

Article

Phenotypic Diversity and Fermentative Potential of Patagonian *Torulaspora delbrueckii* Strains for Cider Production

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Abstract

Torulaspora delbrueckii is a promising non-*Saccharomyces* yeast for beverage diversification, although its diversity and application in cider production remain poorly explored. This study characterized Patagonian *T. delbrueckii* isolates from wine- and cider-associated environments and evaluated their fermentative potential in apple must. Mitochondrial DNA-RFLP analysis showed that mitochondrial profiles varied according to the isolation source, while physiological characterization revealed differences in temperature, sulfite, and ethanol responses among isolates from different sources. Compared with wine isolates, cider isolates generally exhibited greater tolerance to high temperatures, lower sulfite tolerance, and shorter lag phases under ethanol stress. Eleven representative strains completed apple must microfermentations. Compared with a commercial *Saccharomyces cerevisiae* strain, *T. delbrueckii* produced higher glycerol and erythritol concentrations while achieving comparable or higher ethanol levels. Two contrasting strains were further evaluated in sequential mixed fermentations with *S. cerevisiae*. NPCC1340 enhanced glycerol and erythritol production, whereas NPCC1608 increased malic and succinic acids while maintaining low acetic acid levels. In mixed fermentations, ciders retained the characteristic metabolic profiles of each *T. delbrueckii* strain, whereas *S. cerevisiae* improved fermentation performance, producing intermediate fermentation kinetics. These findings reveal phenotypic diversity among Patagonian *T. delbrueckii* strains and suggest differences associated with their isolation sources, highlighting their potential as starter cultures for producing ciders with distinct chemical characteristics.

Keywords: *Torulaspora delbrueckii*; cider; stress tolerance; apple must fermentation; mixed-culture fermentation



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1. Introduction

Non-*Saccharomyces* yeasts are increasingly valued as functional components of beverage fermentation. Their broad metabolic diversity can reshape aroma, acidity, mouthfeel, ethanol yield, and microbial stability, extending the comparatively narrow range of outcomes obtained with conventional *Saccharomyces cerevisiae* starters [1–3]. This potential is commonly exploited through simultaneous or sequential inoculation with *S. cerevisiae*, a strategy that pairs the distinctive metabolic contribution of the non-*Saccharomyces* partner with reliable fermentation completion. However, the magnitude—and even the direction—of these effects is species and strain-dependent, and is further shaped by inoculation ratio,

timing, temperature, nutrient availability, and interspecies interactions [4]. Selecting starter cultures for a given beverage therefore requires characterization at the strain level, under conditions that reproduce the target fermentation process.

Cider is a particularly suitable matrix for this approach, since its chemical and sensory identity depends not only on apple cultivar and processing practices but also on the composition and succession of the fermenting microbiota [5,6]. Moreover, its moderate ethanol concentration may favor the persistence and metabolic activity of non-*Saccharomyces* yeasts. In Argentina, cider production is concentrated in North Patagonia, the country's main apple- and pear-growing region, which has historically accounted for approximately 70–75% of national output [7–9]. There has traditionally relied on dessert apple cultivars and wine-derived commercial starters, a combination that limits product differentiation. Recovering and selecting yeasts from local orchards, traditional beverages, and cider-associated environments offers a way to link this microbial biodiversity to regional typicity and added value. Beyond their technological potential, the recovery and characterization of yeasts from regional fermentative environments can contribute to the valorization of microbial biodiversity as a local biotechnological resource. In North Patagonia, where fruit production and cider making are closely linked to the regional economy, locally recovered yeast strains may provide a reservoir of genetic and phenotypic diversity that can be explored to obtain starter cultures with distinctive technological properties. Their use could contribute to differentiating fermented beverages by introducing strain-specific characteristics associated with regional microbial resources, while also supporting the development of cultures adapted to the environmental and technological conditions of local production systems. This perspective is particularly relevant in the growing interest in the geographical origin and territorial identity of fermented foods and beverages, where microbial resources associated with a production region may contribute to product differentiation and the expression of regional typicity [9]. Previous studies in Patagonia have already applied this strategy: cryotolerant *Saccharomyces uvarum* and *Saccharomyces eubayanus* strains have been recovered from natural and fermentative habitats; indigenous *S. uvarum* strains and hybrids with strong low-temperature performance have been selected for cider and evaluated in apple cider and perry, a cider-like beverage made from pear juice [7,8,10]. Together, these studies establish regional yeast bioprospecting as a practical route toward distinctive Patagonian fermented beverages. Although most have focused on *Saccharomyces* species, a recent study from our laboratory extended this approach to the non-*Saccharomyces* yeast *Pichia kudriavzevii* in cider fermentation [11]. However, the diversity and cider-making potential of other non-*Saccharomyces* species recovered from regional fermentative environments remain largely unexplored. Among non-*Saccharomyces* yeasts, *Torulaspora delbrueckii* is one of the most extensively studied species for controlled alcoholic fermentation. Relative to many other non-*Saccharomyces* species, it combines a comparatively high fermentative capacity with the ability to lower volatile acidity and increase glycerol and other polyol production, mannoprotein release, and aromatic complexity, although the magnitude—and even the direction—of these effects varies markedly among strains and fermentation conditions [4,12–15]. This technological maturity is reflected in the availability of commercial wine starters, including the pure *T. delbrueckii* culture LEVEL2 BIODIVA™ for sequential use with *S. cerevisiae*, *T. delbrueckii*-containing preparations for prefermentative bioprotection, and other strains such as ZYMAFLORE™ Alpha [3]. Commercial formulations and most published applications remain focused on wine, where *T. delbrueckii* has been used to modulate volatile acidity, esters, mouthfeel, and aroma in still, sweet, and sparkling wines [16,17].

The technological interest in *T. delbrueckii* extends significantly beyond winemaking. This species has been detected or evaluated in bread, dairy products, cocoa and other food fermentations, as well as in beer, tequila, mezcal, and fruit-based alcoholic beverages [12]. Evidence in cider, however, remains comparatively limited. *T. delbrueckii* has been identified during spontaneous cider fermentation and evaluated in pure or mixed cultures for cider and sparkling-cider production, where strain-dependent effects on fermentation kinetics, organic acids, volatile compounds, antioxidant properties, and sensory attributes have been reported [18–22]. These studies confirm the relevance of *T. delbrueckii* to apple fermentation, but each has generally examined a small number of strains, so the available evidence cannot be generalized to the species as a whole and offers little insight into how ecological origin and intraspecific genetic diversity relate to cider-relevant phenotypes. Screening locally sourced isolates is therefore needed to determine whether this diversity can be exploited for cider production.

From an ecological standpoint, *T. delbrueckii* is a cosmopolitan species without a single recognized geographical origin: it has been recovered from natural environments such as soil, plants, fruits, and insects as well as from human-associated environments such as wineries, bakeries, dairies, and diverse food and beverage fermentations [7,8,12,23]. Its diversity is structured by both ecology and human activity. Microsatellite data distinguished wild populations with clear geographical differentiation from clusters associated with wine and other bioprocesses, consistent with an early stage of domestication [12,24]. Not all *T. delbrueckii* isolates, therefore, can be assumed to share equivalent fermentative capacity; whether isolates recovered from regional cider environments display ecological differentiation or technological adaptation relative to those from wine environments remains an open question.

In this context, the present study used a bioprospecting framework to investigate the intraspecific diversity and cider-making potential of *T. delbrueckii* strains recovered from wine- and cider-associated environments in North Patagonia. Finally, genetically, ecologically, and phenotypically contrasting strains were selected for evaluation in sequential mixed fermentations with a commercial *S. cerevisiae* starter. By integrating these complementary approaches, this work provides a strain-level basis for developing regional *T. delbrueckii* starters to diversify Patagonian cider production.

2. Materials and Methods

2.1. Yeasts

A total of 21 *Torulaspora delbrueckii* isolates from the North Patagonian Culture Collection (NPCC) were included in this study. All isolates were recovered from fermentative environments in Río Negro Province, Patagonia, Argentina. The sampling sites comprised wineries and cideries located within an approximately 50 km radius. Of the 21 isolates, 15 originated from wine-related environments, including grape surfaces and fermenting grape must, whereas the remaining six were isolated from cider-associated environments, including fermenting apple and pear juices and fruit surfaces (Figure 1). The isolates were preserved at -80°C in 20% glycerol. For reactivation, cultures were grown in GPY medium at room temperature prior to further experimentation.

