Kloeckera*), *Pichia*, *Candida*, *Metschnikowia*, *Kluyveromyces*, and *Saccharomyces*. Additionally, species from other genera, such as *Zygosaccharomyces*, *Saccharomycodes*, *Torulaspora*, *Brettanomyces*, and *Schizosaccharomyces*, may also be present [17]. While many non-*Saccharomyces* species, particularly *Hanseniaspora*, *Candida*, *Pichia*, and *Metschnikowia*, initiate the early stages of indigenous alcoholic fermentation, they are soon overtaken by *S. cerevisiae*, which dominates the mid to final stages of the process, often becoming the only species present in the fermenting juice [18]. Historically, non-*Saccharomyces* yeasts were regarded as spoilage organisms due to their association with stuck fermentations, low ethanol tolerance, reduced acidity production, high residual sugar levels, and off-flavors [19–21]. However, the role of non-*Saccharomyces* yeasts in winemaking has been re-evaluated. Nowadays, winemakers demand yeasts as starter cultures with a whole range of specific properties that largely differ according to the type and style of wine to be made, as well as the technical requirements of the winery [22]. Controlled mixed fermentations involving both *Saccharomyces* and non-*Saccharomyces* species have shown numerous benefits [23]. This approach enhances both the complexity and specificity of wine, as non-*Saccharomyces* species contribute by providing specific compounds during the early stages of fermentation before *S. cerevisiae* takes over, ensuring the optimal progression of alcoholic fermentation and ultimately improving wine quality [19,24,25]. Mixed fermentations have been shown to increase the production of desirable metabolites, such as acetate esters [26] and glycerol [27,28], while certain non-*Saccharomyces* yeasts release more polysaccharides than *S. cerevisiae*, improving mouthfeel and improving wine stability [29]. Among these, *Torulaspora delbrueckii and Metschnikowia pulcherrima* have attracted particular attention for their unique contributions. *T. delbrueckii* has been demonstrated to boost the presence of volatile compounds [30,31], produce higher polysaccharide levels compared to *S. cerevisiae*, exhibit strong β-glucosidase activity that releases aromatic compounds [32], and reduce volatile acidity. Similarly, *M. pulcherrima* produces β-glucosidase, which not only lowers volatile acidity but also increases the production of higher alcohols, esters,

terpenols, and glycerol [27,33]. Furthermore, *M. pulcherrima* has been reported to reduce titratable acidity, enhancing the sensory balance of the final wine product [27,34].

In this study, we investigated the effect of ethanol stress on the growth, membrane fluidity, and surface morphology of industrial oenological yeast strains, including both *S. cerevisiae* and non-*Saccharomyces* strains with distinct fermentative profiles. Specifically, we assessed the growth of selected strains in both solid and liquid media with varying ethanol concentrations, monitored membrane fluidity changes using Laurdan generalized polarization (GP), and examined morphological alterations on the cell surface through atomic force microscopy (AFM). The results of this study provide novel insights into the effects of ethanol in *Saccharomyces* and non-*Saccharomyces* yeasts that can contribute to optimizing oenological industrial fermentation processes.

2. Materials and Methods

2.1. Yeast Strains and Culture Conditions

In the present study, eleven strains of commercial active dry yeast (ADY) were used, which were produced and supplied by the AEB Group (Brescia, BS, Italy). Specifically, nine strains belonged to the *Saccharomyces cerevisiae* species, while the other two were non-*Saccharomyces*. All the strains used in this study were deposited at the Unimore Microbial Culture Collection (UMCC) of the University of Modena and Reggio Emilia, Italy. The yeast strains, their commercial names, and corresponding UMCC codes are listed in Table 1. ADY strains were revitalized in YPD broth (1% yeast extract, 2% peptone, and 2% glucose) (Biolife, Milan, Italy) at the temperature specified in the technical data sheet for each strain. Cultures were stored for long-term preservation at −80 °C in cryovials containing YPD broth supplemented with 25% glycerol (*v*/*v*) as a cryopreservative. A working copy of the culture strains was kept on YPDA medium (1% yeast extract, 1% peptone, 2% glucose, 2% agar) (Biolife, Milan, Italy) and stored at +4 °C.

Table 1. Yeast strains used in this study.

Original Code	UMCC Code	Species	Commercial Name
PB2171	UMCC 3066	*Saccharomyces cerevisiae*	Fermol Fleur
PB2151	UMCC 3065	*Saccharomyces cerevisiae*	Fermol Tropical
PB2101	UMCC 3064	*Saccharomyces cerevisiae*	Fermol Lime
PB2018	UMCC 2592	*Saccharomyces cerevisiae*	Fermol Red Fruit
PB2530	UMCC 263	*Saccharomyces cerevisiae*	Fermol Sauvignon
PB2010	UMCC 24	*Saccharomyces cerevisiae*	Fermol Arome Plus
PB2023	UMCC 20	*Saccharomyces cerevisiae*	Fermol Rouge
PB2019	UMCC 19	*Saccharomyces cerevisiae*	Fermol Blanc
PB2590	UMCC 6	*Saccharomyces cerevisiae*	Fermol Mediterranèe
BBMV3FA5	UMCC 5	*Torulaspora delbrueckii*	Levulia Torula
MCR 24	UMCC 15	*Metschnikowia pulcherrima*	Levulia Pulcherrima

