

Article

The Nutritional Quality of the Culture Medium Influences the Survival of Non-*Saccharomyces* Yeasts Co-Cultured with *Saccharomyces cerevisiae*

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Abstract: Yeast-yeast interactions have been studied mainly using wine yeasts. However, studies are rare for native yeasts from agave juice fermentation. Therefore, this work used strains isolated from the alcoholic fermentation of agave to study the survival of non-*Saccharomyces* yeasts co-cultivated with *Saccharomyces cerevisiae* in media of different nutritional qualities. First, the feasibility of using simple and low-cost culture media was demonstrated to study the interactions between *Saccharomyces cerevisiae* and non-*Saccharomyces* yeasts. The results presented here demonstrated the antagonistic effect exerted by *S. cerevisiae* on *Torulaspora delbrueckii*, which showed a more significant loss of viability. However, the nutritional composition of the culture medium also influences this effect. It was clear that a nutritionally rich medium improved the survival of non-*Saccharomyces* yeasts. Lastly, the change in the survival of non-*Saccharomyces* yeasts also entails a variation in the concentration and diversity of minor volatile compounds produced during fermentation. This was observed in the variety and relative abundance of compounds belonging to the most numerous chemical families, such as alcohols, esters, and terpenes.

Keywords: *Torulaspora delbrueckii*; *Zygosaccharomyces bailii*; yeast-yeast antagonism



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1. Introduction

The agave fermentation process varies depending on the geographical area where the mezcal is made [1], by different production methods, and by diverse yeast populations in the fermentations. Gallegos-Casillas et al. [2] analyzed the diversity of yeasts from different geographical areas of Mexico, concluding that yeast diversity is maintained throughout fermentation. Still, some taxa change their abundance over time. This agrees with previous reports on the possibility of finding different species of non-*Saccharomyces* yeasts in low proportions at the end of agave fermentation, such as *Kluyveromyces marxianus*, *Torulaspora delbrueckii*, *Candida diversa*, *Pichia kluyveri*, and *Zygosaccharomyces bailii* [3–5].

Torulaspora delbrueckii's impact on fermented product quality has been widely studied [6]. It has also been observed that the interaction between *T. delbrueckii* and *Saccharomyces cerevisiae* increases the content of aromatic compounds and reduces acetic acid concentration [7], thus enhancing the quality of the fermented products. This interaction also decreases the production of higher alcohols and fatty acids [8]. On the other hand, *Z. bailii* is a yeast known for its resistance to ethanol and low pH, which is why it has been considered a spoilage yeast [9].

The diversity of yeast during fermentation causes the fermented product to have unique sensory qualities. Therefore, it is vital to understand how the yeast community works and how they interact with each other. The interaction between yeasts has been observed to affect the volatile composition of different beverages, such as wines [10] and

cider [11]. However, it is expected to observe that non-*Saccharomyces* yeast populations decline in the first days of fermentation [12].

The dominance of *S. cerevisiae* over non-*Saccharomyces* yeasts has been observed, regardless of the amount of inoculum used [13]. However, interactions between yeasts can affect their dominance during fermentation [14]. In turn, the dominance of one species over another is closely related to the consumption of nutrients as well as the production of compounds with antimicrobial effects, such as killer toxins, short-chain fatty acids, antimicrobial peptides, and even ethanol itself [14–16].

On the other hand, nutrient limitation during fermentation can affect interactions between yeasts. It has been widely studied under nitrogen limitation, particularly in stuck fermentation [17–21]. It has been observed that nitrogen supplementation is not sufficient to avoid stuck fermentation [22,23]. However, it has been observed that the addition of vitamins and trace elements can affect reactivating stuck fermentation and the dominance of the yeasts involved [23–25].

Considering that little is known about how the native yeasts from the alcoholic fermentation of agave juice interact with each other, this work used yeasts isolated from this origin to investigate the survival of non-*Saccharomyces* yeasts co-cultured with *Saccharomyces cerevisiae* in media of different nutritional qualities. Likewise, we evaluated the effect of changes in yeast populations on the production of minor volatile compounds.

2. Materials and Methods

2.1. Yeasts Strains

This work used two non-*Saccharomyces* strains (*Torulaspora delbrueckii* and *Zygosaccharomyces bailii*) and one *Saccharomyces cerevisiae* strain. The three strains were isolated from alcoholic fermentation of agave to produce mezcal in Durango, Mexico, and were identified by restriction fragment length polymorphisms, as reported previously [4]. Then, the strains were incorporated into the yeast collection of the Microbial Biotechnology Laboratory at the Technological Institute of Durango and preserved in 30% glycerol at $-20\text{ }^{\circ}\text{C}$. Once reactivated, yeast cells were maintained at $4\text{ }^{\circ}\text{C}$ on YPD agar plates containing, per liter: yeast extract, 10 g; peptone, 20 g; dextrose, 20 g; and agar, 15 g. The starter cultures were prepared from an isolated colony transferred from the agar plate to 50 mL of liquid YPD medium containing, per liter: yeast extract, 10 g; peptone, 20 g; dextrose, 20 g. Incubations were carried out overnight at $28\text{ }^{\circ}\text{C}$ with shaking at 150 rpm. Then, the cells were taken to inoculate the cultures described below.