Strain	Isolation source	mtDNA-RFLP	Reference
NPCC1028	Red grape surface	A	(Lopes et al., 2009)
NPCC1029	Wine vats surfaces	A	(Lopes et al., 2009)
NPCC1030	Wine vats surfaces	A	(Lopes et al., 2009)
NPCC1031	Wine vats surfaces	B	(Lopes et al., 2009)
NPCC1032	Fermenting Merlot must	A	(Lopes et al., 2009)
NPCC1033	Fermenting Merlot must	A	(Lopes et al., 2009)
NPCC1034	Fermenting Merlot must	A	(Lopes et al., 2009)
NPCC1035	Fermenting Merlot must	A	(Lopes et al., 2009)
NPCC1036	Fermenting Malbec must	D	(Lopes et al., 2009)
NPCC1049	Fermenting Malbec must	E	(Lopes et al., 2009)
NPCC1339	Fermenting Malbec must	E	(Lopes, 2004)
NPCC1340	Red grape surface	C	(Lopes, 2004)
NPCC1341	Red grape surface	A	(Lopes, 2004)
NPCC1342	Fermenting Malbec must	D	(Lopes, 2004)
NPCC1534	Fermenting Malbec must	E	(Lopes, 2004)
NPCC1608	Red delicious cider fermentation	F	(González Flores et al., 2019)
NPCC1609	Red delicious cider fermentation	F	(González Flores et al., 2019)
NPCC1629	Granny Smith cider fermentation	F	(González Flores et al., 2019)
NPCC1630	Granny Smith cider fermentation	F	(González Flores et al., 2019)
NPCC1844	Pear cider fermentation	F	(González Flores et al., 2021)
NPCC1845	Pear cider fermentation	A	(González Flores et al., 2021)
Commercial <i>S. cerevisiae</i>	Wine	G	-

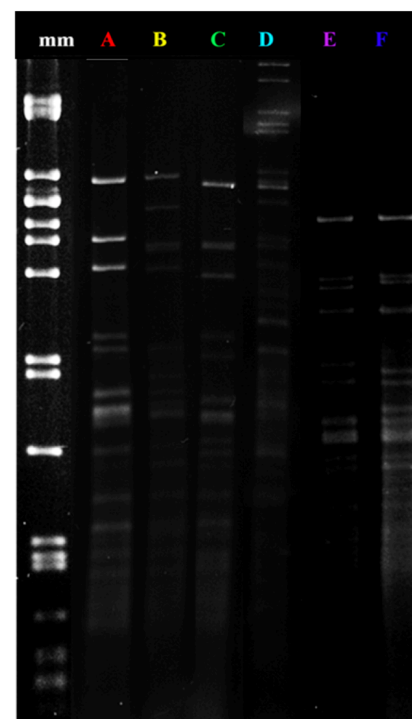


Figure 1. Genetic diversity of *Torulaspora delbrueckii* isolates. (Left), *T. delbrueckii* isolates recovered from wineries and cideries in Río Negro Province, Patagonia, Argentina, with their isolation source, mtDNA-RFLP profile, and reference [7,8,25,26]. (Right), Representative mtDNA-RFLP patterns (A–F) obtained after *HinfI* digestion of mitochondrial DNA. The mtDNA-RFLP profile of the commercial *Saccharomyces cerevisiae* (Sc) strain used in the fermentation assays is also included for comparison. The lane labeled mm indicates the molecular size marker (*PstI*λ).

2.2. DNA Extraction and mtDNA-RFLP Analysis

Total cellular DNA, including mitochondrial DNA, was extracted from all 21 *Torulaspora delbrueckii* isolates using a modified version of the protocol described by López et al. (2001) [27]. Briefly, yeast isolates were grown in 1 mL of liquid GPY medium for 24 h. Cells were collected by centrifugation at $3000 \times g$ for 5 min, washed three times with sterile distilled water, and treated with 30 µL of Zymolyase 20T (2 mg/mL) for 1 h at 37 °C and 200 rpm. Cell lysis was completed with 10% SDS at 65 °C for 15 min. Proteins and cellular debris were precipitated with potassium acetate, and DNA was recovered from the clarified supernatant by isopropanol precipitation. The DNA pellet was washed three times with 70% ethanol, dried, resuspended in 20 µL of Milli-Q water, and stored at −20 °C until use.

Genetic diversity was assessed by mitochondrial DNA restriction fragment length polymorphism (mtDNA-RFLP) analysis. For restriction digestion, 15 µL of purified total DNA was mixed with 2 µL of RNase, 1.5 µL of *HinfI*, 2.5 µL of the corresponding reaction buffer, and 4 µL of sterile distilled water. The mixture was incubated overnight at 37 °C. Restriction fragments were separated by horizontal electrophoresis in 1% agarose gels at a constant voltage of 90 V. Gels were stained and visualized as described above. *PstI*-digested λ DNA, with fragment sizes ranging from 300 to 11,000 bp, was used as the molecular size marker.

2.3. Phenotypic Characterization Assays

2.3.1. Growth at Different Temperatures

Growth at 8, 13, 25, 35 and 37 °C was evaluated by spot assay on GPY agar according to the method described by González Flores et al. (2019) [8]. A commercial *Saccharomyces cerevisiae* control strain, which exhibited greater tolerance to high temperatures than the

wine-associated *T. delbrueckii* isolates, and a cryotolerant *Saccharomyces uvarum* strain were included as reference strains.

2.3.2. Sulfite Tolerance Assays

Sulfite tolerance was assessed by spot assay on YEPD agar supplemented with 0.075 M tartaric acid and increasing concentrations of sodium metabisulfite ($\text{Na}_2\text{S}_2\text{O}_5$; 0, 1, 2, 3, and 4 mM), following the methodology of Park et al. (1999) [28]. The reference strains described in Section 2.3.1 were included as representatives of intermediate (*S. cerevisiae*) and high sulfite tolerance (*S. uvarum*).

2.3.3. Growth Under Ethanol Stress

Based on mtDNA-RFLP profiles, sampling origins, and phenotypic responses in the preceding assays, 11 representative *T. delbrueckii* strains were selected for ethanol tolerance evaluation. Assays were conducted in 96-well microtiter plates containing 200 μL of Yeast Nitrogen Base (YNB) supplemented with 0%, 3%, 5%, 7%, 8%, or 10% (*v/v*) ethanol. Each well was inoculated with a standardized cell suspension to a final concentration of 6×10^6 cells mL^{-1} . Growth was monitored by measuring optical density at 630 nm (OD_{630}) using a Rayto RT-2100C microplate reader (Rayto Life and Analytical Sciences Co., Ltd., Shenzhen, China). Assays were performed in triplicate using randomized plate layouts and incubated at 25 °C until the optical density reached a plateau. The *S. cerevisiae* reference strain mentioned in Section 2.3.1 was included as control of high ethanol tolerance.

2.3.4. Killer Assays

Antagonistic activity was evaluated following the method described by Sangorrín et al. (2002) [29]. The *Torulaspora delbrueckii* isolates selected in Section 2.3.3 were evaluated for their antagonistic activity. The commercial *Saccharomyces cerevisiae* strain and the sensitive *Candida glabrata* strain were used as lawn cultures at an approximate concentration of 10^5 cells/mL. *S. cerevisiae* K2 was included as the killer reference strain. Cell suspensions were uniformly spread onto YEPD-MB agar plates containing 3 mg of methylene blue per 100 mL of medium, buffered at pH 4.6. The selected *T. delbrueckii* isolates were then streaked onto the plates. After incubation, the presence or absence of growth inhibition zones surrounding the yeast streaks was recorded as an indicator of antagonistic activity.

2.4. Apple Must Microfermentations

Pure culture microfermentations were carried out in sterile 35 mL glass bottles containing sterile Granny Smith apple must (12.5 °Brix) and incubated at 20 °C. The initial apple must composition was characterized by HPLC. Eleven representative *T. delbrueckii* isolates, selected as described in Section 2.3.3, were used as starter cultures. Before inoculation, the apple must was sterilized by autoclaving at 105 °C for 30 min to eliminate indigenous microorganisms and ensure that the fermentation process was exclusively driven by the inoculated yeast strain.

The must was inoculated at an initial cell concentration of 2×10^6 cells mL^{-1} . Microfermentations were performed with the selected *T. delbrueckii* isolates, while the same commercial *Saccharomyces cerevisiae* control strain was included as a reference.

Fermentation kinetics were monitored daily by measuring soluble solids (°Brix) with a digital refractometer. Carbon dioxide production was estimated from the daily loss of flask weight, which was determined using an analytical balance with a precision of ± 0.01 g. Fermentations were considered complete when residual reducing sugars reached approximately 2 g L^{-1} and no further consecutive weight loss was observed. At the end of fermentation, the physicochemical composition of the fermented apple must, including residual sugars and fermentation metabolites, was determined by high-performance liquid chromatography (HPLC). All fermentations were carried out in duplicate.

2.5. Mixed-Culture Fermentations Assays in Sterile Apple Must

Mixed-culture fermentations were carried out in 300 mL of sterile *Granny Smith* apple must (13.1 °Brix) treated by autoclaving at 105 °C for 30 min. The initial must composition was determined by HPLC. One *T. delbrueckii* strain from each isolation origin (cider- and wine-associated environments) was selected based on the complementary and contrasting phenotypes identified during the previous screening stages. Selection considered their assimilation profiles, tolerance to temperature, sulfite, and ethanol stress, fermentation kinetics, and production of metabolites of technological interest to the cider industry. The selected strains were co-inoculated with the same commercial *Saccharomyces cerevisiae* reference strain used in the pure-culture microfermentation assays (Section 2.4).

Mixed-culture fermentations were performed following the sequential inoculation strategy described by Mazzucco et al. (2025) [11], in which *T. delbrueckii* was inoculated first at an initial concentration of 1×10^6 cells mL⁻¹, followed 48 h later by inoculation with the commercial *S. cerevisiae* reference strain at the same cell concentration (1×10^6 cells mL⁻¹). Pure cultures, used as references, were inoculated with 2×10^6 cells mL⁻¹ of the corresponding yeast strain.

All fermentations were carried out at 20 °C and monitored daily by measuring soluble solids (°Brix). Samples from both mixed-culture fermentations and the corresponding pure-culture controls were collected at 0, 26, 72, 142, 188, 235, 312, 380, and 477 h after inoculation. Viable yeast populations were quantified by plating on WL Nutrient Agar and incubating at 25 °C for 4 days. Results were expressed as log CFU mL⁻¹.

In mixed-culture fermentations, *T. delbrueckii* and *S. cerevisiae* were preliminarily differentiated based on colony morphology on WL medium. Multiple colonies representing each morphotype were randomly selected at each sampling time and screened for growth at 25 and 37 °C, taking advantage of the inability of *T. delbrueckii* to grow at 37 °C under the conditions tested. Isolate identity was subsequently confirmed by mtDNA-RFLP analysis as previously described.

Fermentations were considered complete when residual reducing sugars reached approximately 2 g L⁻¹ and soluble solids (°Brix) remained stable. At the end of fermentation, the physicochemical composition of the fermented must, including residual sugars and the main fermentation metabolites, was determined by high-performance liquid chromatography (HPLC). All fermentations were performed in duplicate.

