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Mezcal as a Novel Source of Mixed Yeasts Inocula for Wine Fermentation

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Abstract: Mezcal yeasts were evaluated for their potential as grape-juice fermenters, characterizing their fermentation performance, both in terms of primary and volatile metabolites. Experiments were first carried-out in a semi-synthetic medium and then on grape juice, and population dynamics of the chosen mixed inoculum was assessed in grape juice. Accordingly, we initially tested 24 mezcal yeasts belonging to ten different species, and chose those that were more productive and stress tolerant for the mixed (dual) inoculum, having a final selection of three *Saccharomyces cerevisiae* strains (plus Fermichamp, a commercial wine strain) and three non-*Saccharomyces* strains, belonging to *Kluyveromyces marxianus*, *Torulaspora delbrueckii*, and *Zygosaccharomyces bailii* species. For the combination *S. cerevisiae*/*T. delbrueckii* (Sc/Td) mixed inoculum, we observed increasing isoamyl alcohol and phenyl ethyl acetate concentrations, as compared with the use of individual *Saccharomyces* strains, which resulted in a fruitier aroma profile. Alcohol final concentration was in average lower for the Sc/Td inoculum (fermentation power, FP, 13.6) as compared with the individual mezcal *Saccharomyces* strains (FP 14.3), and it was the highest when Td was co-cultured with the commercial strain (FP 14.6). Overall, our results show the feasibility of using yeasts isolated from mezcal as a novel source of inoculum for wine-type fermentation.

Keywords: yeasts mixed inoculum; mezcal; *Saccharomyces*; non-*Saccharomyces*

1. Introduction

Worldwide, two main phenomena are increasingly being reported for wine fermentation, first is the occurrence of stuck fermentations, as fructose becomes the main carbohydrate during the late stages of alcoholic fermentation, and the yeasts have to ferment under conditions of high ethanol concentration and nitrogen limitation. And second, a higher alcohol content and less aroma complexity [1,2]. Some authors have used the positive response to a certain stress as a selection tool for yeast with potential for use in wine production. For example, Zuzuarregui and del Olmo [3] analyzed the resistance to oxidative, osmotic and ethanol stresses among a collection of commercial (winery) and non-commercial *S. cerevisiae* strains, and correlating fermentative behavior with resistance to oxidative stress (by exposure to H_2O_2) and to ethanol stress as the most relevant. García and collaborators [4] analyzed the tolerance to osmotic pressure, ethanol, and pH stresses in a warm climate region DO

'Vinos de Madrid' (Spain). These authors identified some *Saccharomyces* and non-*Saccharomyces* strains adapted to these fermentation stresses, concluding that these yeasts are important in the quality wine in these warm areas.

The use of non-conventional yeast as inoculum for wine making has increased in importance in the last decades, as it has been observed that some strains can increase the aroma complexity of the fermented products [5]. Our research has explored the potential use of novel yeast strains obtained from mezcal, which is a traditional Mexican liquor that involves a very stressful alcoholic fermentation [6]. These yeasts are proposed since the musts of cooked agave plants contain a high fructose content (around 90% of fermentable sugars), Maillard compounds, furfural and even toxic saponins, and mezcal fermentation is carried out without temperature control, making this a very stressful fermentation system [2]. In addition, these yeasts are part of a different domestication event as compared to other *S. cerevisiae* wine strains [7], and their phenotypic characteristics may be different, particularly, when submitted to high fructose concentration [6,8,9]. Novel yeast applications to increase the aroma complexity in wine may be supported by the used of mixed starters. As an example, co-inoculation of *S cerevisiae* strains changes wine composition regarding to monoculture [1] and mixed starters with *Saccharomyces* and non-*Saccharomyces* (*Hanseniaspora vineae*) strains enhances aromatic profile compared to simple mixed inoculation and increased the wine quality [10].

In the current study, fermentative profiles of mezcal yeasts (*Saccharomyces* and non-*Saccharomyces*) in terms of primary and volatile metabolites production were compared when cultivated in a semi-synthetic medium (M3) to simulate wine fermentation. The results were used to choose *Saccharomyces* and non-*Saccharomyces* strains to be cultivated in grape juice individually, or as a mixed inoculum, and their fermentative performance and potential as starters for wine production were evaluated.

2. Materials and Methods

2.1. Yeast Strains and Inoculum Growth Conditions

The 24 yeast strains used belong to the mezcal LCBG yeast collection (which comprises 96 different strains, belonging to ten different yeast genera) and are conserved in 60% glycerol at −70 °C. The commercial wine strain *Saccharomyces cerevisiae* Fermichamp (DSM Food Specialties B.V., The Netherlands) was used as a control for its fructophilic character, which is used to reactivate stuck fermentations as indicated by the manufacturer. The strains used were selected based on both their level of stress tolerance [6] and to be representative of the yeast diversity found in the fermentation of mezcal from Tamaulipas (Mexico). For all the strains, their 26S nucleotide sequences are available in the GenBank and are presented on Table 1, along with the fermentation stage from where they were originally isolated.

Table 1. Molecular identification and mezcal fermentation stage of isolation of the yeasts used in this study.

Species	Strain ID	GenBank Accession Number	Fermentation Stage of Isolation *
Saccharomyces cerevisiae	Fermichamp		-
	Sc3Y2	JQ824877	Final
	Sc3Y3	JQ824872	Final
	Sc3Y4	JQ824875	Final
	Sc3Y5	JQ824869	Final
	Sc3Y8	JQ824874	Final
	Scmosca3	KT945088	fruit fly on the vat surface
	Sc3D6	JQ824876	Final
	Sc3D5	KT945085	Final
	Sc3D4	KT945086	Final
	Sc3D2	JQ824871	Final
	Sc4Y3	KT945087	Trapiche

Table 1. *Cont.*

Species	Strain ID	GenBank Accession Number	Fermentation Stage of Isolation *
Kluyveromyces marxianus	Km4D3	KT945094	Trapiche
	Km1D5	KT945093	Trapiche
	Km1Y9	KT945092	Early
Torulaspora delbrueckii	Td1AN9	KT945090	Early
	Td1AN2	KT945089	Early
	Td1AN1	KT945091	Early
Pichia kluyveri	Pk4D6	KT945083	Trapiche
Yamadazyma mexicana	Pm1AN3	KT945081	Early
Meyerozyma guilliermondii	Pg1Y12	KT945082	Early
Clavispora lusitaniae	Cl4Y4	KT945080	Trapiche
Candida parapsilosis	Cp1Y7	KT945079	Early
Rhodotorula mucilaginosa	RmP12	KT945095	Early
Zygosaccharomyces bailii	Zb3Y1	KT945084	Final

* Fermentation stage periods: Trapiche (pressing of *Agave* spp. cooked stalks), early (1–3 days), and final (7–10 days) stages.

An initial preculture of the tested yeasts was grown on yeast extract-peptone-dextrose agar YPD, Difco Laboratories, France) agar plates containing 10 g/L yeast extract, 20 g/L peptone, 20 g/L D-glucose, plus 20 g/L bacteriological agar (Difco Laboratories, France), and incubated at 30 °C for 48 h. A loop of this preculture was used as inoculum for liquid YPD broth incubated for 24 h at 30 °C with shaking at 200 rpm, and final yeast concentrations (total and viable) were quantified using a Neubauer chamber, adjusting if needed using sterile isotonic solution (9 g/L NaCl solution) and used immediately as inoculum at in the fermentation experiments carried out as described below.