2.2. Microvinification Assay in Grape Juice

For the microvinification experiments, 1 g of ADY culture was rehydrated in 10 mL of sucrose solution (20 g/L) at a controlled temperature according to the manufacturer-recommended range. Specifically, *S. cerevisiae* strains were rehydrated at 37 °C, while non-*Saccharomyces* strains were rehydrated at 30 °C, as these temperatures were optimized for their physiological characteristics. Microvinifications were performed in triplicate using 100 mL glass bottles filled with 90 mL of commercial filtered grape juice (pH 3.36, Titratable Acidity 5.7 g/L, and sugars 170.83 g/L). Subsequently, 5 mL of the rehydrated ADY was inoculated, and the flasks were sealed with 5 mL of paraffin oil (Carlo Erba, Milan, Italy) to ensure anaerobic conditions. Filtered grape juice without the addition of ADY was used as a control. The incubation was conducted at 25 °C under static conditions, and the weight loss of the flasks was measured daily to monitor the progress of fermentation over 12 days. The released carbon dioxide (CO_2) was calculated based on the observed weight loss, which

directly corresponds to the amount of CO_2 produced during the fermentation process. At the end of the fermentation, the samples were collected and immediately analyzed for pH and titratable acidity. Samples for high-performance liquid chromatography (HPLC) analysis were stored at −20 °C until use. The fermentative fitness, defined as the ability to ferment in relation to the CO_2 produced, was evaluated using the method described by Bonciani et al. [35]. This was achieved through the interpolation of the fermentation curves, performed with a fifth-degree polynomial function using the software GraphPad Prism v.10.1.1 (GraphPad Software Inc., San Diego, CA, USA). Two kinetic parameters were then defined as follows:

- $tR_{1/2}$: the time (in days) required to release half of the total CO_2 produced by the best fermenter;
- $tF_{1/2}$: the time (in days) required to release half of the total CO_2 at the end of fermentation for each strain.

Both these terms were calculated for each strain, considering the average values of the three replicates. The ratio (fermentative ratio, FR) between these variables was termed FR = $tR_{1/2}/tF_{1/2}$. An arbitrary cutoff value, FR $\geq$ 0.90, was used to designate high-performance strains. Additionally, another value was obtained for each fermentation replicate by measuring the amount of CO_2 developed (g) after 2 days of fermentation, referred to as fermentative vigor (FV).

Analytical Methods

The pH and titratable acidity of the samples were determined with an XSPH 80 PRO STIRRER (Securlab, Roma, Italy). Titratable acidity was measured by titration with a 0.1 M NaOH solution. After filtration through 0.45 µm nitrocellulose membranes, 20 µL of each filtered sample collected at the end of the fermentation trial were injected into a Jasco LC-Net II/ADC HPLC system (Jasco Inc., Hachioji, Japan), equipped with a Jasco PU-2080 Plus pump, for the analysis of sugars and organic acids. Isocratic elution was carried out using a 300 × 7.8 mm Aminex® HPX-87H column (Bio-Rad Laboratories, Segrate, Italy) maintained at 40 °C with an Eldex CH-150 oven (Eldex Corp., Napa, CA, USA). The mobile phase consisted of 0.005 N H_2SO_4 and 5% v/v acetonitrile, with a flow rate of 0.6 mL/min. Calibration curves for the standards were generated using Jasco ChromNav software v. 1.18.03 (Tokyo, Japan), which was also used for peak integration and adjustment.

2.3. Phenotypic Growth Test

2.3.1. Assay in Culture Media at Different Ethanol Concentrations

The preliminary screening of the yeast strains involved evaluating their growth ability in a selective medium supplemented with different ethanol concentrations. The culture strains were rehydrated from −80 °C storage and subsequently cultured in YPD broth at 27 °C for 24 h. Cell counts were performed using a Bürker chamber (Brand, Wertheim, Germany) to standardize the inoculum concentration for plating. Growth tests were carried out on YPDA medium supplemented with various ethanol concentrations (0, 10, 12, 13, 14, 16, and 18% v/v). For each strain, 5 µL of culture at a standardized concentration of 10^6 cells/mL were spotted onto the agar plates, which were then incubated at 27 °C for 96 h. All tests were performed in triplicate.

2.3.2. Assay in Broth at Different Ethanol Concentrations

Pre-cultures of each strain were prepared in YPD broth and incubated at 27 °C for 16 h. A concentration of 0.5 OD_{600} for all strains was inoculated into 20 mL YPD broth with different ethanol percentages (0, 10, and 14% v/v). Measurements were taken at 600 nm with a spectrophotometer (Jasko V-550, Tokyo, Japan) after 0, 4, 6, 24, 32, 48, 56, and 72 h. The experiment was carried out in triplicate.

2.4. Laurdan Membrane Fluidity Assay

Yeast cultures preserved at −80 °C were rehydrated in YPD broth and incubated at 27 °C for 16 h. Subsequently, 0.5 OD_{600} of the cultured strains was inoculated into 5 mL of YPD broth containing ethanol at concentrations of 10%, 14%, 16%, and 18% (*v*/*v*). YPD broth without ethanol was also inoculated as a control. After 24 h of fermentation, a cell aliquot was standardized by diluting to 0.4 OD_{600}. The samples were centrifuged (8800× *g* for 5 min), and the cells were washed with phosphate-buffered saline (PBS) (Sigma-Aldrich St. Louis, MO, USA) at pH 7.4 and resuspended in fresh PBS. To incorporate the fluorescent probe, the standardized samples were incubated with 5 µM Laurdan (Cayman Chemical, Ann Arbor, MI, USA) for 1 h at 30 °C [3]. Membrane fluidity measurements were performed using a Jasco FP-6200 spectrofluorometer (Jasco Inc., Hachioji, Japan). A cuvette containing an unlabeled cell suspension at the same cell density was used to measure background fluorescence, which was subtracted from the fluorescence readings obtained from the labeled cell suspension. The experiment was carried out in triplicate. The results were expressed as Generalized Polarization (GP), calculated using the following equation:

$$GP = (I_{440} - I_{490})/(I_{440} + I_{490})$$

where

- I_{440}: Emission intensity at 440 nm;
- I_{490}: Emission intensity at 490 nm.