2.6. Physicochemical Analysis of Pure and Mixed-Culture Fermentations

The concentrations of ethanol, glycerol, methanol, sorbitol, glucose, fructose, erythritol and organic acids (citric, malic, acetic, lactic, and succinic acids) were determined by high-performance liquid chromatography using a 1260 Infinity HPLC system (Agilent Technologies, Santa Clara, CA, USA) [8]. Quantification was performed using analytical standards (99% purity; Sigma-Aldrich, St. Louis, MO, USA), and all measurements were carried out in duplicate.

2.7. Statistical Analysis

Growth under ethanol stress and fermentation kinetics were modeled in RStudio [30]. Yeast growth under ethanol stress was described using the modified Gompertz model [31]:

$$y = A \exp \exp \left\{ -\exp \left[\frac{\mu_{\max} e}{A} (\lambda - t) + 1 \right] \right\}$$

where y is the $\ln(N_t/N_0)$, N_0 is the initial OD_{630nm} and N_t is the culture OD_{630nm} measured at time t (h); $A = \ln(N_\infty/N_0)$ is the maximum population value reached with N_∞ as the asymptotic maximum of OD_{630nm}, $\mu_{\max}$ is the maximum OD_{630nm}.

Fermentation kinetics, based on cumulative CO₂ production, were also fitted to the modified Gompertz model [31]:

$$y = A \exp \exp \left\{ -\exp \left[\frac{\mu_{\max} e}{A} (\lambda - t) + 1 \right] \right\}$$

where y is the accumulated CO₂ production (g), A is the asymptotic CO₂ production (g), $\mu_{\max}$ is the maximum CO₂ production rate (g h⁻¹), and λ is the lag phase duration (h).

Changes in soluble solids (°Brix) during fermentation were modeled using an exponential decay equation [32]:

$$y = D + S \cdot \exp(-k \cdot t)$$

where y is the measured °Brix value, t is time (h), D is the asymptotic °Brix value as t approaches infinity, S is the estimated change in °Brix, and k is the decay constant (h⁻¹).

Kinetic parameters and physicochemical data were analyzed by one-way analysis of variance (ANOVA), followed by the Tukey test at a significance level of $\alpha = 0.05$, using RStudio [30].

Heatmaps of the ethanol tolerance assay parameters were generated in R version 3.0.2 (R Core Team, Vienna, Austria) through RStudio version (Posit Software, PBC, Boston, MA, USA, 2025) [30]. For each strain, kinetic parameter values obtained at each ethanol concentration were normalized by the corresponding value measured under ethanol-free conditions (0% ethanol). Hierarchical clustering was performed using Euclidean distance and complete linkage based on the normalized parameter values obtained at ethanol concentrations ranging from 3 to 10% (v/v). For the lag phase heatmap, normalized values were log-transformed for color scaling.

Principal component analysis (PCA) was performed using RStudio with the base R function `prcomp` [30]. The analysis was applied to the physicochemical parameters measured during pure-culture microfermentations. For mixed-culture fermentations, PCA was performed using both the physicochemical parameters determined by HPLC and the fermentation kinetic parameters.

3. Results

3.1. Intraspecific Characterization of *T. delbrueckii* Isolates

The mtDNA-RFLP analysis of the 21 *T. delbrueckii* isolates revealed six distinct mitochondrial profiles (A–F) (Figure 1). Isolates recovered from wine-associated environments exhibited greater mitochondrial diversity, encompassing five different profiles (A–E), whereas isolates from cider-associated environments were considerably less diverse and were almost exclusively assigned to profile F. The only exception was strain NPCC1845, isolated from a perry fermentation, which displayed profile A. Interestingly, the minor mitochondrial profiles (B–E) were consistently associated with specific wine-related substrates. Profile B was detected only on wine vat surfaces, profile C exclusively on red grape surfaces, whereas profiles D and E were restricted to fermenting Malbec musts. In contrast, profile A was the predominant mitochondrial genotype among wine isolates and was also identified in the single perry isolate.

3.2. Phenotypic Characterization of *T. delbrueckii* Isolates

3.2.1. Temperature Tolerance

Growth at 25 °C was highest for all 21 *T. delbrueckii* isolates, whereas no growth was detected at either 8 or 37 °C (Table A2). The greatest intraspecific variation was observed at 13 and 35 °C; therefore, these temperatures were used to calculate a thermal tolerance index (TTI), defined as the difference between the spot-assay growth scores at 35 and 13 °C (TTI = score₃₅ °C – score₁₃ °C). Positive TTI values indicated a relative growth preference for the higher temperature, negative values indicated a relative preference for the lower temperature, and a value of zero indicated comparable growth at both temperatures. Accordingly, strains were classified as having high-temperature-biased, low-temperature-

biased, or balanced thermal responses, respectively (Figure 2A). The commercial *S. cerevisiae* and one *S. uvarum* strain were included as comparative references for growth at high and low temperatures, respectively.

The *T. delbrueckii* strains were distributed across the three thermal-response categories. Most cider isolates carrying mitochondrial profile F showed a relative preference for growth at 35 °C, with the exception of the NPCC1608 strain, which showed a preference for 13 °C. In contrast, most wine isolates assigned to mitochondrial profile A grew comparatively better at 13 °C. The cider isolate NPCC1845, which also carried mitochondrial profile A, exhibited a balanced thermal response. The NPCC1340 strain, isolated from the surface of red grapes, was the only *T. delbrueckii* strain unable to grow at 35 °C. It exhibited the lowest TTI value (−3), representing the strongest relative preference for growth at 13 °C within the collection (Figure 2A).

3.2.2. Sulfite Tolerance

Sulfite exposure revealed a marked differentiation among 21 *T. delbrueckii* isolates according to their isolation origin (Figure 2B). To provide a semiquantitative measure of this response, a sulfite tolerance index (STI) was calculated based on the highest Na₂S₂O₅ Concentration at which growth was detected ($C_{\max}$) and the growth Score recorded at that concentration ($S_{\max}$): $STI = [C_{\max} + (S_{\max}/10)]$. In this index, $C_{\max}$ represents the primary measure of tolerance, whereas $S_{\max}/10$ serves as a decimal adjustment to distinguish among strains capable of growing at the same maximum concentration without outweighing the contribution of $C_{\max}$.

All cider isolates exhibited limited tolerance and grew only up to 1 mM Na₂S₂O₅. In contrast, wine isolates displayed a broader range of responses, from strong growth at 1 mM to detectable growth at 4 mM. Within the wine group, strains carrying mitochondrial profiles B and C showed the highest tolerance, whereas those assigned to profiles D and E grew only at 1 mM. Wine isolates with mitochondrial profile A exhibited intermediate tolerance, growing at concentrations of 2–3 mM, while NPCC1845, a cider isolate carrying the same mitochondrial profile, showed only weak growth at 1 mM. Similarly, all cider isolates carrying mitochondrial profile F exhibited low tolerance.

Overall, sulfite tolerance was strongly associated with isolation origin, while mitochondrial profiles accounted for part of the phenotypic variation observed among wine isolates.

3.2.3. Growth Kinetics Under Ethanol Stress Conditions

Because the preliminary assays revealed no significant physiological differences among isolates sharing the same mitochondrial profile and sampling origin, eleven *T. delbrueckii* strains representing the different mitochondrial profiles, isolation sources, and physiological responses were selected to evaluate ethanol tolerance. Increasing ethanol concentrations markedly affected the growth kinetics of all strains, causing a progressive reduction in relative maximum specific growth rate ($\mu_{\max}$) and a concomitant extension of the lag phase (λ) (Figure 2C,D). The greatest variability in relative $\mu_{\max}$ was observed at 3% (v/v) ethanol, at which some strains exhibited higher values than under the control condition, whereas others already showed a reduction. At higher ethanol concentrations, relative $\mu_{\max}$ values progressively decreased and tended to converge among most strains.

The main exception was NPCC1342, which maintained the highest relative growth rates across nearly all concentrations evaluated. No clear separation according to isolation origin was observed at low or intermediate ethanol concentrations. However, at the highest concentration tested (10%, v/v), wine isolates tended to retain higher relative growth rates than cider isolates, suggesting that differences associated with isolation origin become apparent only under the most severe stress conditions (Figure 2C).