2.2. Setup of Minifermentation Conditions

All fermentation experiments were carried out in minibioreactor tubes of 50 mL with 4-hole vent caps (Corning Science de México, Reynosa, TAM, Mexico), but covering 3 of the 4 holes available in the cap with cellotape just before inoculation, to allow semianaerobic fermentation conditions and also to diminish loss of water. The minibioreactors contained 20 mL of either the semi-synthetic medium M3 (Oliva-Hernández et al., 2013) or grape juice medium. Medium M3 contained 200 g/L of total sugars (glucose/fructose, 1:1), 1 g/L of yeast extract, 2 g/L of $(NH_4)_2SO_4$, 0.4 g/L of $MgSO_4$ $7H_2O$, 5 g/L of KH_2PO_4, dissolved distilled water and with the pH adjusted to 5 before autoclaving. For wine-type fermentations, pasteurized red grape juice was used (Carrefour, Toulouse, France), which was typically around 200 g/L of total sugars, and also adding a small volume of ammonium sulfate sterile solution to have a final concentration of 2 g/L of $(NH_4)_2SO_4$ in the grape juice, to avoid nitrogen limitation during fermentation. The inoculum used was 3×10^6 cells/mL, either when using individual or a mixed inoculum. In the latter case, a ratio of 1:9 of *S. cerevisiae*/non-*Saccharomyces* strains was used. Incubation was performed at 30 °C using an agitated Minitron HG incubator (Infors AG, Switzerland) at 75 rpm. Each experiment was run in triplicate, hence withdrawing and analyzing three different tubes per sampling time, and measurements for each minibioreactor were performed at least two times. Average values and standard deviation are reported accordingly.

2.3. Mixed Yeasts Populations Quantification

Quantification of the yeasts populations during fermentation was performed on Wallerstein Differential Agar WLD (Sigma-Aldrich, St. Louis, MO, USA) for following the non-*Saccharomyces* populations, as *S. cerevisiae* is unable to grow on such media. This allowed an easy verification of the viable count of the non-*Saccharomyces* species. Colony counts for *S. cerevisiae* were obtained by

subtracting the WLD count number to the count obtained on on Wallerstein Nutrient Agar (WL Sigma-Aldrich, St. Louis, MO, USA) for whole yeasts population counts, and are reported as colony forming units per milliliter CFU/mL. Total and viable cell counts were determined by counting on a Neubauer chamber, using methylene blue staining as an indicator of viability of the whole population (*Saccharomyces* and non-*Saccharomyces*). All the samples were analyzed in triplicate.

2.4. Biomass and CO_2 Production Quantification

Biomass was quantified as dry weight by centrifuging 2 mL of each sample in dry and pre-weighed 2 mL Eppendorf tubes for 10 min at 14,000 rpm. The supernatant was recovered and filtered for further high performance liquid chromatography HPLC analysis, and tubes containing the biomass pellet were dried half-open at 60 °C overnight, placed in a desiccator for at least 4 h, and then weighed. Biomass production was calculated as the difference in the weight of the tube divided by the volume of the centrifuged sample. Duplicate samples were taken from each of the three minibioreactor tubes per sampling time.

The release of carbon dioxide was used as an indicator of fermentation progress and to decide when to stop the experiments; hence, weight loss was followed for each minibioreactor every 24 h. At the experimental conditions tested, both in the semi-synthetic medium M3 and in grape juice, the rate of water loss in the minibioreactors per open hole in the cap was measured to be 0.0034 g_{water}/h per hole (R^2 = 0.999), and this value was used as a correction factor to assess the CO_2 liberated per liter of medium.

2.5. Sugar Consumption and Metabolite Quantification by HPLC

The consumption of sugars and the production of metabolites (ethanol, glycerol and acetic acid) in the centrifuged (15 min at 10,000 rpm at 4 °C) and filtered (Millex-GV13 0.22 µm pore size, Millipore Sigma, Burlington, MA, USA) sample supernatants were measured with an Accela HPLC (Thermo Scientific, France) coupled to an auto sampler and using a Phenomenex ROA-Organic acid column (250 mm × 4.6 mm; 8-µm diameter beads). The mobile phase was 5 mM H_2SO_4. The volume of the injection loop was 25 µL with each run lasting around 30 min with a flow rate of 0.17 mL/min at 30 °C. The peaks were detected by infra red IRD and/or ultra violet UVD, depending on the compound measured. Calibration curves were constructed using ethanol, glycerol, acetic acid, fructose, and glucose standards ranging from 0.125 to 5 g/L.

2.6. Volatile Compound Quantification by GC-MS

The production of volatile metabolites relevant for the organoleptic profile characterization of each strain was assessed by GC-MS in a TraceGC machine (Thermo Finnigan, Villebon Sur Yvette, France). Fermentation samples were centrifuged at 7000 rpm (5697× *g*) for 15 min at 10 °C in a Sigma 6K15 centrifuge, and 10 mL were taken and extracted by SPME (PDMS fiber assembly, SUPELCO, Bellefonte, PE, USA) at 40 °C and adding 3 g of NaCl, and the volatile compounds were measured by using a ZB-5ms Phenomenex column (30 m length × 0.25 mm internal diameter, 0.25 µm film thickness). The carrier gas was helium at a flow rate of 1 mL/min, the injector was set at 240 °C, and the following temperature program was used: 10 min at 35 °C, first ramp of 2 °C/min up to 60 °C, isothermal at 60 °C for one minute, second ramp of 2.5 °C/min up to 90 °C, third ramp of 10 °C/min up to 130 °C, isothermal at 130 °C for 2 min, and fourth ramp of 20 °C/min up to 240 °C. The transfer line was set at 250 °C. Internal standard was 3-octanol. The MS was performed in a PolarisQ ion trap machine (Thermo Finnigan, Villebon Sur Yvette, France) with a source temperature of 200 °C, ionization of 70 eV, and the multiplier offset was 0 volts.

2.7. Statistical Analyses

Statistical analysis was performed using the Analyze-it software for Microsoft Excel (version 2.20) and the JMP routine of the SAS software for ANOVA analysis.

3. Results

3.1. Sampling and Yeast Identification

Yeasts used on this work, presented on Table 1, were isolated from a very rustic mezcal production winery, from freshly pressed cooked agave must to the final stage of fermentation, spanning in total a ten-day process. Due to the working conditions on the winery, it was possible to sample only at pressing and early fermentation stages, when the vats are being fed fresh agave must during a couple of days, and at end of the fermentation, as the producer covers the fermentation vats with straw and mud, and it is not possible to open them mid-process. Nonetheless, we found a very high yeast diversity in these samples. Strains used represent the productive diversity of this fermentation.

As it can be noticed on Table 1, *S. cerevisiae* strains were found typically at the end of the fermentation, as expected, while non-*Saccharomyces* strains were isolated at the pressing and early fermentation stages.

3.2. Fermentation Performance of the Mezcal Yeast Strains in Semi-Synthetic Medium M3

On medium M3, the two hexoses were rapidly consumed during the first 24 h, but, by 72 h of fermentation, less fructose was consumed as compared to glucose, as previously reported [8]. Control strain Fermichamp completed the fermentation after 96 h of inoculation. Hence, for this first part of screening, the fermentations of all the 24 tested strains were sampled at this time, to compare their productivity (Table 2) and volatile metabolite profile (Table 3).

Table 2. Primary metabolite profiles and hexose consumption for all the yeasts tested at 96 h of fermentation in synthetic medium M3, 200 g/L of initial sugars.