To better compare the data, GP values are expressed as:

$$GP\ (relative\ value) = GP\ sample\ treated\ with\ ethanol/GP\ control$$

2.5. Atomic Force Microscopy

The local morphology and surface topography of yeasts subjected to different concentrations of ethanol for 24 h was assessed by AFM measurements. Samples were processed according to Canetta et al. [36] with minor modifications. Briefly, after inoculating 0.5 OD_{600} of cultured strains in YPD broth medium supplemented with 0%, 10%, and 18% (*v*/*v*) ethanol, the cells were centrifuged at 8800× *g* for 5 min, then washed and resuspended in filtered distilled water (Arium® Pro system, Sartorius AG, Göttingen, Germany) (0.22 µm). Then, 250 µL of cell suspensions were spread on the surfaces of empty Petri dishes. The cells were then left to dry overnight at room temperature. AFM was performed using a Bioscope I microscope equipped with a Nanoscope IIIA controller (Veeco Metrology, Plainview, NY, USA), operating in a noncontact mode in air and at room temperature. A silicon cantilever with a nominal spring constant of 0.8 Nm^{-1} and a nominal resonance frequency of 40 kHz (MikroMaschHQ: CSC37/NoAl, MikroMasch, Germany) was used. For each sample, different sets of height and error mode images of decreasing size (from 25 × 25 μm^2 to 5 × 5 μm^2) were acquired on different areas of the surface at relatively low scanning speeds. The images were analyzed using Gwiddion 2.61 free software (http://gwyddion.net/, accessed on 6 May 2024). The Sq root mean square (RMS) roughness was extracted from 1 × 1 μm^2 cropped topographic images (obtained from different 5 × 5 μm^2 images) after data leveling by mean plane subtraction and removal of a third-order x-y polynomial background.

2.6. Statistical Analyses

Statistical analysis was carried out by analysis of variance (ANOVA) using SPSS software v. 20.0.0 (IBM, Chicago, IL, USA). Tukey's multiple comparison test was used to identify significant differences between strains ($p < 0.05$). All experiments were performed in triplicate.

3. Results and Discussion

This study examined the effects of ethanol stress on the physiological and morphological characteristics of oenological yeast strains. The use of well-documented, commercially

available strains provided a solid basis for investigating tolerance mechanisms. We evaluated growth in media supplemented with different ethanol concentrations and assessed membrane fluidity and cell surface changes to optimize industrial fermentation processes.

3.1. Evaluation of the Fermentative Performance of Yeast Strains

In this study, the yeast strains were subjected to fermentation trials in grape juice to assess their winemaking potential on a laboratory scale. Microvinification tests revealed that, among tested strains, four *S. cerevisiae* strains (UMCC 3065, 3064, 20, and 19) demonstrated strong fermentative performance (FR > 0.90), as indicated by the CO_2 production plot (Figure 1). In contrast, the non-*Saccharomyces* strains showed lower FR and FV values compared to the *S. cerevisiae* strains, reflecting a reduced fermentative capacity. Within the *S. cerevisiae* group, UMCC 3066 reached an FR value of 0.90 but exhibited lower FV than the other *S. cerevisiae* strains. Fermentative vigor at the start of fermentation is essential for technological performance, as it enhances the starter's ability to dominate the fermentation process and outcompete other microbial populations. Among the non-*Saccharomyces* strains, *T. delbrueckii* UMCC 5 and *M. pulcherrima* UMCC 15 showed the lowest fermentative performance. However, UMCC 5 had higher FR and FV values than UMCC 15, indicating a stronger fermentative capacity compared to the *M. pulcherrima* strain (Table 2).

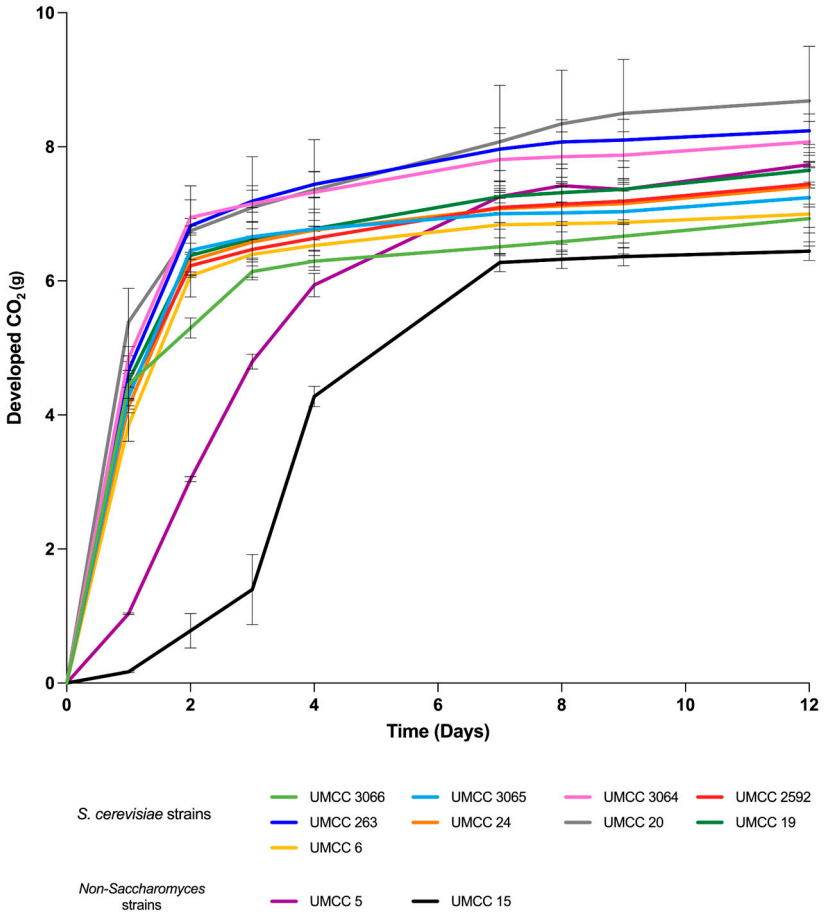

Figure 1. Developed CO_2 over 12 days of microfermentative trials in grape juice. Data are expressed as the mean of triplicate measurements ± standard deviation.