2.2. Yeast Growth and Viability Monitoring

Yeast growth was monitored by cell counting in a Neubauer chamber, and the samples were diluted appropriately in an isotonic saline solution (0.9% *w/v*). Yeast viability was determined by plate counting using the differential medium Wallerstein Laboratory Nutrient agar (WLN, Sigma-Aldrich, St. Louis, MO, USA) and MGYP medium supplemented with two inhibitory compounds. The MGYP medium contained, per liter, dextrose, 10 g; casein peptone, 5 g; yeast extract, 3 g; malt extract, 3 g; agar, 20 g. It was separately supplemented with the two inhibitory compounds. Three crystal violet (CV) concentrations were evaluated: 0.0001%, 0.0005%, and 0.001%. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ was added to the medium at 0.63 g/L. The MGYP medium without inhibitor was used as a control. All media were incubated at $28\text{ }^{\circ}\text{C}$, and counting was performed after 24 h for the MGYP+ CuSO_4 medium and after 48 h for the MGYP+CV and WLN media. All plates were monitored every 24 h for seven days to evaluate changes.

2.3. Culture Medium and Fermentation Kinetics

Mono- and co-cultures were carried out in 250 mL Erlenmeyer flasks containing 150 mL of the medium. The first culture medium was semisynthetic medium M2, designed to simulate the basic composition of diluted agave must [26,27]. This medium contained per liter: glucose, 10 g; fructose, 90 g; yeast extract, 1 g; $(\text{NH}_4)_2\text{SO}_4$, 2 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 g;

KH_2PO_4 , 5 g. The sugars were separately sterilized in an autoclave. The pH of the medium was adjusted to 5. The second culture medium was the YPD medium modified to contain a fructose/glucose ratio similar to that found in cooked agave juice (9/1) and named YPDA. It contained the following per liter: glucose, 10 g; fructose, 90 g; yeast extract, 10 g; and casein peptone, 20 g. Monocultures of each yeast were carried out by inoculating 1×10^6 cells/mL. At the same time, binary co-cultures were initiated using 5×10^5 cells/mL of each yeast. Binary co-cultures were labeled as follows: *S. cerevisiae* with *T. delbrueckii*, Sc/Td; *S. cerevisiae* with *Z. bailii*, Sc/Zb; and *T. delbrueckii* with *Z. bailii*, Td/Zb. Finally, ternary co-cultures were initiated using 3×10^5 cells/mL of each yeast strain. All fermentations were carried out in triplicate. Samples of culture medium (1 mL) were taken every three hours from each fermentation, which were analyzed in duplicate to determine yeast growth and the concentration of fructose, glucose, and ethanol. Samples were centrifuged at $6720 \times g$ for 3 min, followed by filtration through a Nylon membrane (0.45 μm), and stored at -20°C until analysis. The concentrations of residual sugars (glucose and fructose) and ethanol were determined by high-performance liquid chromatography (HPLC). Finally, volatile compounds were identified and semi-quantified after 24 h of fermentation using headspace solid-phase microextraction (HS-SPME) followed by gas chromatography/mass spectrometry (GC/MS) analysis.

2.4. Glucose, Fructose, and Ethanol Quantification

The glucose, fructose, and ethanol concentrations were determined using an Agilent 1200 series HPLC system (Agilent, Santa Clara, CA, USA) equipped with a refractive index detector. The samples were filtered through 0.22 μm nitrocellulose membranes, and 1 μL of the solution was injected into the system. The isocratic separation was performed at 70°C on a Rezex ROA-Organic Acid H^+ (8%) ion exclusion column (7.8 mm $\times$ 300 mm, Phenomenex, Torrance, CA, USA). The mobile phase was 5 mM H_2SO_4 solution at a 0.6 mL/min flow rate. Quantification was performed using an external calibration curve.

2.5. Identification and Semi-Quantification of Minor Volatile Compounds

HS-SPME was performed followed by GC/MS analysis. The filtered sample (5 mL) was placed in a microextraction flask, and 1.5 g of NaCl was added. 1-Pentanol was used as an internal standard (300 mg/L) to semi-quantify the minor volatile compounds. The vial was incubated in a water bath at 35°C for 5 min, and then the SPME fiber (DVB/CAR/PDMS 50/30 μm , Supelco, St. Louis, MO, USA) was exposed in the headspace for one hour. After that time, the fiber was exposed to the injection port of an Agilent 7890A gas chromatograph for 10 min at 250°C for the thermal desorption of volatile compounds. The injector worked in splitless mode using a liner for SPME (0.75 mm id, Supelco). The components were separated on an FFAP column (30 m $\times$ 0.32 mm $\times$ 0.25 μm). Ultra-high-purity helium was used as the carrier gas at a 1 mL/min flow rate. The oven temperature was set to 40°C for 3 min and then increased to 52°C at $3^\circ\text{C}/\text{min}$. After 1 min at 52°C , oven temperature increased to $10^\circ\text{C}/\text{min}$ until it reached 200°C . Finally, the temperature was maintained at 200°C for 15 min. The mass spectrometry detector (Agilent 5975C) was operated at 230°C , an ionization voltage of -70 eV , and SCAN mode (1.6 scans per second). The compounds were identified using the NIST 2011 mass spectral library with a $\geq 80\%$ match and the retention indices were calculated using an alkane series (C7–C30).

The concentration of the minor volatile compounds was determined as described by Kokoti et al. [28] using the equation:

$$C_x = C_i * \frac{A_x}{A_i}$$

C_x is the concentration (mg/L) of the unknown compound, C_i is the concentration of the internal standard (mg/L), A_x is the peak area of the unknown compound, and A_i is the peak area of the internal standard. The results obtained from this equation are semi-quantifications based on the mass of the internal standard and are not absolute

quantities. Then, each compound is expressed as a mass equivalent to 1 µg/L of the internal standard (1-Pentanol). However, this allows us to establish statistical differences between compounds under different experimental conditions.