Strain	Glucose	Fructose	Ethanol	CO_2	Glycerol	Acetic Acid	Dry Weight
	(g/L)						
Fermichamp	0.0 ± 0.0	4.7 ± 1.7	67.2 ± 1.4	97.7 ± 5.7	2.6 ± 0.1	0.5 ± 0.1	6.0 ± 0.3
Sc3Y2	29.9 ± 3.9	54.8 ± 3.1	38.0 ± 0.2	49.8 ± 2.0	3.3 ± 0.2	0.0 ± 0.0	7.0 ± 1.7
Sc3Y3	9.9 ± 1.4	21.7 ± 2.3	58.8 ± 13.5	75.8 ± 19.2	7.3 ± 1.0	0.8 ± 0.1	4.9 ± 0.5
Sc3Y4	3.9 ± 1.4	13.5 ± 2.3	61.4 ± 4.7	83.0 ± 11.4	2. 7 ± 0.1	0.5 ± 0.03	3.9 ± 0.4
Sc3Y5	9.9 ± 6.7	26.9 ± 9.7	57.7 ± 7.3	72.2 ± 11.6	6.7 ± 0.1	0.5 ± 0.3	4.5 ± 0.3
Sc3Y8	9.9 ± 1.5	22.1 ± 1.3	53.3 ± 5.8	84.5 ± 4.36	2.6 ± 0.2	0.6 ± 0.2	3.8 ± 0.2
Scmosca3	0.0 ± 0.0	2.7 ± 0.5	67.9 ± 0.4	94.0 ± 0.46	6.9 ± 0.2	0.3 ± 0.1	5.7 ± 0.2
Sc3D6	0.3 ± 0.5	6.6 ± 2.7	68.4 ± 7.1	90.7 ± 1.42	8.2 ± 0.5	0.5 ± 0.03	5.9 ± 0.2
Sc3D5	1.1 ± 1.6	9.0 ± 10.0	65.1 ± 4.9	90.0 ± 8.0	6.3 ± 0.2	0.3 ± 0.1	5.7 ± 0.1
Sc3D4	0.4 ± 0.6	6.5 ± 5.6	66.0 ± 2.8	89.4 ± 7.3	6.9 ± 0.3	0.5 ± 0.03	5.4 ± 0.2
Sc3D2	1.2 ± 0.3	12.5 ± 1.7	65.4 ± 1.2	88.3 ± 2.0	8.3 ± 0.1	0.9 ± 0.01	5.6 ± 0.1
Sc4Y3	0.0 ± 0.0	3.6 ± 2.9	67.2 ± 0.2	92.9 ± 0.6	7.4 ± 0.1	0.3 ± 0.01	5.7 ± 0.1
Km4D3	30.2 ± 24.2	51.6 ± 17.8	34.5 ± 17.2	53.6 ± 24.2	5.3 ± 2.39	0.5 ± 0.5	4.8 ± 0.2
Km1D5	33.2 ± 0.5	57.2 ± 3.9	29.8 ± 2.1	46.0 ± 2.5	5.8 ± 0.67	0.2 ± 0.2	6.6 ± 0.6
Km1Y9	12.2 ± 12.9	34.8 ± 16.6	45.3 ± 17.2	66.5 ± 19.4	6.2 ± 1.90	0.5 ± 0.3	5.1 ± 0.6
Td1AN9	7.1 ± 5.2	27.8 ±10.3	50.6 ± 7.6	77.9 ± 6.5	6.6 ± 0.7	0.9 ± 0.2	4.5 ± 0.5
Td1AN2	59.1 ± 3.0	67.9 ± 4. 7	20.0 ± 3.1	27.4 ± 4.4	3.8 ± 0.4	0.0 ± 0.0	6. 9 ± 0.8
Td1AN1	35.2 ± 3.9	58.3 ± 4.5	31.2 ± 0.2	37.4 ± 2.6	2.9 ± 0.03	0.0 ± 0.0	8.2 ± 0.4
Pk4D6	37.0 ± 4.8	60.8 ± 7.7	25.2 ± 2.9	34.7 ± 0.5	5.4 ± 0.7	0.2 ± 0.03	6.15 ± 0.5
Pm1AN3	25.9 ± 4.9	51.4 ± 3.6	39.7 ± 3.7	49.6 ± 6.5	2.8 ± 0.2	0.0 ± 0.0	5.86 ± 0.7
Pg1Y12	83.0 ± 0.4	83.0 ± 0.4	6.3 ± 0.2	6.7 ± 1.3	0.4 ± 0.04	0.0 ± 0.0	7.4 ± 1.0
Cl4Y4	24.8 ± 12.4	48.6 ± 10.8	40.0 ± 7.0	49.3 ± 11.7	3.03 ± 0.5	0.0 ± 0.0	6.2 ± 1.0
Cp1Y7	45.8 ± 0.2	64.0 ± 0.3	19 ± 0.01	28.6 ± 5.1	5.7 ± 0.2	0.2 ± 0.0	7.8 ±1.4
RmP12	70.8 ± 19.6	76.2 ± 7.6	11.6 ± 13.9	13.3 ± 9.9	1.4 ± 1.9	0.5 ± 0.03	5.0 ± 1.3
Zb3Y1	46.2 ± 17.7	0.64 ± 0.3	56.3 ± 2.0	66.6 ± 10.0	8.4 ± 0.1	0.3 ± 0.02	6.4 ± 0.6

All data are the average of three different minibioreactor samples taken by duplicate, and standard deviation is reported.

Table 3. Volatile profiles of all mezcal yeast tested at 96 h of fermentation in synthetic medium M3, 200 g/L of initial sugars.