At the end of fermentation (12 days), the resulting wines were analyzed for key oenological parameters, including total acidity (TA), residual sugar, ethanol, glycerol, and organic acids (Table 3). In terms of residual sugars, there were no substantial differences among the strains, with even the non-*Saccharomyces* strains showing low levels of residual sugars. Similarly, no significant differences in ethanol production were observed. Re-

garding total acidity, the lowest value was attributed to *M. pulcherrima* (UMCC 15). Some authors have also reported that *M. pulcherrima* can decrease the titratable acidity of the final wines [27,34]. Notably, UMCC 15 showed the highest glycerol production among the strains, supporting the idea that *Metschnikowia* tends to increase glycerol levels in wines. Glycerol is an important compound for wine quality, enhancing attributes such as flavor persistence and mouthfeel [37]. During fermentation, glycolysis produces energy without affecting the redox balance, but biomass formation and organic acid synthesis lead to excess NADH [38–41]. Maintaining redox balance is essential, yet wine yeasts, including *S. cerevisiae*, lack the enzymes to interconvert NADH and NADPH [42–44]. To manage this, *S. cerevisiae* reduces dihydroxyacetone-3-phosphate to glycerol-3-phosphate, while *M. pulcherrima*, less adapted to anaerobic fermentation conditions, relies heavily on glycerol production for NAD+ regeneration. This shift to glycerol synthesis helps balance excess NADH, increasing glycerol yield in the final wine [45].

Table 2. Fermentative ratio (FR) and fermentative vigor (FV) for the eleven yeast strains. ANOVA with Tukey post hoc test ($p < 0.05$) divided strains into homogeneous subgroups indicated with lowercase alphabetical letters.

Sample	FR	FV (g)
UMCC 3066	0.90	5.30 ^{b}
UMCC 3065	0.92	6.46 ^{a}
UMCC 3064	0.95	6.95 ^{a}
UMCC 2592	0.89	6.23 ^{ab}
UMCC 263	0.67	6.82 ^{a}
UMCC 24	0.85	6.31 ^{a}
UMCC 20	1	7.11 ^{a}
UMCC 19	0.91	6.38 ^{ab}
UMCC 6	0.84	6.08 ^{ab}
UMCC 5	0.29	3.04 ^{c}
UMCC 15	0.19	0.78 ^{d}

Table 3. Values of residual sugars (glucose and fructose, expressed in g/L), ethanol and glycerol yields (both expressed as g/100 g of consumed sugar), tartaric acid (expressed in g/L), and succinic acid, acetic acid, citric acid (each expressed as g/100 g of consumed sugar), along with titratable acidity (TA) (expressed in g/L) for 11 strains. The data are the average of three replicates. ANOVA with Tukey post hoc test ($p < 0.05$) divided strains into homogeneous subgroups indicated with lowercase alphabetical letters.

Sample	Glucose (g/L)	Fructose (g/L)	Residual Sugars (g/L)	EtOH g/100 g Consumed Sugar	Gly g/100 g Consumed Sugar	Tartaric Acid (g/L)	Succinic Acid g/100 g Consumed Sugar	Acetic Acid g/100 g Consumed Sugar	Citric Acid g/100 g Consumed Sugar	TA (g/L)
UMCC 3066	2.47 ^{ab}	3.00 ^{cde}	5.47 ^{abc}	50.66 ^{a}	3.17 ^{ab}	1.31 ^{ab}	1.60 ^{a}	0.22 ^{b}	0.35 ^{ab}	7.37 ^{ab}
UMCC 3065	2.46 ^{ab}	3.27 ^{bc}	5.73 ^{abc}	51.39 ^{a}	2.83 ^{bc}	1.31 ^{de}	1.25 ^{ab}	0.16 ^{e}	0.29 ^{j}	7.63 ^{a}
UMCC 3064	2.53 ^{ab}	3.11 ^{cd}	5.64 ^{abc}	51.62 ^{a}	2.50 ^{c}	1.25 ^{abc}	1.22 ^{ab}	0.09 ^{d}	0.29 ^{cd}	7.47 ^{ab}
UMCC 2592	2.55 ^{ab}	2.76 ^{e}	5.31 ^{c}	50.80 ^{a}	3.00 ^{bc}	1.11 ^{de}	1.21 ^{b}	0.21 ^{b}	0.21 ^{ef}	7.40 ^{ab}
UMCC 263	2.48 ^{ab}	2.87 ^{de}	5.35^{c}	47.68 ^{a}	3.10 ^{ab}	1.15 ^{bcd}	1.16 ^{b}	0.18 ^{c}	0.15 ^{hi}	7.67 ^{a}
UMCC 24	2.34 ^{bc}	3.12 ^{cd}	5.46 ^{abc}	47.07 ^{a}	2.73 ^{bc}	1.17 ^{bcd}	1.13 ^{b}	0.21 ^{b}	0.24 ^{de}	7.40 ^{ab}
UMCC 20	2.41 ^{bc}	2.35 ^{f}	4.76 ^{d}	46.50 ^{a}	2.93 ^{bc}	0.87 ^{d}	1.60 ^{a}	0.31 ^{a}	0.30 ^{bc}	7.83 ^{a}
UMCC 19	2.00 ^{c}	3.44 ^{ab}	5.44 ^{abc}	50.80 ^{a}	2.70 ^{bc}	1.23 ^{abc}	1.19 ^{b}	0.30 ^{a}	0.18 ^{fg}	7.43 ^{ab}
UMCC 6	2.31 ^{bc}	3.62 ^{a}	5.93 ^{a}	50.47 ^{a}	3.00 ^{bc}	1.30 ^{de}	1.38 ^{ab}	0.26 ^{e}	0.21 ^{ef}	7.73 ^{a}
UMCC 5	2.29 ^{bc}	3.51 ^{ab}	5.80 ^{abc}	50.63 ^{a}	2.90 ^{bc}	1.39 ^{a}	1.11 ^{b}	0.21 ^{b}	0.12 ^{h}	7.40 ^{ab}
UMCC 15	2.85 ^{a}	3.01 ^{cde}	5.86 ^{ab}	51.48 ^{a}	3.63 ^{a}	1.26 ^{abc}	1.23 ^{ab}	0.10 ^{d}	0.39 ^{a}	6.53 ^{b}