2.6. Statistical Analysis

One-way analysis of variance ($p < 0.05$) with Tukey's test was used to compare means between the different fermentations using the static Minitab software, version 20.3 (Minitab Inc., State College, PA, USA). The plots were made using the SigmaPlot statistical software, version 15 (Grafiti LLC, Palo Alto, CA, USA). A heat map with clustering was adopted to analyze the variations between different fermentations using the TBtools software [29].

3. Results and Discussion

3.1. Monitoring Culture Medium Selection

Cultures of two or more microorganisms require specific methodologies to differentiate one species from the other. For this, techniques such as flow cytometry [30–32] and procedures based on molecular biology [33] have been used. However, an alternative is plate counting using differential and selective media. This work used Wallerstein agar and two media based on MGY agar.

The antimicrobial properties of crystal violet have been known for a long time [34], but nowadays, it is mainly used in the textile industry [35]. Its use has been suggested to avoid cross-contamination of beers [36] and control yeasts associated with food production [37]. This work tested the usefulness of crystal violet for the recovery of *S. cerevisiae* in co-culture with non-Saccharomyces yeasts. The concentrations of 0.001% and 0.0005% completely inhibited the growth of *T. delbrueckii* and *Z. bailii*. In comparison, a concentration of 0.0001% caused an inhibition of close to 96% in both yeasts. Otherwise, all three concentrations allow the development of *S. cerevisiae*. At a concentration of 0.0001%, 100% of *S. cerevisiae* grows, the concentration of 0.0005% causes inhibition of 2.5%, and, finally, a concentration of 0.001% inhibits the growth of *S. cerevisiae* by 38%.

On the other hand, the selective medium MGY+CuSO₄ completely inhibits the development of *S. cerevisiae*, and the recovery of non-Saccharomyces yeasts varies depending on the species. For *Z. bailii*, an inhibition of 6.7% was observed, and *T. delbrueckii* showed an inhibition of 44.8%. Moreover, in addition to inhibiting *S. cerevisiae*, this medium allows the differentiation of the other two yeasts. The *T. delbrueckii* colonies presented a phase coloration in the colony's center, while *Z. bailii* grows as white colonies. Figure 1 shows the growth of *S. cerevisiae* and *T. delbrueckii* in co-culture in the M2 medium, differentiating both yeasts using the MGY+CuSO₄, MGY+CV, and WLN media.

Figure 1 shows that the MGY+CuSO₄ medium exhibited slightly lower viable cell counts of *T. delbrueckii* compared to the WLN medium. Nonetheless, it is evident that MGY+CV and MGY+CuSO₄ are two low-cost media compared with the WLN medium for the appropriate differentiation of the yeasts tested here. Consequently, they allow the individual monitoring of each population's development during the co-culture of these three yeasts. Regarding the interaction between the yeasts, *T. delbrueckii* coexists without problems with *S. cerevisiae* during the first 12 h of culture. However, co-culture likely triggers a response from *S. cerevisiae* that cannot tolerate *T. delbrueckii* since, in the following 12 h, the latter's viability decreases significantly. This agrees with what Tronchoni et al. [38] reported. These results led to the selection of MGY+CV and MGY+CuSO₄ media to survey the binary and ternary mixed cultures.

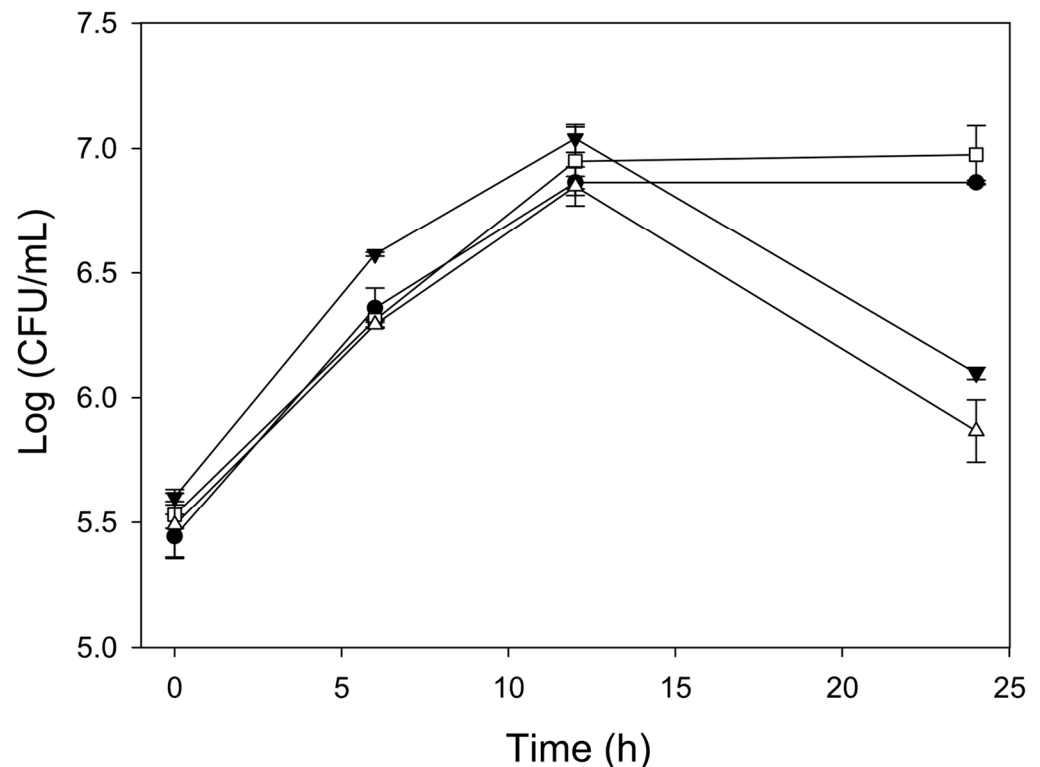


Figure 1. Growth kinetics of *S. cerevisiae* and *T. delbrueckii* co-cultured in M2 medium, differentiating both yeasts using MGYT+CuSO₄, MGYT+CV, and WLN media. Viable count of *S. cerevisiae* in MGYT+CV medium (□) and WLN medium (●). Viable count of *T. delbrueckii* in MGYT+CuSO₄ (△) and in WLN medium (▼). The data are shown as an average of three fermentations. Vertical bars represent standard deviations.