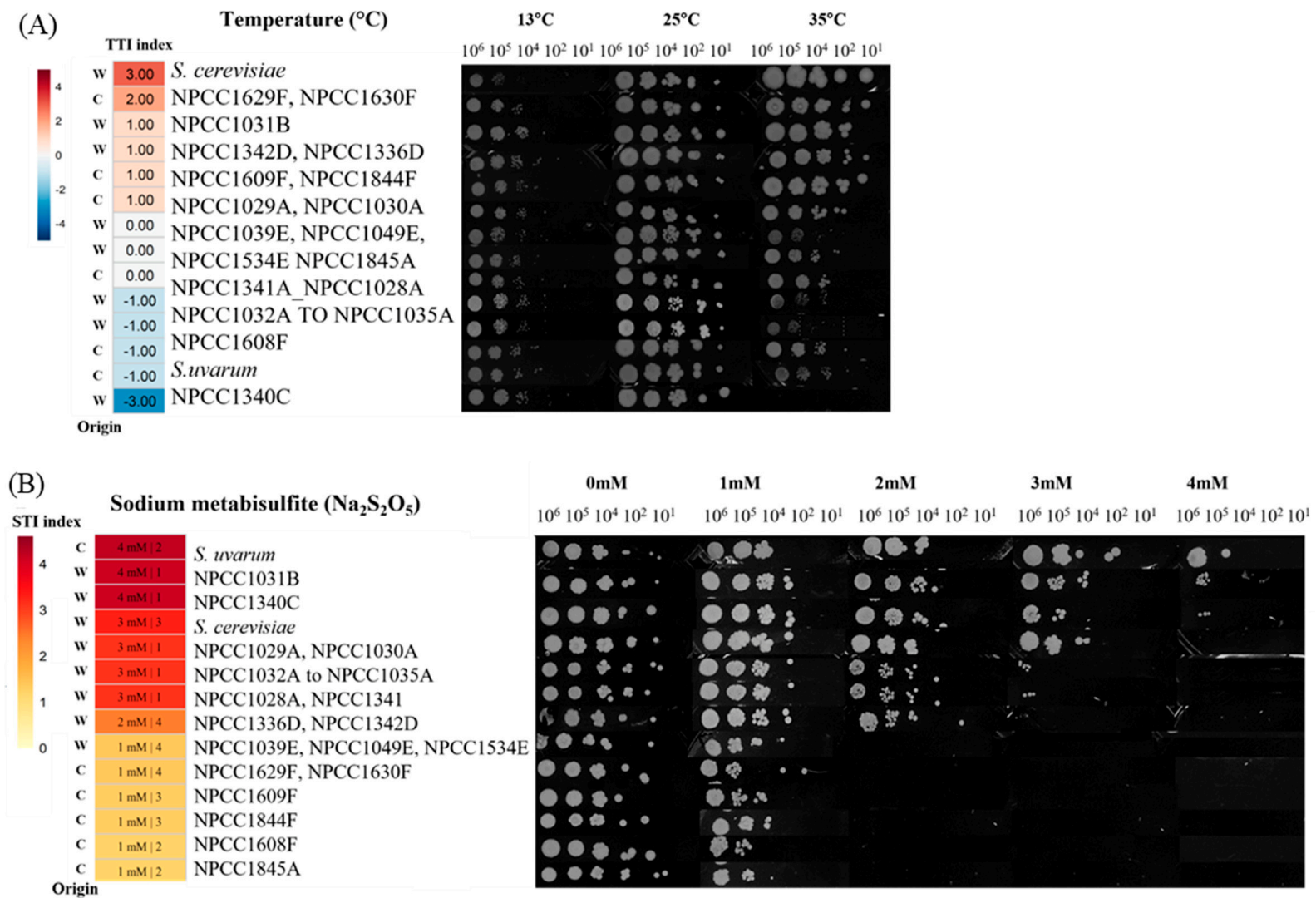


Figure 2. Cont.

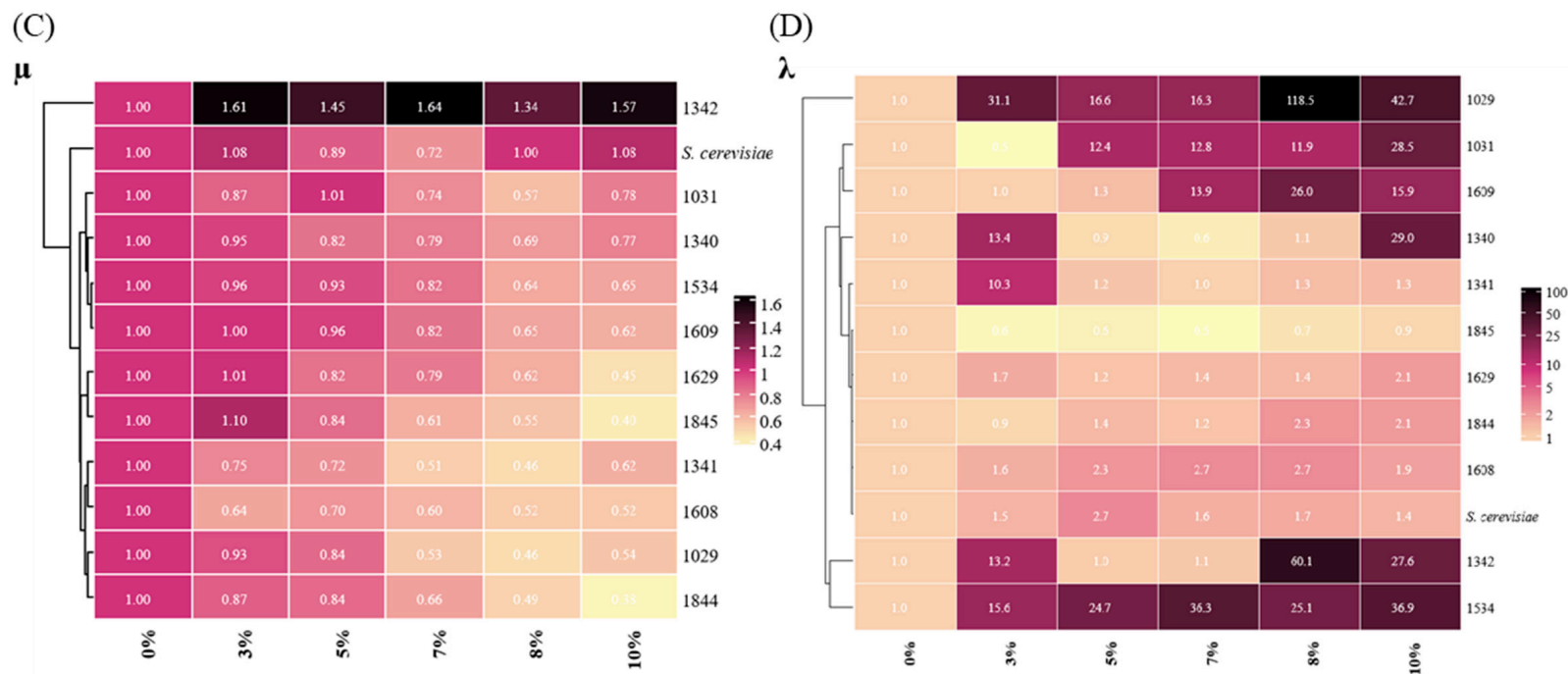


Figure 2. Stress tolerance profiles of *Torulaspora delbrueckii* strains. **(A)** Temperature tolerance assessed at 8, 13, 25, and 35 °C. The thermal tolerance index (TTI) was calculated as the difference between the spot-assay growth scores at 35 and 13 °C (TTI = score₃₅ °C – score₁₃ °C). Positive values (red) indicate thermotolerant phenotypes, negative values (blue) indicate cryotolerant phenotypes, and zero (white) denotes thermal generalists with comparable growth at both temperatures. **(B)** Sulfite tolerance assessed at 0–4 mM sodium metabisulfite (Na₂S₂O₅). The sulfite tolerance index (STI) was calculated as the maximum concentration supporting detectable growth plus one-tenth of the growth score at that concentration (STI = C_{max} + S_{max}/10). Cell colors range from yellow (lower tolerance) to red (higher tolerance), and labels indicate the maximum tolerated concentration and its corresponding growth score. In panels A and B, representative spot assays of serial 10-fold cell dilutions are shown alongside the tolerance indices; strains with equivalent scores are grouped in the same row. The isolation origin is indicated by the letters W (wine-related environments) and C (cider-related environments). *Saccharomyces uvarum* and *S. cerevisiae* were included as reference strains for cryotolerance/high sulfite resistance and thermotolerance/intermediate sulfite resistance, respectively. **(C)** Relative growth rate (μ) under increasing ethanol concentrations (0–10%, v/v), normalized to the ethanol-free condition. Cell colors range from yellow (lower relative growth) to black (higher relative growth), and numerical values are displayed within each cell. Clustering was performed using Euclidean distance and complete linkage based on growth values obtained at 3–10% ethanol. **(D)** Relative lag-phase duration (λ) under increasing ethanol concentrations, expressed as fold change relative to the ethanol-free condition. Cell colors represent log-transformed relative lag values, ranging from yellow (shorter relative lag) to dark magenta (longer relative lag). Strains were grouped using Euclidean distance and complete-linkage hierarchical clustering, and the dendrogram represents similarities in their adaptation to ethanol-induced stress. Commercial *S. cerevisiae* was included as a high-ethanol-tolerance reference strain.

In contrast, lag-phase duration showed marked strain-dependent variation, with several isolates exhibiting pronounced increases at ethanol concentrations $\geq 8\%$ (*v/v*). Hierarchical clustering based on λ revealed a partial association with mitochondrial haplotype: three of the four strains carrying haplotype F, as determined by mtDNA-RFLP—NPCC1608, NPCC1629, and NPCC1844, all isolated from cider—clustered closely and consistently exhibited short lag phases throughout the ethanol concentration range evaluated. Overall, wine isolates displayed greater phenotypic variability and longer lag phases under ethanol stress (Figure 2D).

Strain NPCC1342 showed the best performance in terms of growth rate, matching or exceeding the relative $\mu_{\max}$ of the highly ethanol-tolerant reference strain *S. cerevisiae* NPCC169 at 10% (*v/v*) ethanol (1.57 versus 1.08, respectively). Nevertheless, NPCC1342 also exhibited one of the longest lag phases, particularly at 8% and 10% (*v/v*) ethanol, despite maintaining the highest growth rate. This finding indicates that adaptation-phase duration and subsequent growth rate represent relatively independent components of ethanol tolerance.

3.3. Apple Must Microfermentations of *T. delbrueckii* Isolates

The eleven representative strains selected for the ethanol stress assays were evaluated in apple must microfermentations. All *T. delbrueckii* strains completed fermentation within approximately 19 days and reached residual reducing sugar concentrations below 2 g/L.

The kinetics of soluble-solids consumption showed substantial intraspecific variability, particularly among wine-derived strains. Cider-associated isolates followed relatively homogeneous °Brix-decay trajectories and, during most of the fermentation, tended to reach lower soluble-solids values than most wine-derived strains (Appendix A Figure A1A). Wine-derived strains showed a broader range of D, S, and K values, whereas cider-associated isolates clustered within narrower ranges for these parameters (Table A1). NPCC1031, a wine-derived strain carrying mitochondrial profile B, exhibited the highest kinetic constant for °Brix decrease ($K = 0.008$), while NPCC1844, a pear-cider isolate carrying profile F, showed a similarly high value ($K = 0.007$).