Strain	Isoamyl Alcohol	Isoamyl Acetate	Phenyl Ethyl Acetate	Ethyl Decanoate	Ethyl Octanoate	Ethyl Hexanoate	Ethyl Butyrate
	(mg/L)			(μg/L)			
Fermichamp	74.0 ± 4.2	43.0 ± 2.8	76.5 ± 23.3	13.0 ± 5.0	49.5 ± 17.7	59.0 ± 8.5	ND
Sc3Y2	47.5 ± 4.9	D	12.5 ± 4.95	D	D	ND	ND
Sc3Y3	62.5 ± 24.7	25.5 ± 19.1	28.0 ± 1.4	7.5 ± 2.1	31.0 ± 1.4	53.5± 14.9	ND
Sc3Y4	66.0 ±1.4	36.0 ± 2.8	49.0 ± 4.2	21.0 ± 1.7	58.0 ± 0.01	73.5 ± 6.4	ND
Sc3Y5	52.5 ± 12.0	25.0 ± 14.1	123 ± 5.0	8.5 ± 0.7	15 ± 7.1	48.5± 16.3	ND
Sc3Y8	49.0 ± 4.2	D	16.0 ± 1.4	D	D	ND	ND
Scmosca3	59.5 ± 21.9	28.0 ± 0.0	187 ± 40.0	7.5 ± 5.0	77.0 ± 8.0	76.0 ± 9.0	ND/D
Sc3D6	92.0 ± 7.1	42.0 ± 1.4	32.0 ± 12.7	9.0 ± 2.8	35.5 ± 12.0	69.0 ± 7.1	215 ± 8
Sc3D5	78.0 ± 8.5	27.0 ± 1.4	35.5 ± 12.0	12.0 ± 4.2	83.0 ± 14.1	82.0 ± 8.5	205 ± 8
Sc3D4	112 ± 0.7	46.5 ± 2.1	42.5 ± 3.5	15.5 ± 5.0	74.5 ± 19.1	81.0 ± 8.5	0.0
Sc3D2	67.5 ± 9.2	32.0 ± 2.8	47.0 ± 9.9	10.0 ± 0.01	48.0 ± 1.4	55.0 ± 4.2	0.0
Sc4Y3	74.0 ± 1.4	30.5 ± 0.7	25.5 ± 16.3	13.5 ± 6.4	71.0 ± 18.4	85.5 ± 0.7	230 ± 8
Km4D3	60.5 ± 27.6	33 ± 0.0	1693 ± 574	D	2.0 ± 0.01	ND	ND
Km1D5	15.0 ± 1.4	1774 ± 588	2422 ± 186	D	5.5 ± 0.7	ND	ND
Km1Y9	79.5 ± 23.3	23.5 ± 19.0	2772 ± 743	D	2.0 ± 0.01	ND	ND
Td1AN9	75.5 ± 9.2	13.5 ± 0.7	2594 ± 395	D	D	ND	ND
Td1AN2	44.0 ± 21.2	ND	100 ± 13	D	3.0 ± 0.01	ND	ND
Td1AN1	31.0 ± 4.2	ND	5.0 ± 1.0	D	D	ND	ND
Pk4D6	11.0 ± 0.0	414 ± 136	4754 ± 821	D	2.0 ± 0.01	ND	ND
Pm1AN3	41.0 ± 5.7	ND	19.5 ± 3.5	D	D	ND	ND
Pg1Y12	15.5 ± 0.7	ND	1.0 ± 0.01	D	D	ND	ND
Cp1Y7	ND	353 ± 64	5211 ± 452	D	2.0 ± 0.01	ND	ND
Cl4Y4	45.5 ± 5.0	ND	13.0 ± 6.0	D	D	ND	ND
RmP12	21.0 ± 0.0	ND	± 9.8	D	3.0 ± 0.01	ND	ND
Zb3Y1	61.0 ± 2.8	12.0 ± 4.2	143 ± 36.1	D	D	ND	ND

ND: Not detected; D: concentration below the lower limit of the calibration curve. All data are the average of three different minibioreactor samples, and standard deviation is reported.

As can be seen in Table 2, all the yeast species were able to produce ethanol, with *S. cerevisiae* strains being the most productive, as expected, but some of the non-*Saccharomyces* strains were also able to produce above 45 g/L (Km1Y9, Td1AN9 and Zb3Y1) during this fermentation time. Regarding residual sugars, it is worth noting that, at the 96-h sampling time, fermentations with the *S. cerevisiae* 3D series had lower residual sugar concentrations, but, at the end of fermentation (360 h, data not shown), the fermentations with the 3Y series (except Sc3Y2, which was a high glycerol producer) had almost completely consume both sugars.

Besides some *S. cerevisiae* strains (Sc3Y3, Sc3Y4, Sc4Y3, Scmosca3 and Fermichamp), only strain Zb3Y1 was able to consume fructose almost completely (residual fructose below 2 g/L). However, it did not consume glucose completely (residual of 8.5 g/L) by the end of the fermentations (360 h, data not shown), and it also produced a high amount of glycerol as compared with the other non-*Saccharomyces* strains. The non-*Saccharomyces* strains, although less productive in terms of ethanol, were very interesting from the point of view that acetic acid was not produced, or it was but in very low quantities.

3.3. Volatile Productions of the Mezcal Yeast Strains in Medium M3

Concerning volatile metabolites produced on the semi-synthetic medium M3, marked differences were observed (Table 3) in the production levels of all volatile compounds tested in this work, even among strains of the same species, as observed for the *S. cerevisiae* group.

Most of the *S. cerevisiae* strains produced ethyl hexanoate, but this volatile was not detected in any of the non-*Saccharomyces* strains. Among all the strains (*Saccharomyces* and non-*Saccharomyces*) only three (Sc3D5, Sc3D6, and Sc4Y3) produced ethyl butyrate. Neither hexyl acetate nor 1-pentanol was

detected for any of the yeast strains under the conditions tested. Concerning phenyl ethyl acetate, it was produced in high amounts by the three strains belonging to *K. marxianus* (Km4D3, Km1D5, and Km1Y9) and also by strains Td1AN9 (*T. delbrueckii*), Pk4D6 (*Pichia kluyveri*), and Cp1Y7 (*Clavispora lusitaniae*). For the next stage on grape juice, we tested strains *Saccharomyces* and non-*Saccharomyces* individually and in mixed culture (co-culture). The three chosen *Saccharomyces cerevisiae* strains (Sc3Y3, Sc3Y4, and Sc3Y8, plus control strain Fermichamp) were previously selected based on their global stress tolerance [6]. These mezcal strains belong to tolerance group 1, meaning a high tolerance to ethanol, with or without a hexose present and also tolerant to an initial fructose concentration of 500 g/L. Nonnon-*Saccharomyces* strains were chosen based on their high ethanol production (above 45 g/L, Table 2) and relative fructophilic. According to this, strains Km1Y9, Td1AN9 were selected, and strain Zb3Y1 was also included due to its high ethanol production and for being the most fructophilic (Table 2) of all strains at the sampled time.

3.4. Fermentation Performance and Volatile Production of the Selected Yeasts, Individually and as Mixed Inoculum in Grape Juice Medium

Based on their carbon dioxide profiles, we choose a fixed time of 144 h of fermentation to compare the performance of all strains in grape juice medium, both in individually inoculated (Figure 1 and Table 4) and in mixed cultures (*Saccharomyces*/non-*Saccharomyces*, Table 5). From Figure 2, it is clear that strain Zb3Y1 is the best fructose consumer, but it leaves a high amount of glucose in the medium.

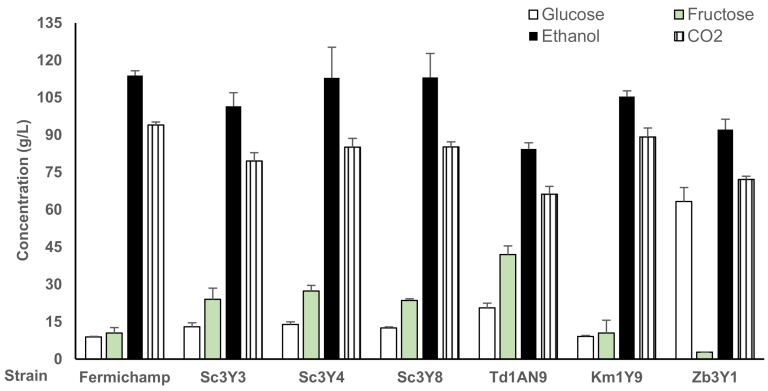

Figure 1. Hexose consumption and primary metabolite production for the selected *Saccharomyces* and non-*Saccharomyces* yeasts fermenting individually in grape juice medium at a sugar concentration of 137 g/L initial glucose and 119 g/L initial fructose at 144 h. Values are the average of six measurements, and standard deviations are presented as error bars.

Table 4. Biomass, acetic acid, and glycerol productions and productivity parameters for the selected *Saccharomyces* and non-*Saccharomyces* yeasts fermenting individually in grape juice medium at a sugar concentration of 137 g/L initial glucose and 119 g/L initial fructose, at 144 h.