3.2. Growth at Different Ethanol Concentrations in Solid Media

Ethanol tolerance differences among yeast strains are crucial in selecting strains for winemaking. In particular, ethanol tolerance is especially important due to its strong correlation with fermentative aptitude. After assessing the fermentative aptitude of the strains, phenotypic tests were conducted to evaluate the ethanol resistance in solid media. The screening of the eleven strains on YPDA medium supplemented with varying ethanol concentrations revealed differences in growth, both within strains of the same species and between different species (Figure 2). All *S. cerevisiae* strains grew up to ethanol concentrations of 14% (v/v), while UMCC 3066, UMCC 3065, UMCC 3064, UMCC 2592, and UMCC 19 grew up to 16% (v/v). All of these strains, except for UMCC 2592 (FR = 0.89), exhibited an FR $\geq$ 0.90. In contrast, non-*Saccharomyces* strains exhibited impaired growth when exposed to ethanol concentrations exceeding 10% (v/v) and showed the lowest FR values. The strong correlation observed between ethanol tolerance and fermentative aptitude suggests that strains capable of withstanding higher ethanol concentrations are more likely to maintain robust fermentative aptitude under stressful conditions typical of the later stages of alcoholic fermentation.

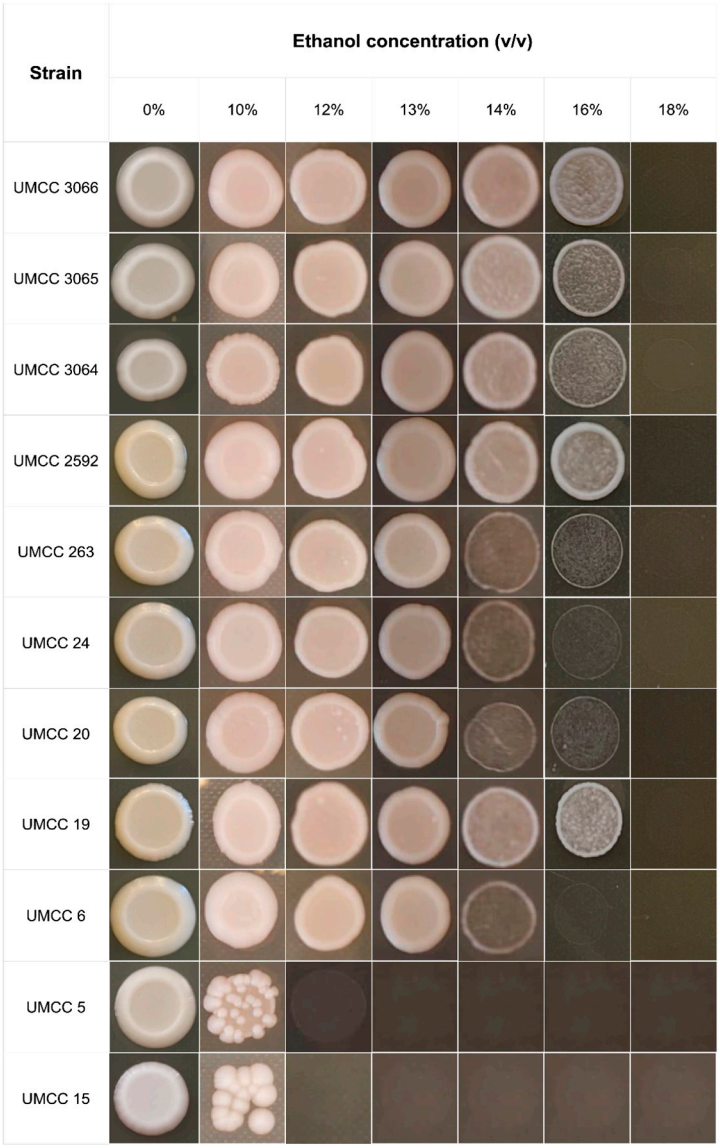

Figure 2. Growth on YPDA medium supplemented with 0, 10, 12, 13, 14, 16, and 18% (v/v) ethanol. The yeast cultures were spotted on plates at a concentration of 10^6 cells/mL.