3.2. Fermentation Kinetics

Figure 2 shows the growth kinetics of the binary and ternary co-cultures performed in the YPDA medium. The graphs in Figure 2 show the data for the biomass produced expressed in logarithmic growth cycles. *Saccharomyces cerevisiae* reached the stationary phase after 15 h of incubation (Figure 1). Figure 2A also shows that *T. delbrueckii* grew well during this period. However, from 15 to 24 h, there was a slight but evident decrease in cell viability. Instead, although the growth of *Z. bailii* was slightly lower than that of *S. cerevisiae*, both yeasts did not show difficulties in coexisting and reaching the stationary phase (Figure 2B).

Like in the Sc/Zb co-culture, during the Td/Zb co-culture, a good coexistence of *T. delbrueckii* and *Z. bailii* occurred (Figure 2C). *Zygosaccharomyces bailii* grew very similarly in this co-culture and when co-cultured with *S. cerevisiae*. However, *T. delbrueckii* showed more robust growth in this co-culture than when coexisting with *S. cerevisiae*. In the ternary co-culture (Figure 2D), the three yeasts grew well and reached the stationary phase. *Saccharomyces cerevisiae* and *Z. bailii* did not show differences in their growth in the other co-cultures. Still, it is noticeable that *T. delbrueckii* did not show the slight loss of viability that was observed in the Sc/Td co-culture. It has been previously reported that the loss of viability depends on the yeast strain used [39].

Table 1 presents some fermentation parameters to complete the analysis of yeast behavior during the cultures. The specific growth rate (μ) values were obtained by fitting the logistic equation to the growth data using the tool Solver in Excel 365 (Microsoft Corporation, Redmond, WA, USA), as reported previously [40]. The parameter μ did not show significant differences ($p > 0.05$) among the cultures carried out, which agrees with the three yeasts' similarities during the exponential growth phase in all cultures. It was also observed that almost all fermentations consumed the total sugars present in the YPDA

medium after 24 h of incubation, with the only exception of the *Z. bailii* monoculture. This agrees with the fact that *Z. bailii* showed the slowest growth, even among the monocultures of the three yeasts (Figure S1C).

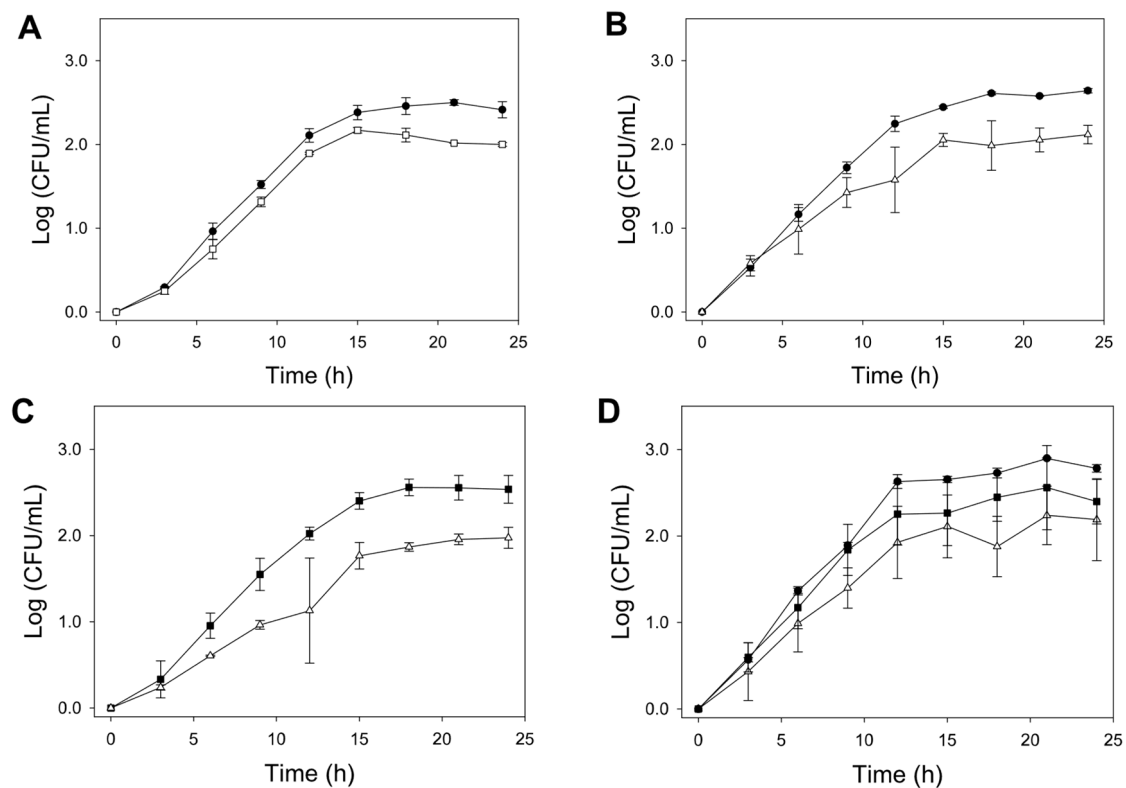


Figure 2. Growth kinetics of co-cultures in YPDA medium. Viable cells of *S. cerevisiae* (●) and *T. delbrueckii* (□) during the Sc/Td co-culture (A). Viable cells of *S. cerevisiae* (●) and *Z. bailii* (△) during the Sc/Zb co-culture (B). Viable cells of *T. delbrueckii* (■) and *Z. bailii* (△) during the Td/Zb co-culture (C). Viable cells of *S. cerevisiae* (●), *T. delbrueckii* (■), and *Z. bailii* (△) during the ternary co-culture (D). The data is shown as an average of three fermentations. Vertical bars represent standard deviations.