In contrast, CO₂ production curves showed considerably less separation according to isolation origin (Appendix A Figure A1B). No significant differences were detected among *T. delbrueckii* strains in maximum cumulative CO₂ production (A) or maximum CO₂ production rate ($\mu_{\max}$), indicating comparable overall fermentative capacities. The commercial *S. cerevisiae* strain displayed the highest numerical $\mu_{\max}$ value. The clearest origin-associated trend was observed for lag-phase duration (λ): cider-associated strains exhibited shorter and less variable adaptation phases (11.357–16.764 h; mean, 14.262 h) than wine-derived strains (13.805–27.185 h; mean, 18.846 h). NPCC1608, isolated from apple-cider fermentation and carrying mitochondrial profile F, showed the shortest lag phase (11.357 h), whereas the wine-derived strain NPCC1341, assigned to profile A, exhibited the longest (27.185 h); these were the only strains that differed significantly for this parameter. Most *T. delbrueckii* isolates also initiated exponential CO₂ production earlier than the commercial *S. cerevisiae* strain ($\lambda = 24.003$ h) (Table A1).

On the other hand, analysis of the final composition of the fermented musts revealed complete utilization of the main fermentable sugars. This high consumption capacity was reflected in ethanol concentrations ranging from 5.42 to 5.90% (*v/v*), with no clear differentiation according to isolation origin or mitochondrial profile.

Comparison with the initial must revealed significant changes in the organic acid profile. All strains produced significantly higher malic acid concentrations than those initially present in the must (1.421 g/L). The commercial *S. cerevisiae* strain increased the malic acid concentration by 32.2%, whereas the *T. delbrueckii* strains produced significantly higher concentrations (Table 1). Under the conditions evaluated, this consistently greater production by the *T. del-*

brueckii strains indicates a species-associated difference relative to the commercial *S. cerevisiae* control. NPCC1341 and NPCC1608, isolated from wine and cider and carrying mitochondrial profiles A and F, respectively, exhibited the highest malic acid concentrations (Table 1).

Regarding citric acid, 8 of the 11 *T. delbrueckii* strains showed a significant increase relative to the initial concentration in apple must (between 100% and 200% higher than the initial concentration), whereas three strains yielded concentrations below the initial level (Table 1). This response was not associated with either isolation source or mitochondrial profile. In comparison, the commercial strain consumed approximately 45% of the initial citric acid and therefore exhibited the greatest consumption.

Acetic acid production was also particularly variable. The wine strains NPCC1341 and NPCC1342 produced the lowest concentrations (0.071 g/L), whereas the wine strain NPCC1534 and the cider isolates NPCC1844 and NPCC1845 showed the highest accumulation, reaching concentrations greater than that produced by the commercial *S. cerevisiae* strain (Table 1).

Significant differences in glycerol production were observed among the ciders; however, no clear association with either isolation origin or genetic background was detected. Notably, the cider-derived strain NPCC1845 produced the highest glycerol concentration. In contrast, wine-derived strains tended to produce higher erythritol concentrations, with NPCC1340 showing the highest value. Overall, all *T. delbrueckii* strains produced significantly higher concentrations of glycerol and erythritol than the commercial *S. cerevisiae* strain (Table 1).

3.4. Selection of *T. delbrueckii* Strains for Mixed Fermentations

Strains NPCC1340 and NPCC1608 were selected for mixed-fermentation assays based on their genetic, ecological, and phenotypic complementarity. NPCC1340 was isolated from the surface of red grapes and exhibited mitochondrial profile C, a minor profile detected exclusively in this substrate. In contrast, NPCC1608 was isolated from cider and belonged to mitochondrial profile F, which predominated among isolates associated with this substrate (Figure 1). Thus, these strains represent distinct mitochondrial lineages and fermentation-associated environments. Both strains also displayed cryotolerant phenotypes compatible with the low temperatures commonly used in cider production, although to different extents. NPCC1340 exhibited the lowest thermal tolerance index within the collection and was the only strain unable to grow at 35 °C, whereas NPCC1608 showed a more moderate cryotolerant phenotype. The latter was also an exception among isolates carrying mitochondrial profile F, most of which were thermotolerant (Figure 2A).

The most pronounced differences between the two strains were observed in their responses to sulfite and ethanol. NPCC1340 showed high sulfite tolerance, with detectable growth at up to 4 mM Na₂S₂O₅, whereas NPCC1608 grew only up to 1 mM, a response characteristic of the cider isolates (Figure 2B). Contrasting responses were also observed under ethanol stress. At 10% (v/v) ethanol, NPCC1340 retained a higher relative growth rate than NPCC1608 but exhibited a considerably longer lag phase. Conversely, NPCC1608 maintained short adaptation phases throughout the concentration range evaluated, although its growth rate declined more markedly at high ethanol concentrations (Figure 2C,D).

Apple must microfermentations further confirmed this complementarity. NPCC1608 exhibited the shortest lag phase among all strains evaluated, whereas NPCC1340 showed an intermediate value. Nevertheless, both strains displayed comparable overall CO₂ production capacities and completed fermentation (Table A1). Regarding the final chemical composition, NPCC1340 produced the highest erythritol concentration (0.024 g/L) and a high glycerol concentration. In contrast, NPCC1608 produced one of the lowest acetic acid concentrations, together with a high accumulation of malic acid and substantial glycerol production. (Table 1).

Table 1. Chemical composition of apple must after microfermentation with different *Torulaspora delbrueckii* strains and *Saccharomyces cerevisiae* as a control strain.

Compounds	Apple Must	NPCC1029	NPCC1031	NPCC1340	NPCC1341	NPCC1342	NPCC1534	NPCC1608	NPCC1609	NPCC1629	NPCC1844	NPCC1845	Commercial <i>S. cerevisiae</i>
Citric acid (g/L)	0.134 ± 0.002 *†	0.358 ± 0.053 ^{ab}	0.408 ± 0.0233 ^a	0.322 ± 0.0213 ^{ab}	0.11 ± 0.015 ^c	0.115 ± 0.005 ^c	0.291 ± 0.012 ^b	0.325 ± 0.044 ^{ab}	0.337 ± 0.022 ^{ab}	0.091 ± 0.004 ^c	0.366 ± 0.005 ^{ab}	0.358 ± 0.007 ^{ab}	0.076 ± 0.004
Malic acid (g/L)	1.421 ± 0.011 *†	2.179 ± 0.021 ^{bc}	2.142 ± 0.101 ^{bc}	2.152 ± 0.016 ^{bc}	2.442 ± 0.048 ^a	2.136 ± 0.024 ^{bc}	2.068 ± 0.012 ^c	2.421 ± 0.055 ^a	2.206 ± 0.007 ^{bc}	2.209 ± 0.009 ^{bc}	2.073 ± 0.038 ^c	2.280 ± 0.016 ^{ab}	1.878 ± 0.009
Acetic acid (g/L)	NM	0.131 ± 0.022 ^{bc}	0.128 ± 0.007 ^{bc}	0.123 ± 0.012 ^{bc}	0.071 ± 0.017 ^c	0.071 ± 0.026 ^c	0.315 ± 0.002 ^a	0.081 ± 0.001 ^{bc}	0.164 ± 0.034 ^{bc}	0.170 ± 0.046 ^b	0.297 ± 0.033 ^a	0.339 ± 0.003 ^a	0.231 ± 0.093
Succinic acid (g/L)	NM	0.608 ± 0.082	0.566 ± 0.024	0.626 ± 0.098	0.628 ± 0.051	0.560 ± 0.012	0.678 ± 0.130	0.693 ± 0.027	0.545 ± 0.127	0.596 ± 0.084	0.557 ± 0.201	0.428 ± 0.270	0.346 ± 0.039
Lactic acid (g/L)	NM	0.575 ± 0.024 ^{bc}	0.496 ± 0.028 ^{cd}	0.464 ± 0.070 ^d	0.511 ± 0.004 ^{cd}	0.497 ± 0.002 ^{cd}	0.502 ± 0.014 ^{cd}	0.563 ± 0.011 ^{bcd}	0.633 ± 0.009 ^b	0.477 ± 0.007 ^{cd}	0.743 ± 0.021 ^a	0.633 ± 0.029 ^b	0.419 ± 0.016
Glucose (g/L)	34.841 ± 0.011 *	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Fructose (g/L)	70.914 ± 0.014 *	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Sorbitol (g/L)	7.623 ± 0.001 *	7.191 ± 0.061	7.131 ± 0.784	6.930 ± 0.456	7.446 ± 0.267	7.289 ± 0.098	7.307 ± 0.049	6.833 ± 0.508	6.771 ± 0.486	7.363 ± 0.002	7.297 ± 0.120	7.395 ± 0.013	7.298 ± 0.038
Erythritol (g/L)	NM	0.022 ± 0.004 ^{ab}	0.018 ± 0.001 ^{abc}	0.024 ± 0.002 ^a	0.015 ± 0.002 ^{abc}	0.016 ± 0.003 ^{abc}	0.008 ± 0.002 ^{de}	0.009 ± 0.002 ^{cde}	0.006 ± 0.002 ^e	0.013 ± 0.001 ^{cde}	0.006 ± 0.001 ^e	0.01 ± 0.001 ^{cde}	0.005 ± 0.002
Glycerol (g/L)	NM	3.162 ± 0.065 ^c	4.205 ± 0.274 ^b	4.27 ± 0.081 ^{ab}	3.122 ± 0.017 ^c	3.797 ± 0.132 ^b	4.355 ± 0.213 ^{ab}	4.072 ± 0.219 ^b	4.369 ± 0.072 ^{ab}	3.876 ± 0.122 ^b	3.925 ± 0.062 ^b	4.810 ± 0.102 ^a	2.882 ± 0.007
Ethanol (% v/v)	NM	5.564 ± 0.202	5.815 ± 0.248	5.597 ± 0.126	5.417 ± 0.153	5.68 ± 0.171	5.899 ± 0.183	5.722 ± 0.188	5.673 ± 0.007	5.515 ± 0.478 ^{ab}	5.663 ± 0.056	5.567 ± 0.118	4.881 ± 0.094

Different superscript letters within the same row indicate significant differences among *T. delbrueckii* strains (Tukey's test, $p < 0.05$). The commercial *S. cerevisiae* control strain was excluded from the ANOVA. † Indicates a significant difference between the unfermented must and fermented musts, as determined by one-way ANOVA followed by Tukey's multiple comparison test ($p < 0.05$). ND, not detected. NM, not measured. * For compounds measured in unfermented must, the standard deviation was calculated from duplicate HPLC injections of the same sample (technical replicates, $n = 2$) and therefore represents analytical variability rather than biological variability.