3.3. Growth at Different Ethanol Concentrations in Liquid Media

The ethanol tolerance of these eleven strains was further evaluated in YPD broth at 27 °C over 72 h of fermentation. All *S. cerevisiae* strains demonstrated a moderate decrease in relative optical density (OD_{600}) at a 10% (*v*/*v*) ethanol concentration, while a drastic reduction was observed at higher concentrations of 14%, 16%, and 18% (*v*/*v*) (Figure 3). This contrasts with the results from ethanol tolerance tests conducted on solid media, where the inhibitory effects were less pronounced. The reduced inhibition observed in solid media is likely due to the immobilizing effect of agar, which provides a more protective environment for yeast cells, as well as the possible evaporation of ethanol from the solid media. Indeed, immobilized yeast cells have been shown to outperform free cells, exhibiting higher ethanol tolerance and reduced substrate inhibition [46]. Moreover, multiple studies have confirmed that immobilized *S. cerevisiae* produces more ethanol compared to free cells [46–49]. Except for *M. pulcherrima* UMCC 15, which showed compromised growth, the other yeast strains, including *T. delbrueckii* UMCC 5, did not exhibit significantly inhibited growth at 10% (*v*/*v*) ethanol concentration. This test allowed for a distinction between the two non-*Saccharomyces* strains. Specifically, UMCC 15 was strongly inhibited at 10% (*v*/*v*), while UMCC 5 exhibited a response similar to the *S. cerevisiae* strains, indicating better ethanol tolerance.

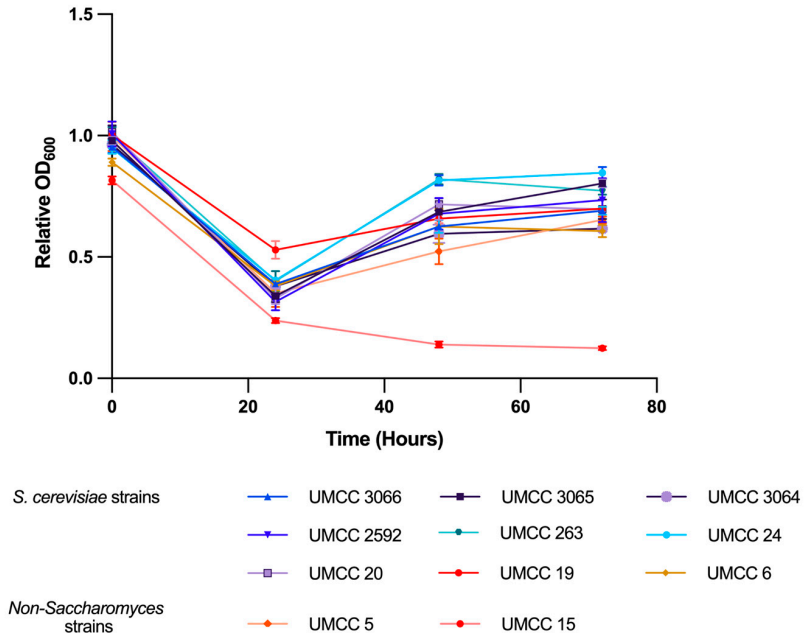

Figure 3. Growth curves of yeast strains in YPD broth supplemented with 10% (*v*/*v*) ethanol. Data are presented as relative OD_{600} values, normalized to the OD_{600} of the control (yeast grown without ethanol).

For subsequent analyses, measurements were conducted after 24 h of fermentation, as this time point was identified as the stage where ethanol exerted the most significant inhibitory effect on growth. This time frame led to the greatest growth inhibition across all strains except for *M. pulcherrima* UMCC 15, where growth remained highly compromised for up to 72 h.

3.4. Evaluation of Membrane Fluidity

Membrane fluidity is a critical factor for maintaining membrane integrity and enabling yeast cells to adapt to ethanol stress. In this study, six yeast strains with varying fermentative aptitudes and ethanol tolerance were selected for membrane fluidity assays. Strain UMCC 3064 was chosen due to its ethanol tolerance up to 16% (*v*/*v*), with an FR > 0.90 and the highest FV value. Moreover, strain UMCC 2592 was included because, despite

having an FR < 0.90, it exhibited moderately high fermentative vigor, while strain UMCC 3066, with an FR = 0.90, displayed lower fermentative vigor. Among the *S. cerevisiae* strains, UMCC 24 was also tested, which showed ethanol tolerance up to 14% (*v*/*v*) and an FR < 0.90. Additionally, the two non-*Saccharomyces* strains, UMCC 5 and UMCC 15, were also tested.

The six yeast strains exhibited varying changes in membrane fluidity at 10% (*v*/*v*) ethanol, with all showing an increase, though to different extents. These variations corresponded to their levels of ethanol resistance and fermentative capacity. Among the *S. cerevisiae* strains, UMCC 3066 displayed the highest membrane fluidity, while the non-*Saccharomyces* strains, *T. delbrueckii* UMCC 5 and, particularly, *M. pulcherrima* UMCC 15, had the lowest values. At higher ethanol concentrations (14%, 16%, and 18% *v*/*v*), UMCC 15 continued to exhibit the lowest membrane fluidity among the strains, indicating a pronounced stiffening of the membrane (Figure 4).

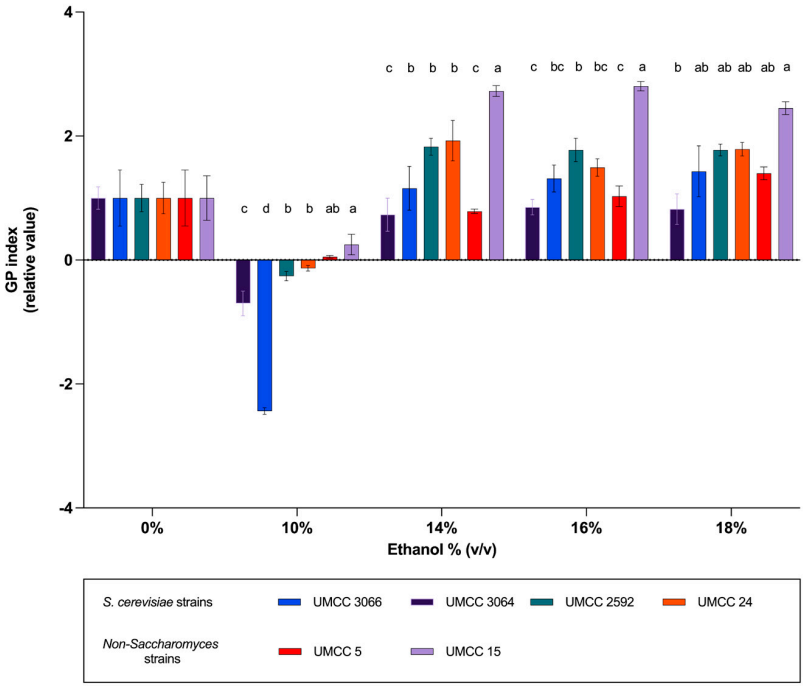

Figure 4. Membrane fluidity expressed as relative GP index values for strains UMCC 3066, UMCC 3064, UMCC 2592, UMCC 5, and UMCC 15. Significant differences among strains at the same ethanol concentration are indicated by different letters ($p < 0.05$).