Table 1. Fermentation parameters of mono- and co-cultures in YPDA medium.

		μ (h ⁻¹)	Residual Fructose (g/L)	Residual Glucose (g/L)	Ethanol Content (g/L)	$Y_{P/S}$ (g/g)
Monoculture	Sc	0.59 ± 0.14 a	0.00 ± 0.00 b	0.00 ± 0.00 b	35.70 ± 0.67 b	0.357 ± 0.007 b
	Td	0.34 ± 0.05 a	0.00 ± 0.00 b	0.00 ± 0.00 b	35.77 ± 0.58 b	0.358 ± 0.006 b
	Zb	0.26 ± 0.09 a	6.74 ± 0.43 a	4.19 ± 0.36 a	25.42 ± 1.56 c	0.285 ± 0.018 c
Co-culture Sc/Td	Sc	0.59 ± 0.01 a	0.00 ± 0.00 b	0.00 ± 0.00 b	38.98 ± 2.30 ab	0.39 ± 0.023 ab
	Td	0.57 ± 0.02 a				
Co-culture Sc/Zb	Sc	0.39 ± 0.02 a	0.00 ± 0.00 b	0.00 ± 0.00 b	39.97 ± 0.67 ab	0.40 ± 0.007 ab
	Zb	0.41 ± 0.06 a				
Co-culture Td/Zb	Td	0.48 ± 0.02 a	0.00 ± 0.00 b	0.00 ± 0.00 b	43.03 ± 3.43 a	0.43 ± 0.034 a
	Zb	0.43 ± 0.32 a				
Ternary co-culture	Sc	0.56 ± 0.08 a	0.00 ± 0.00 b	0.00 ± 0.00 b	37.42 ± 3.7 b	0.374 ± 0.037 b
	Td	0.58 ± 0.03 a				
	Zb	0.44 ± 0.05 a				

The data are average of triplicates ± standard deviation. Different letters indicate significant differences ($p < 0.05$) among values in the same column. Sc: *S. cerevisiae*; Td: *T. delbrueckii*; Zb: *Z. bailii*. $Y_{P/S}$: yield of ethanol produced relative to the sugars consumed.

This table also shows that the *T. delbrueckii* and *S. cerevisiae* monocultures produced similar amounts of ethanol, and the *Z. bailii* monoculture produced the lowest amount. Conversely, the Td/Zb co-culture produced more ethanol among all the fermentations. Moreover, it is noticeable that co-cultures showed higher ethanol production than the monocultures. Similar observations can be made regarding the $Y_{P/S}$ values; *S. cerevisiae* and *T. delbrueckii* monocultures had identical yields, while all co-cultures had higher yields than monocultures. Altogether, these results show that, in the nutritionally rich YPD medium, *S. cerevisiae* causes a slight inhibition of *T. delbrueckii*, which manifests itself mainly in a slight loss of viability after concluding the exponential growth phase. However, *Z. bailii* shows no signs of being inhibited by *S. cerevisiae* in this medium.

The growth kinetics of the co-cultures developed in the M2 medium are shown in Figure 3. As in Figure 2, the graphs in Figure 3 present the biomass production expressed in logarithmic growth cycles. All graphs in this figure show less abundant growth than in the YPD medium, but this can be explained by the fact that the M2 medium is nutritionally limited compared with the YPD medium. Figure 3A shows that *S. cerevisiae* and *T. delbrueckii* grew well during the first nine hours of incubation. Subsequently, a marked loss of viability of *T. delbrueckii* was evident. On the other hand, the Sc/Zb co-culture in the M2 medium (Figure 3B) was carried out in a similar way to that in the YPD medium. The two yeasts developed well, reaching the stationary phase after nine hours of incubation.

Figure 3C shows that *Z. bailii* grew without problem in the M2 medium without reaching the stationary phase. During this co-culture, *T. delbrueckii* reached the exponential growth phase at 12 h, showing a very slight loss of viability from this incubation time. Lastly, the ternary co-culture is depicted in Figure 3D. It displays the growth kinetics of *S. cerevisiae* and *Z. bailii*, similar to those of the other co-cultures in this medium. Conversely, *T. delbrueckii* competed well with the other two yeasts for up to 12 h of incubation during the ternary co-culture. Then, it showed an even more pronounced loss of viability than in the Sc/Td co-culture, where no viability was detected at 24 h.

Table 2 presents the fermentation parameters of the cultures in the M2 medium. Similar to the YPD medium cultures, the μ values in Table 2 show no significant differences ($p > 0.05$), indicating a consistent initial growth pattern for the different yeasts. This agrees with that reported previously by Pourcelot et al. [41], who found that 70% of the co-cultures of 15 yeasts did not show changes in μ values. However, as the exponential growth phase approaches its end, nutrient depletion becomes apparent, exposing some particular differences in yeast growth, as shown by the kinetics in Figure 3.