Strain	Dry Weight	Acetic Acid	Glycerol	Fermentation Power (FP)	Fermentation Purity	$Y_{EtOH/S}$
	g/L			% *v*/*v*	$g_{acet.ac}$/FP	
Fermichamp	9.0 ± 0.3	0.2 ± 0.0	9.1 ± 0.2	14.4	0.014	0.48
Sc3Y3	6.2 ± 0.7	0.7 ± 0.2	10.2 ± 1.1	12.9	0.054	0.46
Sc3Y4	6.0 ± 0.2	0.8 ± 0.1	10.0 ± 0. 9	14.3	0.056	0.53
Sc3Y8	6.9 ± 0.4	0.8 ± 0.1	10.0 ± 0.8	14.3	0.056	0.51
Td1AN9	4.7 ± 0.2	0.5 ± 0.0	8.7 ± 0.4	10.7	0.047	0.44
Km1Y9	3.6 ± 0.3	0.7 ± 0.0	9.9 ± 0.0	13.4	0.052	0.45
Zb3Y1	6.3 ± 0.3	0.3 ± 0.0	7.8 ± 0.5	11.7	0.026	0.49

All data are the average of three different minibioreactor samples taken by duplicate, and standard deviation is reported. Fermentation power, purity, and ethanol yields are calculated using metabolites' average values.

Table 5. Sugar consumption and primary metabolite production for the selected yeasts, fermenting individually, or as mixed inoculum in grape juice medium (103 g/L initial glucose and 105 g/L initial fructose), at 144 h.

Individual or Mixed Inocula Sc/non-Sc (1:9)	Glucose	Fructose	Ethanol	CO_2	Glycerol	Ac. Acid	Dry Weight
	(g/L)						
Fermichamp	6.3 ± 0.09	5.6 ± 1.2	68.0 ± 1.9	99.4 ± 3.0	8.0 ± 0.2	0.5 ± 0.1	11.4 ± 0.2
Fcham/Td1AN9	3.4 ± 2.6	7.5 ± 2.7	82.2 ± 1.1	92.9 ± 1.6	7.9 ± 0.3	0.3 ± 0.1	8.6 ± 0.4
Fcham/Km1Y9	3.5 ± 2.7	6.8 ± 1.7	84.5 ± 2.3	94.5 ± 3.9	8.1 ± 0.2	0.4 ± 0.1	8.4 ± 0.3
Fcham/Zb3Y1	3.8 ± 3.0	7.8 ± 4.1	82.1 ± 1.0	91.8 ± 3.0	4.8 ± 1.0	1.2 ± 0.5	8.3 ± 0.6
Sc3Y3	7.1 ± 0.5	14.0 ± 2.0	66.0 ± 1.2	98.6 ± 2.5	8.0 ± 0.2	0.5 ± 0.0	7.6 ± 0.4
Sc3Y3/Td1AN9	9.2 ± 1.7	24.9 ± 4.7	75.7 ± 1.7	86.4 ± 2.5	8.4 ± 0.4	1.2 ± 0.2	5.9 ± 0.2
Sc3Y3/Km1Y9	11.4 ± 3.4	31.9 ± 7.1	73.4 ± 6.5	77.1 ± 2.0	7.6 ± 1.1	1.2 ± 0.4	5.4 ± 0.2
Sc3Y3/Zb3Y1	14.4 ± 6.0	17.9 ± 6.1	79.1 ± 3.9	84.4 ± 5.9	6.3 ± 2.0	1.2 ± 0.6	6.5 ± 0.6
Sc3Y4	8.1 ± 1.8	16.4 ± 5.9	67.0 ± 3.3	94.7 ± 5.4	8.4 ± 0.3	0.6 ± 0.1	7.5 ± 0.7
Sc3Y4/Td1AN9	11.3 ±2.3	30.0 ± 4.6	73.3 ± 1.7	78.9 ± 3.8	7.5 ± 0.1	0.5 ± 0.2	5.6 ± 0.3
Sc3Y4/Km1Y9	9.2 ± 3.5	25.0 ± 8.9	76.4 ± 3.9	81.3 ± 4.1	7.1 ± 0.4	0.8 ± 0.3	5.1 ± 0.3
Sc3Y4/Zb3Y1	20.8 ± 5.0	23.6 ± 5.1	71.8 ± 4.0	78.4 ± 5.8	5.0 ± 1.2	1.3 ± 0.8	5.2 ± 0.3
Sc3Y8	9.5 ± 2.8	19.5 ± 7.4	65.8 ± 2.7	1010± 6.6	7.3 ± 1.1	0.76 ± 0.3	7.0 ± 0.6
Sc3Y8/Td1AN9	15.4 ± 8.2	35.8 ± 14.3	70.0 ± 7.8	76.4 ± 11.1	7.8 ± 0.3	0.9 ± 0.4	5.6 ± 0.3
Sc3Y8/Km1Y9	9.8 ± 2.3	27.7 ± 6.0	75.9 ± 1.0	80.9 ± 5.6	7.4 ± 0.3	1.0 ± 0.3	5.5 ± 0.4
Sc3Y8/Zb3Y1	17.3 ± 5.2	23.0 ± 4.8	81.6 ± 7.0	79.6 ± 5.5	7.4 ± 1.0	0.7 ± 0.4	6.2 ± 0.6

Values are the average of three different minibioreactor samples taken by duplicate, and standard deviations are reported.

As seen in Table 4, fermentations on grape juice had high yields for all *Saccharomyces* and non-*Saccharomyces* strains.

Next, fermentation performances were compared for the mixed inocula experiments to verify the effect of the co-inoculation on productivity for individually fermenting and on mixed inocula fermentation (Table 5), to assess the effect of the yeasts' combinations. It is worth noting that the natural grape juice medium used in the experiments for Tables 4 and 5 were from different juice stocks; hence, the difference in the total sugar residual concentrations at the same sampling time. As reference, values of metabolite productions of the individual *S. cerevisiae* strains are included again in Table 5 to be compared with their mixed inocula fermentations.

An increase in ethanol production was observed when mixed inocula were used, with respect to the pure *S. cerevisiae* strain used, but, also, some of the mixtures increased their acetic acid production beyond acceptable quality levels (less than 1 g/L), specifically those including *S. cerevisiae* Sc3Y3, *K. marxianus* Km1Y9 or *Z. bailii* Zb3Y1 strains.

Due to its better overall aroma, lower ethanol production, and low acidity and higher fermentation purity, we decided to analyze the non-*Saccharomyces* strain Td1AN9in more detail. The kinetic behavior for primary metabolites (Figure 2) was assessed on mixed cultures with different *S. cerevisiae* strains having in common the Td1AN9 strain as the non-*Saccharomyces* couple. As it can be seen in Figure 2 for the whole fermentation profile for the mixed cultures using Td1AN9 as the non-*Saccharomyces* strain, there was a fast sugar consumption for the three mixed cultures tested, and maximal ethanol production was obtained between 120 and 144 h of fermentation.