The correlation between membrane fluidity and ethanol tolerance has been extensively documented, although some studies yielded inconsistent results. Ethanol-induced stress primarily targeted the cell membrane, and several authors proposed a link between the fatty acid composition of phospholipid membranes and ethanol tolerance. A well-established relationship existed between ethanol tolerance and an increased degree of fatty acid unsaturation in the membrane lipids of *S. cerevisiae* [50–57]. In contrast, other researchers, such as Ishmayana et al. [58], suggest an inverse correlation, proposing that lower membrane fluidity might contribute to higher ethanol tolerance in some strains. Our findings indicated that membrane fluidity increased under low ethanol stress, consistent with the observations of Yang et al. [7], who reported that yeast cells adjusted membrane fluidity to counteract ethanol's disruptive effects. In particular, a correlation between ethanol tolerance and the increase in membrane fluidity at 10% (*v*/*v*) ethanol was observed. Strains with higher ethanol tolerance and superior fermentative performance exhibited a greater increase in membrane fluidity compared to less tolerant strains. This pattern was consistent with the findings of Lairón-Peris et al. [59], who noted that the most ethanol-tolerant strain underwent the greatest changes in fluidity, with membranes becoming significantly more fluid at

10% (*v*/*v*) ethanol compared to the control without ethanol. This aligned with the findings of Jones and Greenfield [60], who demonstrated that elevated membrane fluidity led to increased cell membrane permeability. As a result, cells with higher membrane fluidity could more effectively expel ethanol, maintaining lower intracellular ethanol concentrations and mitigating ethanol-induced stress. In our study, this explanation was further supported by the behavior of *M. pulcherrima* UMCC 15, identified as the least ethanol-tolerant strain. This strain exhibited slower fermentative kinetics, showed the smallest increase in membrane fluidity at 10% (*v*/*v*) ethanol, and experienced a significant reduction in fluidity at higher ethanol concentrations. Its limited ability to adjust membrane fluidity under ethanol stress likely accounted for its reduced ethanol tolerance and impaired fermentation performance.

3.5. Evaluation of Cell Surface Morphology Under Ethanol Stress

Atomic force microscopy is a powerful tool for directly visualizing and quantifying physical, morphological, and structural changes occurring on the yeast cell surface in response to different stress conditions [61]. In this study, two non-*Saccharomyces* strains, UMCC 5 (*T. delbrueckii*) and UMCC 15 (*M. pulcherrima*), alongside two *S. cerevisiae* strains, UMCC 3066 and UMCC 24, have been selected for the AFM investigation as they exhibited different fermentative capacities and membrane fluidity changes under ethanol stress. Figure 5 displays representative AFM error mode images of the surface of the selected yeasts. It should be noted that, in this study, both height and error mode signals have been acquired. However, to better visualize small topographical details, we report AFM images obtained by error mode signal.

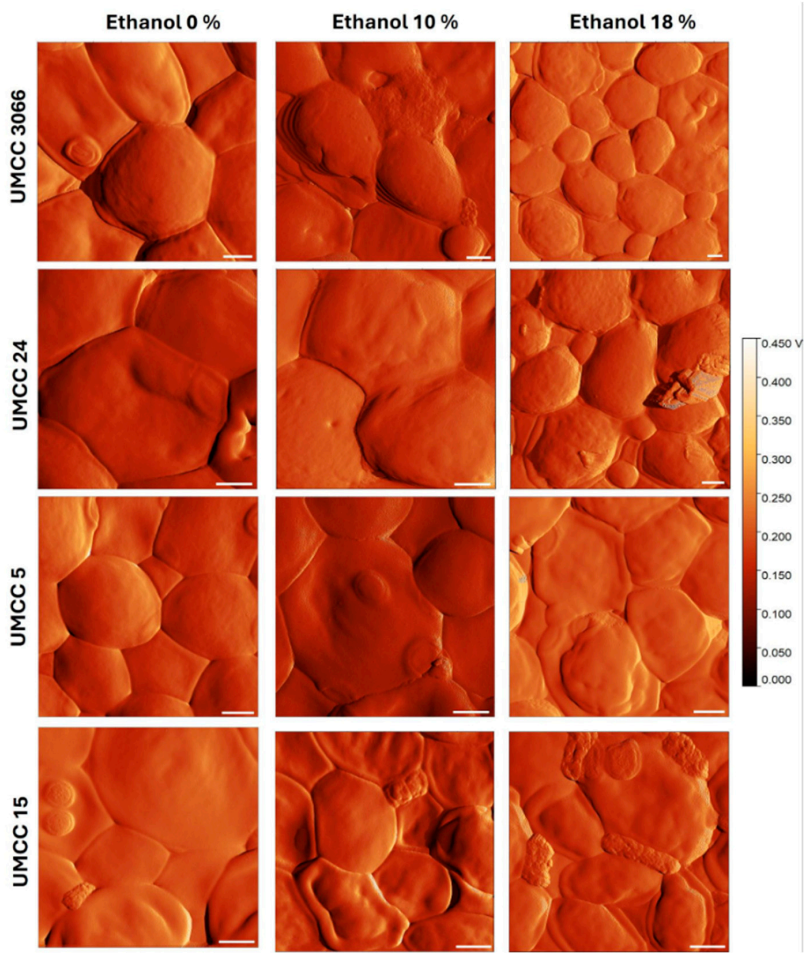

Figure 5. AFM error mode images of UMCC 3066, UMCC 24, UMCC 5, and UMCC 15 after exposure to 0%, 10%, and 18% (*v*/*v*) of ethanol. Scale bars: 1 μm.