In addition, neither strain exhibited killer activity against the commercial *S. cerevisiae* strain (Table A2). The absence of detectable antagonistic activity suggests that these strains may be compatible with the commercial strain, supporting their potential use in mixed fermentations.

In summary, the two strains were selected due to their distinct isolation origins and mitochondrial profiles, their marked cryotolerance—which is advantageous for conducting fermentations under the low-temperature conditions typical of Patagonian cider production—their contrasting tolerance to sulfite and ethanol, and their balanced production of compounds of technological interest to the cider industry.

3.5. Mixed-Culture Fermentations in Sterile Apple Must

As observed in the microfermentation assays, all pure and mixed fermentations were completed within 19 days and left less than 2 g/L of residual reducing sugars. However, fermentation kinetics differed among inoculation strategies. The commercial *S. cerevisiae* strain exhibited the highest °Brix reduction rate constant (K), the greatest overall decrease in soluble solids (S), and the lowest final soluble solids value (D), indicating the most vigorous fermentation. Mixed fermentations showed intermediate D and S values and a greater overall reduction in soluble solids than the corresponding pure *T. delbrueckii* cultures (Table A3).

Viable-cell counts at the end of fermentation (477 h) ranged from 4.15×10^8 to 5.48×10^8 CFU/mL, with an overall mean of $(4.98 \pm 0.93) \times 10^8$ CFU/mL. No significant differences in viable-cell counts were detected among pure and mixed fermentations. Despite these similar total counts, species-specific monitoring revealed that *T. delbrueckii* remained approximately one order of magnitude more abundant than *S. cerevisiae* at all sampling times following the addition of the latter. Thus, although both species were inoculated at 1×10^6 CFU/mL, the commercial strain—added 48 h after *T. delbrueckii* in the sequential inoculation scheme—never surpassed the initially inoculated species. Accordingly, *T. delbrueckii* and *S. cerevisiae* represented approximately 90% and 10% of the viable population, respectively, throughout the mixed fermentations.

The final chemical composition of the ciders showed that the strain-specific metabolic traits previously identified in pure cultures were largely retained in mixed fermentations (Table 2). Fermentations involving NPCC1608 were characterized by higher concentrations of malic and succinic acids. Notably, the *Td* NPCC1608–*S. cerevisiae* mixed culture produced the highest concentration of malic acid, a high concentration of succinic acid, and the lowest concentration of acetic acid among all treatments. In contrast, the commercial *S. cerevisiae* strain produced the highest concentrations of lactic and acetic acids.

Strain NPCC1340 exhibited its characteristic polyol-producing profile in both pure and mixed cultures. The *Td* NPCC1340–*S. cerevisiae* mixed fermentation produced the highest erythritol concentration and approximately 50% more glycerol than the pure *S. cerevisiae* culture (Table 2). These results indicate that the metabolic contribution of *T. delbrueckii* was not masked by the presence of *S. cerevisiae*, which produced the lowest glycerol and erythritol concentrations. Moreover, the low acetic acid production and enhanced polyol accumulation associated with *T. delbrueckii* were also observed in the mixed fermentations. Ethanol concentrations were comparable among treatments, indicating that the metabolic diversification achieved through mixed inoculation did not compromise alcoholic fermentation.

Principal component analysis further supported the occurrence of distinct and complementary metabolic profiles (Figure 3). Fermentations involving NPCC1608 were mainly associated with malic, succinic, and citric acids, as well as sorbitol and ethanol, whereas those involving NPCC1340 were more closely associated with glycerol and erythritol. Pure

S. cerevisiae fermentations were positioned in the direction of greater lactic and acetic acid production and higher values of the kinetic parameters associated with soluble solids reduction. Mixed cultures occupied positions close to their corresponding pure *T. delbrueckii* cultures but showed substantially improved kinetic parameters. Together with the population dynamics, this pattern supports the successful establishment and primary contribution of *T. delbrueckii* to the final chemical composition, while the commercial *S. cerevisiae* strain contributed mainly to fermentation kinetics.

Table 2. Chemical composition of pure and mixed apple must fermentations performed with two selected *Torulaspora delbrueckii* strains.

	Apple Must	TdNPCC1608	TdNPCC1340	TdNPCC1608 + Sc	TdNPCC1340 + Sc	<i>S. cerevisiae</i>
Citric acid (g/L)	0.140 ± 0.002 *	0.074 ± 0.006	0.068 ± 0.004	0.068 ± 0.005	0.060 ± 0.004	0.062 ± 0.001
Malic acid (g/L)	6.960 ± 0.051 *	7.084 ± 0.001 ^a	6.668 ± 0.210 ^{ab}	7.133 ± 0.143 ^a	6.300 ± 0.122 ^b	6.632 ± 0.045 ^{ab}
Succinic acid (g/L)	NM	1.780 ± 0.091 ^c	1.465 ± 0.008 ^{ab}	1.650 ± 0.038 ^{bc}	1.375 ± 0.061 ^a	1.343 ± 0.00 ^a
Lactic acid (g/L)	NM	0.031 ± 0.006 ^a	0.027 ± 0.004 ^a	0.074 ± 0.004 ^b	0.057 ± 0.008 ^b	0.120 ± 0.002 ^c
Acetic acid (g/L)	NM	0.022 ± 0.001 ^{ab}	0.081 ± 0.000 ^{ab}	0.019 ± 0.001 ^a	0.068 ± 0.035 ^{ab}	0.088 ± 0.014 ^b
Glucose (g/L)	43.580 ± 0.052 *	ND	ND	ND	ND	ND
Fructose (g/L)	76.840 ± 0.180 *	ND	ND	ND	ND	ND
Sorbitol (g/L)	10.505 ± 0.068 *	7.483 ± 0.380	7.879 ± 0.308	7.674 ± 0.059	6.683 ± 1.266	7.311 ± 0.109
Erythritol (g/L)	NM	0.021 ± 0.003 ^{abc}	0.038 ± 0.002 ^{bc}	0.014 ± 0.001 ^{ab}	0.044 ± 0.016 ^c	0.005 ± 0.000 ^a
Glycerol (g/L)	NM	3.900 ± 0.093 ^a	4.743 ± 0.229 ^b	3.655 ± 0.012 ^a	4.588 ± 0.146 ^b	2.815 ± 0.026 ^c
Ethanol (% v/v)	NM	6.566 ± 0.124	6.381 ± 0.239	6.334 ± 0.019	6.217 ± 0.027	6.362 ± 0.021

Superscript letters per row indicate significant differences between strains. Tukey's test, $p < 0.05$. The commercial *S. cerevisiae* control strain was excluded from the ANOVA. ND, not detected. NM, not measured. * For compounds measured in unfermented must, the standard deviation was calculated from duplicate HPLC injections of the same sample (technical replicates, $n = 2$) and therefore represents analytical variability rather than biological variability.

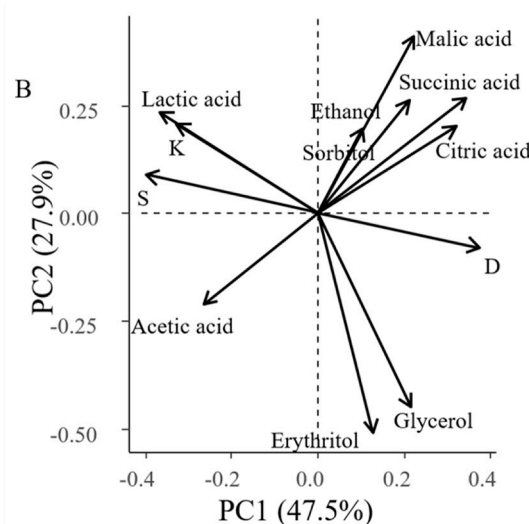
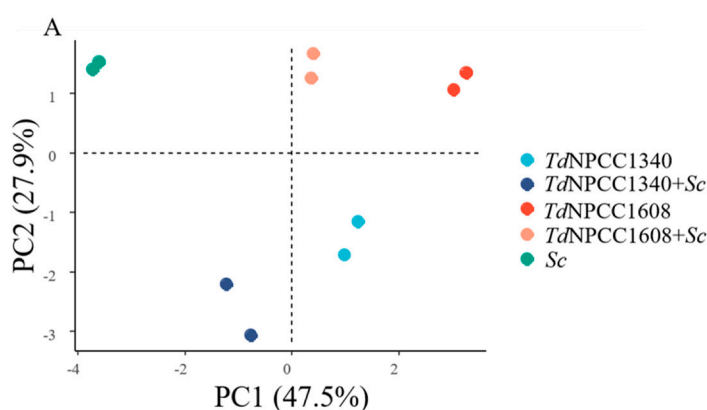


Figure 3. Principal component analysis (PCA) of physicochemical and kinetic data from pure and mixed fermentations involving *Torulaspora delbrueckii* strains NPCC1340 and NPCC1608 and the commercial *Saccharomyces cerevisiae* strain. (A) Score plot showing the distribution of fermentations according to physicochemical variables determined by HPLC—including organic acids, glycerol, erythritol, sorbitol, and ethanol—and the kinetic parameters D, S, and K obtained by nonlinear modeling of the °Brix depletion curves. (B) Loading plot showing the magnitude and direction of each variable's contribution to the first two principal components.