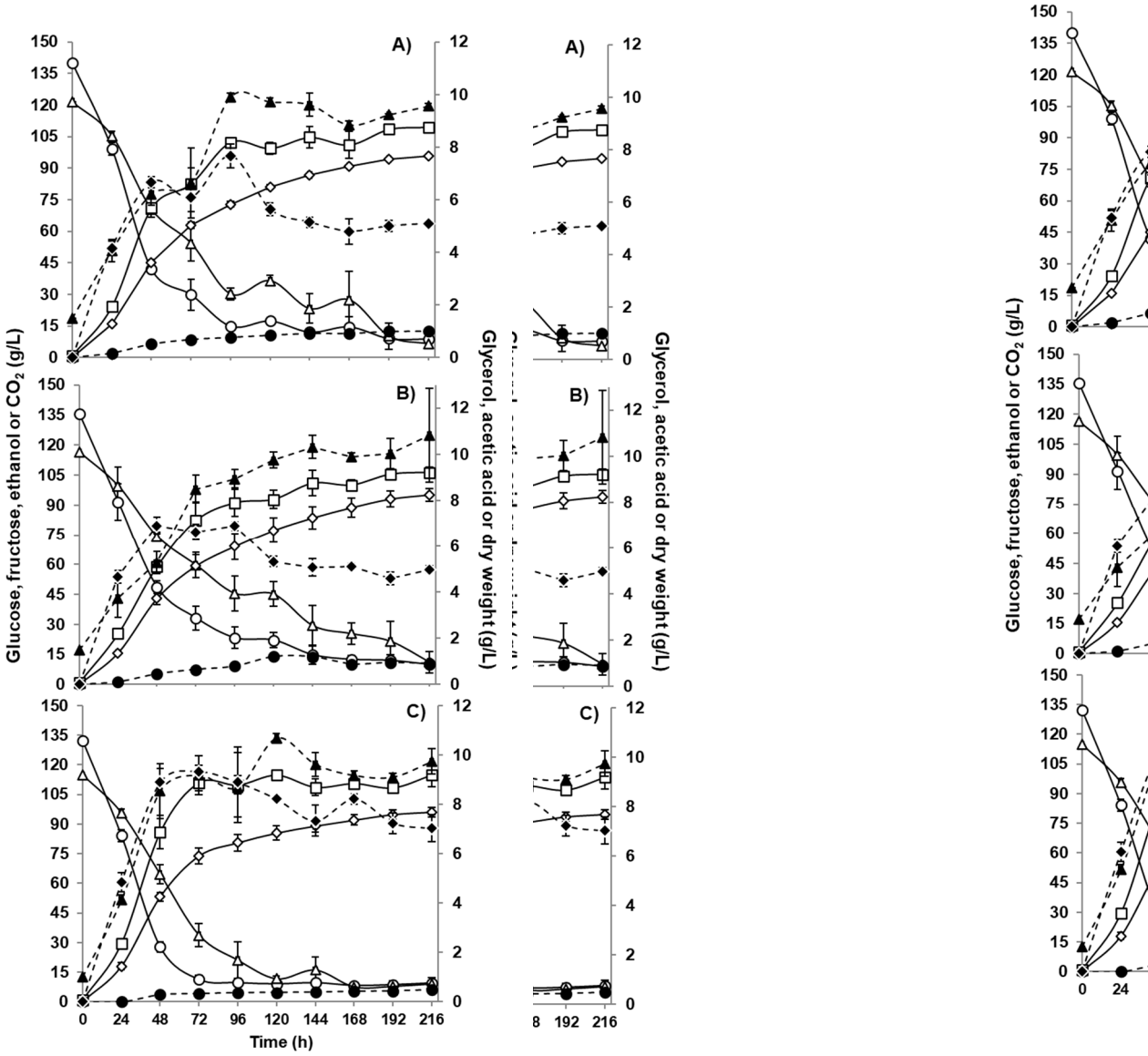

Figure 2. Fermentation kinetics on grape juice medium for the three *Saccharomyces cerevisiae*/*Torulaspora delbrueckii* (Td1AN9) mixed inoculum tested: (**A**) Sc3Y4/Td1AN9, (**B**) Sc3Y8/Td1AN9, and (**C**) Fermichamp/Td1AN9. Markers are average values of: (–○–) Glucose, (–△–) fructose, (–□–) ethanol, (–◇–) CO_2 released, (- -●- -) acetic acid, (- -▲- -) glycerol, and (- -◆- -) dry weight.

Growth characterized as global values of optical densities and viability showed that, mixed culture containing the control strain Fermichamp grew faster (Figure 3A), and this was due to a higher percentage of such *S. cerevisiae* strain thriving on the grape juice (Figure 3B). Although total population remained high (no lysed cells were observed under the microscope, without methylene blue), the viability rapidly declined after 72 h of culture when assessed by methylene blue staining. It is worth noticing that viability of *T. delbrueckii* at 144 h of grape juice fermentations was higher when combined with mezcal yeasts than with the control strain Fermichamp (Figure 3B, full markers), even considering that all three fermentations had the same amount of ethanol, around 100 g/L.

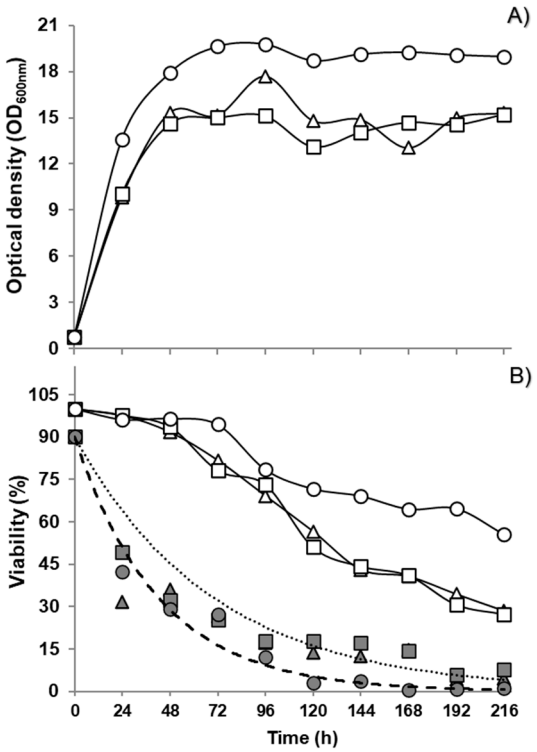

Figure 3. Culture growth on grape juice medium for the three *Saccharomyces cerevisiae/Torulaspora delbrueckii* mixed inoculum fermentations tested, expressed as: (**A**) Optical density at 600 nm, (−Δ−) Sc3Y4/Td1AN9, (−□−) Sc3Y8/Td1AN9; (−O−) Fermichamp/Td1AN9; and (**B**) Percentage of viability of the whole culture (open markers, determined by methylene blue staining) and that of *Torulaspora delbrueckii* as determined on WL Differential and WL Nutrient agar media, (····▲····) Sc3Y4/Td1AN9, (- - -■- - -) Sc3Y8/Td1AN9; (- - -●- - -) Fermichamp/Td1AN9. Exponential decay fittings shown with dotted and slashed lines. Standard deviations were always less than 10%; hence, the deviation bars are omitted in the graphs for clarity.

Population kinetics analysis (Figure 4) of the mixed inoculum fermentations showed that, for the first 24 h of fermentation the two populations grew rapidly and maintained a high population from 24 to 48 h of fermentation. The *S. cerevisiae* strains in general maintained their population at around 5×10^7 cells/mL until the end of the culture, whilst the population of the non-*Saccharomyces* strain Td1AN9 declined by around 90% (between 1 to 5×10^6 cell/mL). This strain was most affected by control strain Fermichamp (Figure 4C), but never completely disappearing. At the end of fermentation, the whole viability (*Saccharomyces* and non-*Saccharomyces*) of the culture remained at values around 50% as shown in Figure 3B. This meant that viable yeast population was comprised mainly by the *S. cerevisiae* strain inoculated, but, still, the non-*Saccharomyces* strains was present and metabolically active in high quantities.