As can be observed, the degree of compactness of cell aggregates, as well as the morphology of the cell surface, were barely altered for UMCC 3066 and UMCC 24 upon exposure to ethanol, even at the highest tested concentration. In contrast, deformation of cell morphology and loss of smoothness were found for UMCC 5 and UMCC 15, especially when exposed to 18% (*v*/*v*) ethanol. These findings corroborate the RMS data. In fact, the two non-*Saccharomyces* strains exhibited larger RMS values when exposed to 18% (*v*/*v*) ethanol (Figure 6). Conversely, the lowest RMS values at the various ethanol concentrations were found for UMCC 24 and UMCC 3066, which are the most tolerant strains tested in this study (Figure 6). Notably, UMCC 3066 consistently had the lowest RMS values across ethanol concentrations. Consistent with the findings of Canetta et al. [36], the more ethanol-tolerant strains showed minimal morphological changes compared to less tolerant strains. The significant changes in cell surface morphology observed in the non-*Saccharomyces* strains suggest a greater loss of membrane integrity under ethanol-induced stress than that observed for *S. cerevisiae* strains. Similar responses have been observed under other stressors, such as osmotic and thermal stress [62]. In summary, results from AFM assays confirm that *S. cerevisiae* may possess greater resilience to ethanol toxicity. This pattern further indicates that non-*Saccharomyces* strains may experience increased variability or instability under high ethanol stress. In contrast, the superior stability and tolerance to ethanol of the *S. cerevisiae* strain UMCC 3066 were further confirmed by AFM analysis.

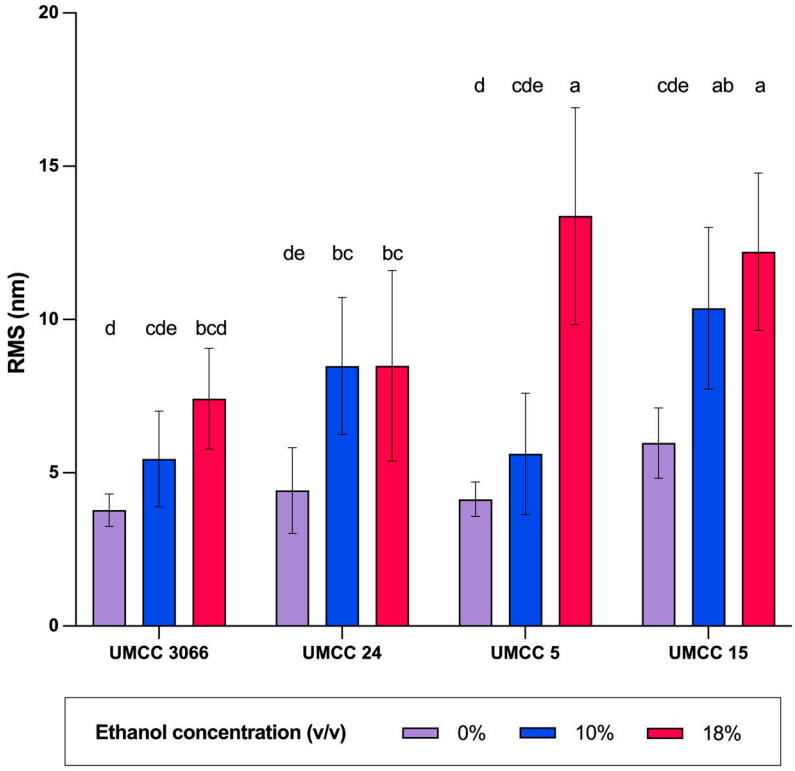

Figure 6. RMS values of UMCC 3066, UMCC 24, UMCC 5, and UMCC 15 after exposure to 0%, 10%, and 18% (*v*/*v*) of ethanol for 24 h. Data are expressed as mean ± standard deviation, obtained from 10 images for each sample. Significant differences are indicated by different letters ($p < 0.05$).

4. Conclusions

In this study, *Saccharomyces* and non-*Saccharomyces* wine yeasts, each with unique fermentative characteristics and profiles in secondary compound production, displayed varied responses in growth, membrane fluidity, and cell morphology under ethanol stress. Notably, strains capable of tolerating ethanol concentrations up to 16% (*v*/*v*) and demonstrating superior fermentative performance exhibited increased membrane fluidity at the lowest ethanol concentration tested. In contrast, less tolerant strains with lower fermen-

tative capacity but with significant contributions to final product complexity—such as *T. delbrueckii* UMCC 5 and *M. pulcherrima* UMCC 15—showed smaller increases in fluidity. At the highest ethanol concentration tested (18% *v*/*v*), these less tolerant strains displayed higher surface roughness, suggesting greater instability, whereas more tolerant strains exhibited lower RMS values, indicative of enhanced adaptability. These findings contribute to the development of multiple selected starters for oenological applications, optimizing *S. cerevisiae* strains for essential fermentative functions while harnessing the distinct attributes of non-*Saccharomyces* yeasts to enrich product complexity, thereby expanding the range of commercial options available. Based on the outcomes of this work, these strains can be further explored to understand their genetic background related to the pathway involved in preserving membrane fluidity, especially under ethanol-induced stress.

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