4. Discussion

The present study highlights the substantial intraspecific diversity of *Torulaspora delbrueckii* and its relevance to the selection of strains for cider production. By integrating ecological origin, mitochondrial diversity, stress responses, fermentation kinetics, metabolite production, and performance in sequential cultures, we identified traits associated with isolation origin as well as traits that varied primarily among strains. These findings reinforce the importance of combining genetic and phenotypic characterization to identify strains with complementary properties suited to cider fermentation.

The greater mitochondrial diversity detected among wine-associated isolates, compared with the limited diversity observed in the cider-associated group, may reflect the evolutionary history of the grape/wine lineage. This lineage was globally dispersed with the expansion of viticulture and remains only weakly specialized, thereby retaining substantial ancestral genetic diversity [12,24]. In contrast, cider-associated *T. delbrueckii* populations remain poorly characterized. Although this species has been recovered from cider fermentations and its use in aromatic cider production is emerging as a promising application [20,21,33], current evidence is insufficient to determine whether cider isolates constitute a differentiated population or belong to the broader genetic group associated with other bioprocesses.

The phenotypic data further showed that mitochondrial similarity did not necessarily predict thermal performance. Growth assays revealed intraspecific variability between 13 and 35 °C, whereas no isolate grew at either 8 or 37 °C. Consequently, the collection examined here cannot be strictly characterized as cryotolerant or thermotolerant. Nevertheless, growth at 37 °C is not uniformly absent in *T. delbrueckii*, because phenotypic surveys have reported strain-dependent growth at this temperature, frequently weak or slow [4]. The recurrent coexistence of *T. delbrueckii* with different *Saccharomyces* species could contribute to thermal niche partitioning. Most cider-associated *T. delbrueckii* isolates showed greater relative fitness at high temperature, whereas *S. uvarum*, with which this species coexists in Patagonian cider environments, is characterized by greater fitness at low temperatures [7,8]. Conversely, wine-associated *T. delbrueckii* isolates, which coexist predominantly with the more thermotolerant *S. cerevisiae* [25], displayed either low-temperature-biased or intermediate thermal responses. These contrasting patterns resemble the thermal differentiation described among sympatric *Saccharomyces* species, including *S. cerevisiae* and *S. paradoxus*, *S. kudriavzevii*, or *S. uvarum*, in which temperature-dependent fitness favors different species at high and low temperatures [34–39]. Accordingly, the high-temperature-biased response of cider-associated *T. delbrueckii* and the greater relative fitness of wine-associated isolates at low or intermediate temperatures may reflect ecological differentiation that reduces direct competition with their respective *Saccharomyces* counterparts.

Although thermal responses also differed according to isolation origin, sulfite tolerance showed an even clearer association with the ecological source of the isolates. All cider-associated isolates grew only up to 1 mM Na₂S₂O₅, whereas wine isolates spanned a broader range of tolerance and strains carrying mitochondrial profiles B and C grew at concentrations up to 4 mM. The weak response of NPCC1845, despite sharing profile A with wine isolates, further supports an ecological rather than strictly mitochondrial association. Sulfite is a major selective agent in wine environments, and recurrent exposure has driven convergent adaptation in wine strains of *S. cerevisiae* and *S. uvarum* [40,41]. Although the molecular basis of sulfite tolerance in our *T. delbrueckii* isolates remains unknown, the observed origin-associated pattern is compatible with analogous selection imposed by winemaking practices. From an applied perspective, the high tolerance of NPCC1340 could facilitate implantation in sulfited must, whereas the uniform sensitivity of cider isolates indicates that sulfite dose and inoculation timing should be considered when developing regional starters.

Ethanol stress revealed a different and more nuanced form of variation. Increasing ethanol concentration reduced relative growth rates and prolonged lag phases in all strains, but the two response variables were not coupled. NPCC1342 retained the highest relative growth rates across most concentrations and performed similarly to the *S. cerevisiae* control at 10% (v/v) ethanol, yet it also displayed one of the longest adaptation phases at 8 and 10%. At the highest ethanol concentration, wine isolates tended to retain greater relative growth rates, whereas several cider isolates carrying profile F maintained comparatively short lag phases. Thus, wine origin was associated mainly with growth after adaptation, while cider origin was associated with faster entry into growth rather than greater ethanol tolerance per se. This distinction is technologically relevant because rapid adaptation can favor early establishment, whereas sustained growth under increasing ethanol becomes more important toward fermentation completion. The partial independence of these traits also agrees with the multigenic and condition-dependent nature of ethanol tolerance described in *T. delbrueckii* [42].

The fermentation assays translated some, but not all, of these stress-response patterns into apple must. Cider-associated strains displayed shorter and less variable lag phases for CO₂ production and tended to remain lower on the °Brix decay curves during most of fermentation.

All isolates completed fermentation and produced comparable ethanol concentrations. Fermentation chemistry was even more strongly strain dependent. All *T. delbrueckii* strains produced significantly more glycerol and erythritol than the commercial *S. cerevisiae* strain, consistent with the recognized capacity of *T. delbrueckii* to redirect carbon toward polyol production [43]. The higher glycerol production may be related to a more developed glycerol–pyruvic pathway in *T. delbrueckii* compared with other yeasts, as previously suggested [13,44,45]. In addition, the higher erythritol production may be associated with differences in the availability of precursors derived from the pentose phosphate pathway, which plays a central role in erythritol biosynthesis in yeasts [46]. However, glycerol production was not associated with isolation origin or mitochondrial profile: the highest concentration was produced by the cider isolate NPCC1845. Erythritol showed a tendency toward higher values among wine-derived strains, with NPCC1340 reaching the maximum concentration. Organic-acid patterns likewise resisted broad species-level generalization, but each acid can be rationalized through known aspects of yeast central carbon metabolism. Regarding malic acid, all *T. delbrueckii* strains, and even the commercial *S. cerevisiae* control, ended fermentation with higher concentrations than those initially present in the apple must. Yeasts of the genus *Saccharomyces* and related species are generally poor consumers of extracellular malate, but they are able to synthesize malate de novo through an anaplerotic route in which pyruvate is carboxylated to oxaloacetate and subsequently reduced to malate, a reductive pathway that operates during active fermentation independently of the malic acid already present in the medium [47,48]. Because this synthetic flux coexists with only a weak and strain-dependent malate-consuming activity in *T. delbrueckii* [49,50], net accumulation rather than net degradation of malic acid is expected, particularly in a matrix such as apple must where the naturally high malate content further limits the relative impact of any consumption. NPCC1341 and NPCC1608 exceeding the other strains in final malic-acid concentration therefore reflects strain-level differences in the balance between this synthetic route and their limited catabolic capacity, rather than a uniform species response. Citric acid, another TCA-cycle-derived organic acid, showed a similarly heterogeneous pattern: eight of the eleven *T. delbrueckii* strains produced significantly more citric acid than the initial must, whereas three strains and the commercial *S. cerevisiae* control consumed it. Citrate occupies a branch point in yeast anaplerotic metabolism, being both a product of citrate synthase activity and a potential carbon/energy source once fermentable sugars and

nitrogen become limiting; whether a given strain nets synthesis or consumption depends on nitrogen availability, the activity of TCA-cycle and glyoxylate-shunt enzymes, and citrate transport, all of which are known to vary among yeast strains and species [51,52]. The markedly greater citric acid consumption by the commercial *S. cerevisiae* strain is consistent with this species' more complete depletion of nitrogen and more active late-fermentation catabolism of TCA-cycle intermediates relative to the *T. delbrueckii* isolates evaluated here. Acetic acid production followed a comparable strain-dependent trend. Although low volatile-acidity production is one of the most consistently reported oenological traits of *T. delbrueckii* [13–15,53], this property is not fixed at the species level: recent screenings across genetically diverse *T. delbrueckii* strains have shown substantial intraspecific variation in acetate and acetate-ester metabolism, with some strains behaving similarly to conventional *S. cerevisiae* [15]. Accordingly, the elevated acetic-acid concentrations observed here in NPCC1534, NPCC1844, and NPCC1845—exceeding that of the commercial *S. cerevisiae* strain—together with the low values recorded for NPCC1341 and NPCC1342, indicate that low volatile acidity should not be treated as an intrinsic property of every *T. delbrueckii* strain, but rather as a trait requiring strain-specific verification, particularly when transferring evidence generated in grape must to a compositionally distinct matrix such as apple must. The combined results instead demonstrate that polyol production is a robust species-associated contribution in this collection, whereas the magnitude of glycerol production and the organic-acid profile require strain-specific selection.

This multidimensional variation guided the selection of NPCC1340 and NPCC1608 for mixed fermentations. The strains represented distinct ecological origins and mitochondrial profiles and combined complementary technological traits. NPCC1340 showed the strongest low-temperature bias, high sulfite tolerance, relatively strong growth at 10% ethanol after a prolonged lag, and the highest erythritol production. NPCC1608 combined a short adaptation phase, weak sulfite tolerance, substantial glycerol production, high malic acid accumulation, and low acetic acid production. Importantly, neither strain exhibited killer activity against the commercial *S. cerevisiae* strain nor against the sensitive *Candida glabrata* strain used as the K2 killer toxin control. This result is consistent with that reported by Renault et al. (2009) [54], who evaluated the killer activity of *Torulaspora delbrueckii* strains against a *S. cerevisiae* strain sensitive to the K2 killer toxin. Although a negative killer assay does not exclude other forms of interaction, it ruled out one potential antagonistic mechanism and supported the evaluation of these strains as compatible partners in sequential fermentation.