Finally, volatile compounds produced by the mixed inocula fermentations in grape juice medium were strongly influenced by the two strains used (Table 6). The presence of the *S. cerevisiae* strains determined, mainly, the isoamyl alcohol, ethyl octanoate, and ethyl decanoate concentrations, whilst *T. delbrueckii* strain 1AN9 determined most of the phenyl ethyl acetate concentrations. For the ethyl hexanoate, it was the average contribution of both strains. Although Td1AN9 is capable, as individual inoculum, to produce isoamyl acetate, only its combination with control strain Fermichamp (which also produces isoamyl acetates individually) produced this compound, but its production by Td1AN9 was inhibited when mixed with the *S. cerevisiae* strains from mezcal. For the mixed culture with Sc3Y4 and Sc3Y8, isoamyl acetate concentrations were present but below the detection limit. In general terms, when using mixed inocula, we observed an increase on isoamyl alcohol levels as compared to pure

inoculum of *S. cerevisiae* strains, as well as a decrease in phenyl ethyl acetate concentration in the fermented product, as compared to the use of the non-*Saccharomyces* strain as pure inoculum.

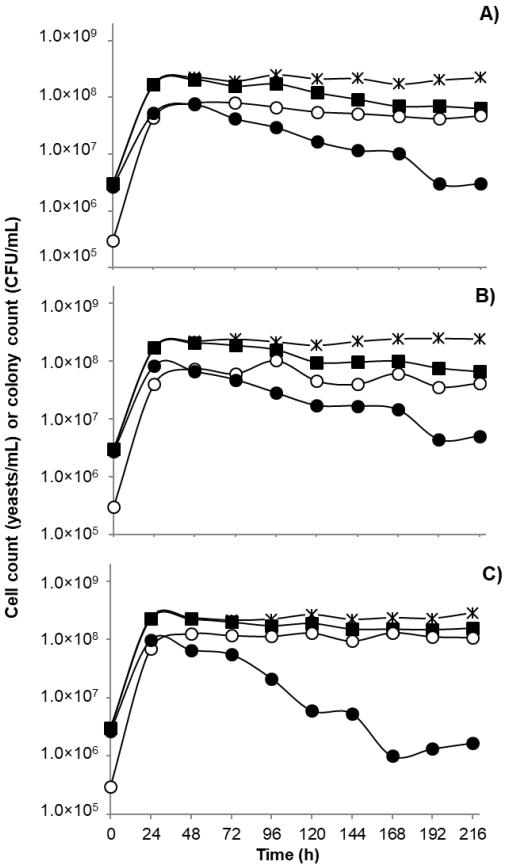

Figure 4. Population kinetics on grape juice medium for the three *Saccharomyces cerevisiae/Torulaspora delbrueckii* mixed inoculum tested: (**A**) Sc3Y4/Td1AN9, (**B**) Sc3Y8/Td1AN9 and (**C**) Fermichamp/Td1AN9; Markers show: (−Ж−) total cells count, (−■−) viable cell count, both determined by a Neubauer chamber without or with methylene blue, respectively; (−O−) *S. cerevisiae* colonies count, and (−●−) *T. delbrueckii* colonies count, determined by solid (WL Differential and WL Nutrient) media. As data is presented in logarithmic scale, standard deviation bars are masked by marker size.

Table 6. Main volatile compounds profiles of the selected *Saccharomyces* and non-*Saccharomyces* yeasts fermenting individually (upper panel) or as a mixed culture (bottom panel) in grape juice medium at a sugar concentration of 137 g/L initial glucose and 119 g/L initial fructose, data at 144 h of culture.

Strain	Isoamyl Alcohol	Isoamyl Acetate	Phenyl Ethyl Acetate	Ethyl Decanoate	Ethyl Octanoate	Ethyl Hexanoate
	(mg/L)			(μg/L)		
Fermichamp	174 ± 5.0	111 ± 9.2	133 ± 14.1	133 ± 2.1	61 ± 4.9	40 ± 2.1
Sc3Y3	77 ± 4.5	D	70 ± 16.5	140 ± 5.3	64 ± 4.2	38 ± 3.5
Sc3Y4	78 ± 0.6	D	63 ± 5.7	103 ± 9.3	63 ± 2.1	33 ± 2.3
Sc3Y8	82 ± 4.0	D	82 ± 20.5	115 ± 27.6	63 ± 2.7	45 ± 10.4
Km1Y9	131 ± 6.0	30 ± 11.2	5069 ± 291.2	39 ± 3.6	38 ± 1.0	D
Td1AN9	143 ± 90	45 ± 14.2	3349 ± 166.8	32 ± 0.6	D	16 ± 1.5
Zb3Y1	213 ± 200	D	139 ± 9.1	32 ± 2.1	34 ± 0.0	15 ± 0.0
Fcham/Td1AN9	141 ± 18.8	55 ± 9.9	543 ± 92.4	105 ± 16.5	41 ± 8.3	23 ± 3.5
Sc3Y4/Td1AN9	93 ± 3.2	D	475 ± 34.9	84 ± 11.5	37 ± 8.5	33 ± 7.0
Sc3Y8/Td1AN9	85 ± 9.1	D	544 ± 8.0	102 ± 16.5	33 ± 4.0	26 ± 2.3

4. Discussion

One of the main objectives of this work was to compare the capabilities of production of aromatic volatile (flavor) compounds of the different mezcal strains when fermenting on a wine-type synthetic medium (M3), and in real grape juice, also, with a single strain inoculum or co-inoculated in a mixed fashion. Semi-synthetic medium M3 allowed us to compare the individual productive behavior of all the 24 strains. We found that ethanol was produced by all the strains, being maximal (but variable amongst strains) for *S. cerevisiae* as expected, but it also was produced in good quantities by some of the non-*Saccharomyces* strains belonging to *Kluyveromyces*, *Torulaspora*, and *Zygosaccharomyces* genera, which made them candidates to be tested in the grape juice medium as part of mixed inoculum with different *S. cerevisiae* strains, in terms of their displayed natural tolerance to this alcohol.

Concerning specifically to *S. cerevisiae* strains, Camarasa et al. [11] analyzed in a high glucose (240 g/L) synthetic medium the phenotypic variability, including the production of aromatic compounds, of a collection of 72 *S. cerevisiae* strains obtained from seven different ecological niches: bakery, laboratory, natural isolates (plants and soil), clinical isolates, fermentative processes (beer, sake, palm wine), vineyard, and commercial wine. They observed that the larger differences amongst the strains are in their biomass production and formation of by-products but, interestingly, not in their ethanol production levels, different to what was observed in this work (Table 2), specifically for ethanol. These authors concluded that commercial wine strains are characterized by high biomass concentration and good fermentative performance, low acetate production, and low ethyl butyrate synthesis. More recently, and similar to the work presented here for *S. cerevisiae* mezcal strains, Franco-Duarte et al. [12] established that, for their 24 *S. cerevisiae* strains, ethanol and organic acids (in particular acetic acid) concentration explained most of the metabolic differences among strains. The *S. cerevisiae* strains studied in more detail here produce comparable amounts of ethanol as the commercial strain Fermichamp, and the selected strains also led to high glycerol levels and were able to almost completely consume glucose and fructose during fermentation. In general, primary metabolites were produced in higher amounts in the grape juice medium than in the semi-synthetic medium M3.