This table also shows that all cultures only partially consumed the sugars in the M2 medium. However, a better consumption of glucose was observed compared to fructose. Glucose consumption was between 56.5% (Td/Zb co-culture) and 96.4% (Sc/Zb co-culture). In comparison, fructose consumption ranged from 22.1% (Td monoculture) to 50.3% (Zb monoculture). Regarding ethanol production, the highest concentrations were produced by the cultures where *S. cerevisiae* was present: monoculture, Sc/Zb co-culture, and ternary culture. Considering the high capacity of *S. cerevisiae* to produce ethanol, it is noticeable that the Sc/Td culture produced the lowest ethanol concentration. The highest value of $Y_{P/S}$ was obtained by the Td/Zb co-culture, which presented intermedium ethanol production but the lowest sugar consumption.

The growth kinetics of the monocultures in both media are shown in Supplementary Figure S1. This figure shows that *Z. bailii* grows similarly in both media during 24 h of incubation. On the other hand, the growth of *S. cerevisiae* and *T. delbrueckii* in the M2 medium was similar to that in the YPD medium only up to 9–12 h of culture. Afterward, *S. cerevisiae* showed a slight loss of viability, while *T. delbrueckii* entered the stationary phase. Therefore, the loss of viability of *T. delbrueckii* observed in M2 medium in co-cultures with *S. cerevisiae* is due to the interaction with the latter yeast; the low nutritional quality of the M2 medium certainly influences but is not the main reason for, the population decline of *T. delbrueckii*. Actually, the survival of *S. cerevisiae* in the M2 medium is better in co-cultures than in its monoculture. This suggests that interaction with non-*Saccharomyces*

yeasts activates physiological responses in *S. cerevisiae*, increasing its ability to compete and survive in nutritionally poor media. However, this is a matter for future research. Lastly, all the tests reported here were carried out with one strain of each yeast species. Thus, some variation in the response would be expected from other strains of *T. delbrueckii*, *Z. bailii*, and *S. cerevisiae*.

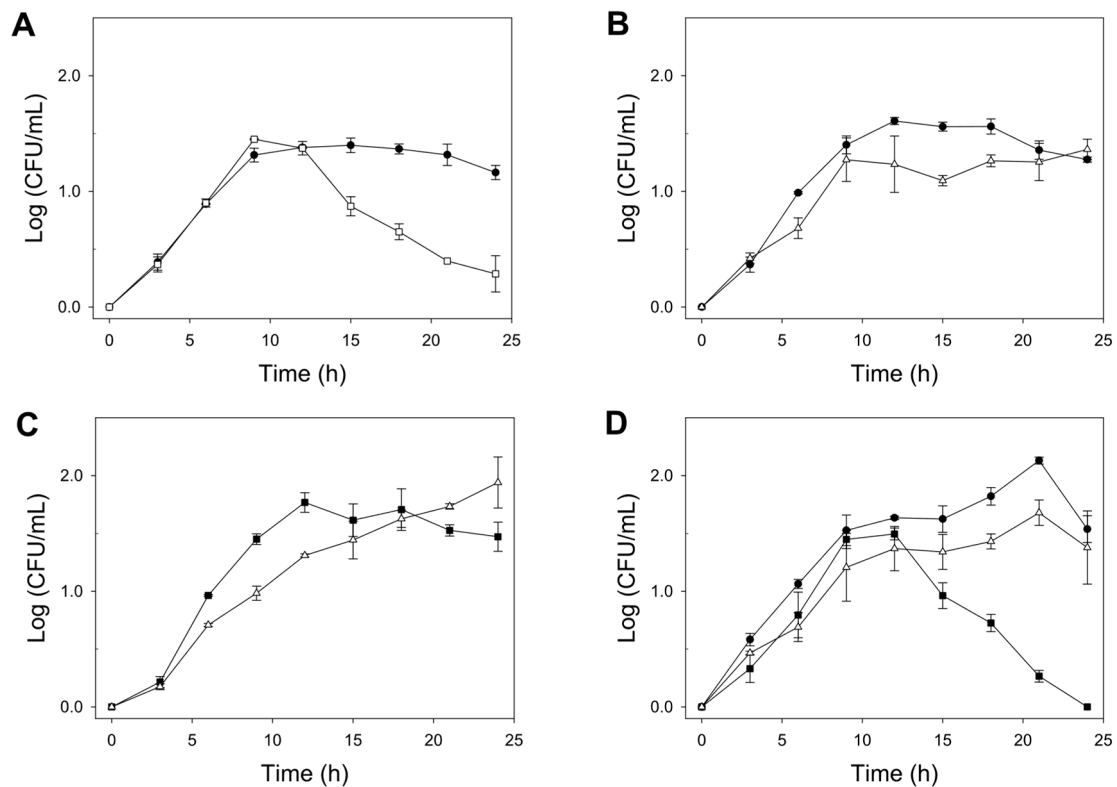


Figure 3. Growth kinetics of co-cultures in M2 medium. Viable cells of *S. cerevisiae* (●) and *T. delbrueckii* (□) during the Sc/Td co-culture (A). Viable cells of *S. cerevisiae* (●) and *Z. bailii* (△) during the Sc/Zb co-culture (B). Viable cells of *T. delbrueckii* (■) and *Z. bailii* (△) during the Td/Zb co-culture (C). Viable cells of *S. cerevisiae* (●), *T. delbrueckii* (■), and *Z. bailii* (△) during the ternary co-culture (D). The data is shown as an average of three fermentations. Vertical bars represent standard deviations.

Table 2. Fermentation parameters of mono- and co-cultures in M2 medium.