For the mixed inocula fermentations, we observed an increased glycerol and acetic acid productions in the mixed cultures as compared with data obtained in pure cultures as reported by Reference [13]. In mixed cultures, the *S. cerevisiae* strains and *T. delbrueckii* 1AN9 reached their maximal populations at 24 h, similar to what was also reported by Reference [13]. The comparison between the cell concentrations obtained in both nutrient WL (non-selective) and differential WL (no growth of *S. cerevisiae*) agar media clearly shows that at the beginning of the fermentation process the majority of the population belongs to the non-*Saccharomyces* strain, as inoculated in higher amounts, but as time proceeds, *S. cerevisiae* becomes dominant up to the end of fermentation, similar to that reported by Reference [13]. However, unlike these authors, who report a low percentage (<1%) of viability for their non-*Saccharomyces* strains (*C. zemplinina* and *H. uvarum*), our strain Td1AN9 have a viability between 10 and 15% at the end of fermentation, and it is most affected by co-inoculation with control strain Fermichamp, being not due to ethanol concentration, as it was similar in all inoculum combinations. We observed the same phenomenon of a major inhibition due to the presence of control strain Fermichamp for the *K. marxianus* and *Z. bailli* strains (data not shown).

Overall, at the conditions tested in this work, the presence of a *S. cerevisiae* strain reduces the growth capacity of Td1AN9 when it is mixed from 48 h of culture. At this time, the concentration of ethanol is around 65 g/L (Figure 2), which is lower to the maximum ethanol production capability by the pure Td1AN9 inoculum fermentation (Figure 1) and where the cell viable count is the same as the individual *S. cerevisiae* strains, around 1×10^8 cells/mL. Hence, we cannot attribute solely to ethanol the inhibition/damaging effect over *T. delbrueckii* cells at this time, although we know that concentrations above 8% ethanol are stressing on solid media as previously determined for this strain in YPD [6], and that the *S. cerevisiae* strain is taking advantage of the cellular contents leaked by the non-*Saccharomyces* strain. This is in contrast to what has been reported for *H. guilliermondii* [14]. These authors tested not only different ratio of species in the mixed inocula but also aerobic conditions and different media

and *S. cerevisiae* strains (data not shown by the authors) and concluded that inhibition and death of their non-*Saccharomyces* strain was due to some unknown compound present and accumulated in the supernatant of *S. cerevisiae* cultures, different than the killer toxins already reported for *S. cerevisiae*. Similarly, it was reported that the main inhibitory mechanism towards their *K. thermotolerans* and *T. delbrueckii* strains was the physical presence of *S. cerevisiae* cells [15]. Our results seem to support this latter explanation, although it is clear that such effect most probably is species-specific, as in the work of Kosel et al. [16]. These authors did not find any effect of the cell-to-cell contact of the commercial *S. cerevisiae* EC1118 over the growth kinetics of the cultures of *Dekkera bruxellensis*, which is a spoilage yeast of low growth and a low tolerance to high sugar concentration, as well a weak producer of ethanol. All this indicates that more work is needed to clarify the specific properties of *S. cerevisiae* with *T. delbrueckii* co-cultures.

Concerning volatile metabolites production, we found that different esters and higher alcohols were produced, and there were differences between pure and mixed cultures, being strain dependent as observed also by Reference [17] and, in general, being higher for the mixed rather than for the individually inoculated fermentations. Pure cultures of *S. cerevisiae* strains showed the highest total ester content, except for phenyl ethyl acetate, as compared to the non-*Saccharomyces* strain Td1AN9. Maturano et al. [17] obtained, for phenyl ethyl acetate, values from 30 to a maximum of 310 μg/L for the varieties of wine analyzed, while, in the results presented here, the non-*Saccharomyces* yeasts showed values ranging from 130 to 5069 μg/L and, when used a mixed inocula, values were high, ranging from 450 to 540 μg/L, higher than those reported by Reference [17,18]. This is a positive feature due to the great importance of this compound for its very pleasant floral aroma. As reported by Reference [1], which also worked in wine-type synthetic medium, the production of certain volatile compounds that influence wine aroma was strain-dependent, and they observed that the concentrations of the measured compounds (except acetic acid) varied significantly in function of the inoculated strain, to the point that it permitted their identification, concluding that the combined use of two or more yeast strains or species is an interesting alternative for improving wine quality [19]. In the work of Kosel et al. [16], the non-contact (cultures separated by a membrane) co-culture of *S. cerevisiae* with *D. bruxellensis* in a synthetic wine must resulted in a higher production of aromatic ethyl ester compounds, as compared with the pure cultures of the two yeasts. These authors propose a hybrid computational pheno-metabolomic approach to classify and select those *S. cerevisiae* strains with an increased performance on wine making, correlating this selection with a good growth on cycloheximide, on iprodion, and a temperature of 18 °C, the presence of two homozygous alleles (ScAAT6-256 and ScAAT5-256), and a high production of 2-phenylethyl acetate, ethyl butanoate, ethyl hexanoate, and ethyl octanoate.

The co-inoculation of Td1AN9 with any of the two *S. cerevisiae* mezcal strains prevented/inhibited the production of isoamyl acetate but not when mixed with control strain Fermichamp, which is also capable of producing it. This may indicate a level of recognition and/or compatibility amongst mezcal strains, *Saccharomyces* and non-*Saccharomyces*, as this compound (and isoamyl alcohol) has been recently reported as possessing a wide antimicrobial feature when present in a fermented (sake) beverage [20], but also evidenced here by the relatively higher viability of the Td1AN9 strains at the end of the fermentation, when in presence of the two *S. cerevisiae* mezcal strains, but not with control, wine strain Fermichamp. Overall, isoamyl alcohol concentrations varied between *S. cerevisiae* yeasts as reported in other studies in wine [17,18] and other fermented beverages [20].

In winemaking, the use of pure yeast cultures allows a better control of the fermentations; however, it can also reduce the production of some desired metabolites, both from the yeast's metabolism itself and from transformation of precursors present in the grape must. For this purpose, it is increasingly seen more convenient to use different yeast genera and species, which can contribute or influence the chemical composition and the flavor of wines [4,5,12,13,17,19,21]. The volatile compounds produced by the strains analyzed in this study are of great aromatic value, especially the production of ethyl hexanoate and ethyl octanoate (apple note), isoamyl acetate (banana note), and phenyl ethyl acetate

(fruity, floral notes), compounds which could render (in the appropriate amounts) good organoleptic characteristics to a wine.

5. Conclusions

The main aim of this work was to assess the technological feasibility of using mixed inoculum of yeasts, originally isolated from mezcal, to be used in wine-type grape juice fermentations. We observed that some of the mezcal yeast strains were competitive in terms of primary metabolites and volatile compounds production. Fermentation performance comparisons in a semi-synthetic medium allowed us to choose those strains, *Saccharomyces* and non-*Saccharomyces*, with complementary metabolic characteristics to be tested in a real, wine-type fermentation at a small scale and to propose a mixed inoculum prepared with a *S. cerevisiae* and a *T. delbrueckii* mezcal strains to obtain balanced aromatic profiles in a model wine product.

Author Contributions: Conceptualization, C.P.L-C. and P.T; methodology, F.J.D.l.T-G., P.T. and C.P.L-C.; formal analysis, J.A.N-Z and C.P.L-C.; investigation, F.J.D.l.T-G. and C.P.L-C.; resources, C.P.L-C., J.A.N-Z and P.T.; data curation, J.A.N-Z. and C.P.L-C.; writing—Original draft preparation, F.J.D.l.T-G.; writing—Review and editing, J.A.N-Z and C.P.L-C.; visualization, J.A.N-Z. and C.P.L-C.; supervision, C.P.L-C.; project administration, C.P.L-C.; funding acquisition, C.P.L-C., J.A.N-Z and P.T. All authors have read and agreed to the published version of the manuscript.

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