		μ (h ⁻¹)	Residual Fructose (g/L)	Residual Glucose (g/L)	Ethanol Content (g/L)	$Y_{P/S}$ (g/g)
Monoculture	Sc	0.36 ± 0.01 a	60.52 ± 3.22 b	1.37 ± 0.28 cd	12.28 ± 1.14 a	0.325 ± 0.052 ab
	Td	0.39 ± 0.01 a	70.09 ± 8.31 a	2.5 ± 0.39 bc	6.00 ± 0.38 cd	0.242 ± 0.084 bc
	Zb	0.22 ± 0.01 a	44.72 ± 1.19 c	2.43 ± 0.23 ab	6.73 ± 0.48 c	0.129 ± 0.007 d
Co-culture Sc/Td	Sc	0.46 ± 0.08 a	59.66 ± 2.46 b	1.31 ± 0.22 cd	5.36 ± 0.42 d	0.138 ± 0.015 d
	Td	0.42 ± 0.03 a				
Co-culture Sc/Zb	Sc	0.52 ± 0.20 a	46.22 ± 5.28 c	0.36 ± 0.07 d	12.56 ± 0.95 a	0.238 ± 0.039 c
	Zb	0.40 ± 0.10 a				
Co-culture Td/Zb	Td	0.48 ± 0.02 a	65.91 ± 3.98 ab	4.35 ± 0.30 a	9.69 ± 0.63 b	0.332 ± 0.058 a
	Zb	0.22 ± 0.06				
Ternary co-culture	Sc	0.43 ± 0.04 a	50.20 ± 3.19 c	3.12 ± 0.33 ab	11.53 ± 0.45 a	0.248 ± 0.021 abc
	Td	0.48 ± 0.07 a				
	Zb	0.35 ± 0.14 a				

The data are averages of triplicates ± standard deviation. Different letters mean significant difference ($p < 0.05$) among values in the same column. Sc: *S. cerevisiae*, Td: *T. delbrueckii*, Zb: *Z. bailii*. $Y_{P/S}$: yield of ethanol produced concerning sugars consumed.

Harlé et al. [31] reported co-cultures in synthetic grape juice of *S. cerevisiae* with *Metschnikowia pulcherrima* (Mp), *M. fructicola* (Mf), *Hanseniaspora uvarum* (Hu), and *H. opuntiae* (Ho). Although *S. cerevisiae* dominated all the co-cultures, the μ values for the co-cultures were variable. The μ value for the Sc/Mf co-culture was higher than the corresponding values for the monocultures. The co-cultures Sc/Ho and Sc/Mp showed μ values lower than those of monocultures, and the value for Sc/Hu was between the monoculture values. On the other hand, Su et al. [21] reported that the μ value was influenced by the nitrogen sources in the culture medium and the yeast species rather than by the amount of available nitrogen.

Closely to the present work, González-Robles et al. [42] performed co-cultures of *S. cerevisiae* and two non-*Saccharomyces* yeasts (*Hanseniaspora vineae* and *H. uvarum*) using *Agave tequilana* juice as culture medium. They reported higher μ and $Y_{P/S}$ values for co-cultures than for monocultures. On the other hand, Nolasco-Cansino et al. [3] carried out monocultures and co-cultures in filtered agave juice and agave juice with bagasse. They observed that the monoculture of *S. cerevisiae* obtained a higher $Y_{P/S}$ compared to the binary co-cultures of *Kluyveromyces marxianus* and *Pichia kudriavzevii*. Only the ternary co-culture in filtered agave juice yielded a higher $Y_{P/S}$ ratio than the monoculture.

The nutritionally rich medium used in the present work was a blend of casein peptone and yeast extract. Yeast extract is widely used to enrich culture media; it is rich in nucleotides, proteins, amino acids, trace elements such as magnesium and zinc, and vitamins, especially group B vitamins such as thiamine [43]. It has been reported that zinc deficiency can result in incomplete fermentation due to its function as a cofactor in numerous enzymes [44]. On the other hand, thiamine is involved in different metabolic processes and is essential for yeast growth [45]. Thiamine has even been reported to offer protection against oxidative, osmotic, and thermal stress [46,47]. Adding amino acids and zinc has proven to be especially useful for completing sequential fermentation [23].

On the other hand, Mencher et al. [48] demonstrated that the expression of the HXT2 gene by *S. cerevisiae* is a typical response of this yeast to the presence of non-*Saccharomyces* yeasts. HXT2 encodes a hexose-transporting enzyme, so it is suggested that one of the competition mechanisms of *S. cerevisiae* is to increase hexose consumption. In addition, Curiel et al. [49] reported the overexpression of genes involved in the glucose-fermentative pathway.

Although the sugar content in the M2 medium was not exhausted, the growth of the yeasts ceased. These data suggest that the loss of viability of non-*Saccharomyces* yeast compromises ethanol production performance in their respective co-cultures with *S. cerevisiae*. The presence of *S. cerevisiae* does not translate into higher ethanol production, and other factors, such as the composition of the culture medium, are involved. Then, some other nutrients were exhausted, and there was no reason to extend fermentation. Spontaneous agave fermentation begins with a combination of high sugar concentrations (more than 150 g/L) and low yeast populations, proceeding without temperature control during fermentation [1]. This leads to slow fermentation with low ethanol concentrations (25–50 g/L) [50] and high residual sugar content that can reach 50–60 g/L [3,51]. Therefore, the medium M2 used in the present work helps to simulate the spontaneous agave fermentation conditions.

3.3. Production of Minor Volatile Compounds

After 24 h of fermentation, 101 compounds were identified (Table S1), which were distributed into the following chemical families: acetal (1), acids (6), alcohols (20), aldehydes (10), ketones (7), esters (24), terpenes (14), pyrazines (11), and others (8), including phenols, furans, lactones, pyrroles, and sulfur compounds. These results are summarized in the heat maps shown in Figure 4.



Figure 4. Semi-qualitative analysis of volatile compounds in M2 and YPDA media (A) Cluster heatmap of volatile compounds produced by monocultures in both media. (B) Cluster heatmap of volatile compounds produced by co-culture in both media.

The most representative group of compounds was higher alcohols. In general, the YPDA medium produced more alcohol than the M2 medium. The Sc/Td, Sc/Zb, and ternary co-cultures may have fewer higher alcohols than the Td/Zb co-culture, which showed a more significant number of higher alcohols within group M2. Nevertheless, it was also observed that each yeast strain was affected differently by the nutritional composition of the two media. For instance, *T. delbrueckii* produced the highest quantity of higher alcohols (5.197 ± 0.286 mg/L), even more so than in co-cultures in nutritionally rich YPDA medium. Conversely, this yeast produced the lowest amount (1.652 ± 0.32 mg/L) in nutritionally poor M2 medium. The nutritional content and variable loss of viability could explain the changes in the levels of some alcohols, such as 2-phenylethanol. This alcohol is related to cell-cell communication, which is dependent on cell density and the nutritional status of the environment [52]. Therefore, the lower production of higher alcohols in general, but particularly 2-phenyl ethanol, 2-methyl-1-propanol, and 1-propanol, could be related to the loss of viability of *T. delbrueckii*.

The production of aldehydes and ketones is related to the ability of yeasts to carry out oxidation-reduction reactions to achieve the biotransformation of alcohols to their respective ketones or aldehydes and back [53]. Ketone production was higher in *Z. bailii* monocultures, regardless of the culture medium. In contrast, the production of aldehydes was better in the *Z. bailii* monoculture, but only in the M2 medium. *Zygosaccharomyces bailii* stands out for its high production of 2-heptanone and its corresponding alcohol, 2-heptanol. These compounds have previously been reported to contribute to the flavor of Maotai liquor by providing fruity and spicy notes, such as cinnamon aroma (2-heptanone) and fruity flavor (2-heptanol) [54].

Many pyrazines were identified, mainly in the YPDA medium. It is explained because the yeast extract is rich in this type of compound [43]. Therefore, these compounds originate in the components of the culture medium rather than from yeast metabolism. The production of ethyl esters was poor in M2 medium, especially in monocultures of *T. delbrueckii* and *Z. bailii*. Instead, in the YPDA medium, the production of esters was better in all fermentations than in the *S. cerevisiae* monoculture. This result suggests a synergistic effect on ester production mediated by the yeast species involved. The production of esters has been related to the presence of precursors (fatty acids) and to the activity of the enzymes acetyl-CoA carboxylase and fatty acid synthase complex [55].

The fermentation time at which the measurements were performed (24 h) may be related to the abundance of esters because these usually occur in the late stages of fermentation [56]. This could be the case for M2 fermentation, where incomplete fermentation produces poor ester production.

The Sc/Zb co-culture in the YPDA medium obtained the highest number of terpenes among all fermentations. However, the other fermentations in the YPDA medium showed terpene production below that obtained in the M2 medium. Terpene compounds originate from raw materials and are generally bound to carbohydrates; therefore, yeasts must express glucosidase enzymes to release these compounds [57].

Higher concentrations of fatty acids were produced in Sc/Zb and ternary co-cultures in the YPDA medium, as well as in the Sc/Td and ternary co-cultures in the M2 medium, compared to *S. cerevisiae* monocultures in both media. It is noted that the monoculture of *Z. bailii* and the monoculture of *T. delbrueckii* did not produce fatty acids after 24 h of fermentation in the M2 medium. Nonetheless, these monocultures showed the production of fatty acids in the YPDA medium, specifically isobutyric acid and heptanoic acid. The highest production of fatty acids in co-culture has been previously reported for Sc/Td during agave juice fermentation for mezcal production [58]. The production of fatty acids and most volatile compounds is related to the strains used and the medium in which fermentation occurs. For example, a reduction in fatty acid content has been observed using mixed fermentation with *Lachancea thermotolerans* and *Starmerella bacillaris* using Pinot Grigio grape juice [59]. Fatty acids are related to unwanted odors in fermented products if their concentration is above 20 mg/L [60]. The family of fatty acids is known for its antimicrobial capacity since they can interact with the cell membrane, causing the loss of cellular structure and leading to cell death in yeast [61]. However, the fermentations with the most significant production of fatty acids coincided with the co-cultures, showing a lower loss of viability. Therefore, although the overproduction of fatty acids may be related to the loss of viability of non-*Saccharomyces* yeasts, it is not the only determining factor.

4. Conclusions

The feasibility of using simple and low-cost culture media was demonstrated to monitor interactions between *S. cerevisiae* and other yeasts, such as *T. delbrueckii* and *Z. bailii*, isolated from the alcoholic fermentation of agave juice. However, testing these culture media with more yeast species is necessary. The results presented here demonstrate that *Saccharomyces cerevisiae* exerts an antagonistic action on the non-*Saccharomyces* yeast *Torulaspora delbrueckii*, which shows a more significant loss of viability. Nevertheless, the nutritional quality of the culture medium influences this effect since a nutritional-rich

medium modulates negative responses and can improve the survival of non-*Saccharomyces* yeasts. Finally, the survival time of non-*Saccharomyces* yeasts influences the concentration and diversity of the minor volatile compounds produced. The prolonged survival of non-*Saccharomyces* yeasts in the YPDA medium was associated with increased production of higher alcohols and esters. This was in stark contrast to the M2 medium, where the output of these two groups of compounds was poor. Likewise, the production of fatty acids was found to be influenced by the presence of *S. cerevisiae* in the co-cultures.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/fermentation10080400/s1>, Figure S1: Growth kinetics of mono-cultures; Table S1: Semi-quantification of volatile compounds ($\mu\text{g/L}$).

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