The sequential fermentations supported a complementary interaction between the two species: *S. cerevisiae* improved fermentation kinetics, whereas *T. delbrueckii* remained metabolically and numerically relevant throughout the process. The persistence of *T. delbrueckii* contrasts with the population dynamics reported by Taillandier et al. (2014) [55], who observed a progressive decline in its viability after the addition of *S. cerevisiae*. However, Renault et al. (2016) [56] found that sequential inoculation favored the development and prolonged dominance of *T. delbrueckii*, whereas simultaneous inoculation allowed *S. cerevisiae* to become dominant at an earlier stage. The persistence observed in our mixed cultures is therefore consistent with a priority effect promoted by sequential inoculation and indicates that the outcome of the interaction depends strongly on the strains, fermentation matrix, nutritional conditions, and inoculation strategy. A comparable effect of inoculation order was observed by Mazzucco et al. (2025) [11] in Patagonian cider fermentations with *Pichia kudriavzevii* and *S. cerevisiae*. In that study, *P. kudriavzevii* persisted until the end of all mixed fermentations, but its relative abundance depended on the inoculation strategy: comparable populations of both species were obtained when *P. kudriavzevii* was inoculated first, whereas *S. cerevisiae* predominated when it was established before

the non-*Saccharomyces* partner. In comparison, the sustained numerical predominance of *T. delbrueckii* observed here suggests a stronger priority effect under our sequential fermentation conditions.

This prolonged implantation may also explain why the distinctive metabolic profiles of the selected *T. delbrueckii* strains remained evident in the mixed ciders despite the kinetic contribution of *S. cerevisiae* [54–56].

The persistence of *T. delbrueckii* during sequential fermentation was also reflected in the chemical differentiation of the mixed ciders. Rather than converging toward a common intermediate composition, each mixed fermentation remained associated with the metabolic profile of the corresponding *T. delbrueckii* strain, demonstrating that the contribution of this species was strongly strain-dependent. Mazzucco et al. (2025) [11] similarly found that population dynamics were linked to the chemical composition of mixed ciders: the sequential treatment dominated by *P. kudriavzevii* resembled its pure culture, whereas fermentations in which *S. cerevisiae* predominated showed greater contributions of lactic and succinic acids and lower acetic acid production. Nevertheless, the direction of malic-acid modulation differed between the two studies. Mixed fermentations with *P. kudriavzevii* enhanced malic-acid consumption, consistent with the malate-degrading ability of that species, whereas our fermentations involving *T. delbrueckii* were associated with higher malic-acid concentrations. Together, these findings show that the persistence of a non-*Saccharomyces* partner does not produce a uniform effect on cider acidity; rather, the resulting profile depends on the species, strain, and population dynamics established by the inoculation strategy. Intraspecific variation has also been reported in wine, where sequential fermentations involving different *T. delbrueckii* strains produced distinct chemical and aromatic profiles, and broader screenings confirmed that fermentation outcomes depend on the specific combination of *T. delbrueckii* and *S. cerevisiae* strains [57,58]. Evidence for cider remains more limited: Tocci et al. (2023) [20] reported strain-dependent behavior for two commercial *T. delbrueckii* strains during cider and sparkling-cider production, whereas other mixed-cider studies evaluated a single *T. delbrueckii* strain [21]. Our results extend this evidence by showing that regional *T. delbrueckii* strains can generate clearly differentiated mixed ciders even when combined with the same *S. cerevisiae* starter and fermentation strategy. Consequently, starter development should focus on selecting compatible strain pairs rather than assigning a uniform technological effect to *T. delbrueckii*.

Overall, this study supports regional yeast bioprospecting as a strategy for developing differentiated Patagonian ciders. The selected *T. delbrueckii* strains generated distinct chemical profiles in sequential fermentation with *S. cerevisiae* while maintaining satisfactory fermentation performance. Further validation at pilot and industrial scales, together with volatile-aroma and sensory analyses, will determine the technological and commercial potential of these strain combinations.

5. Conclusions

Patagonian *T. delbrueckii* isolates exhibited substantial intraspecific genetic and phenotypic diversity. Differences among isolation sources were observed for several technological traits, particularly sulfite tolerance, adaptation behavior, and, to a lesser extent, thermal response. Cider-associated isolates were uniformly sensitive to sulfite, whereas wine-derived isolates displayed broader sulfite tolerance. Nevertheless, overall fermentative capacity and most metabolite concentrations were primarily strain dependent and could not be consistently predicted from isolation origin or mitochondrial profile. Thus, while the isolation source was associated with specific technological traits, strain-level variability was the main determinant of fermentative and metabolic performance.

All *T. delbrueckii* strains produced more glycerol, erythritol, and malic acid than the commercial *S. cerevisiae* strain in apple must fermentations, although glycerol and organic-acid profiles varied markedly among isolates. In mixed fermentations of apple must, *S. cerevisiae* improved fermentation kinetics, while NPCC1340 and NPCC1608 contributed their distinctive metabolic signatures and *T. delbrueckii* remained the dominant population throughout fermentation.

These findings identify NPCC1340 and NPCC1608 as promising regional starter candidates for producing differentiated Patagonian ciders. Further validation at pilot and industrial scales, together with volatile-aroma profiling and sensory evaluation, will be required to establish the technological and commercial potential of these strain combinations.

Author Contributions: V.K. performed the experimental work and prepared the original draft of the manuscript. M.G.F. contributed to the experimental design, data analysis and interpretation, and writing and revision of the manuscript. M.E.R. contributed to data interpretation and critically reviewed the manuscript. C.A.L. conceived and supervised the study, contributed to the experimental design and interpretation of the results, and reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

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Appendix A

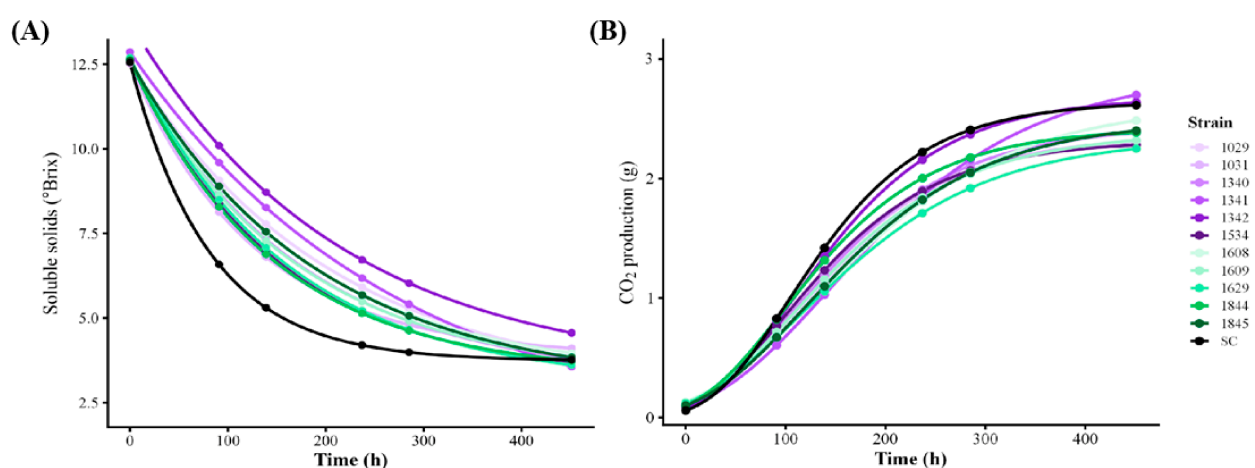


Figure A1. Kinetics of soluble solids consumption (°Brix, (A)) and CO₂ production (B) and during apple must microfermentations performed with different *Torulaspora delbrueckii* strains. A commercial *Saccharomyces cerevisiae* strain (Sc) was included as a positive control. CO₂ production was fitted using the modified Gompertz model, whereas soluble solids consumption was described using an exponential decay model. Purple shades indicate isolates from wine-related environments, whereas green shades indicate isolates from cider-related environments.

Table A1. Kinetic parameters of cider microfermentations conducted with *Torulaspora delbrueckii* strains and *Saccharomyces cerevisiae* as a control strain.

Strain	CO ₂ Production			°Brix Decrease		
	A	μ_{max}	λ	D	S	K
NPCC1029	2.461 ± 0.249	0.009 ± 0.001	18.850 ± 1.842 ^{ab}	2.883 ± 0.010 ^b	9.671 ± 0.003 ^{bc}	0.005 ± 3.94 × 10 ^{−4} cde
NPCC1031	2.361 ± 0.017	0.010 ± 0.001	16.194 ± 2.214 ^{ab}	3.845 ± 0.054 ^a	8.670 ± 0.070 ^c	0.008 ± 2.06 × 10 ^{−4} a
NPCC1340	2.442 ± 0.029	0.010 ± 0.001	16.751 ± 1.846 ^{ab}	3.169 ± 0.051 ^b	9.480 ± 0.050 ^{bc}	0.006 ± 1.33 × 10 ^{−4} cd
NPCC1341	2.894 ± 0.106	0.009 ± 0.001	27.185 ± 1.760 ^a	1.385 ± 0.2331 ^c	11.472 ± 0.261 ^a	0.005 ± 1.11 × 10 ^{−4} e
NPCC1342	2.558 ± 0.177	0.010 ± 0.002	20.291 ± 2.513 ^{ab}	3.335 ± 0.304 ^{ab}	10.403 ± 1.249 ^{ab}	0.005 ± 1.01 × 10 ^{−4} e
NPCC1534	2.320 ± 0.059	0.010 ± 0.001	13.805 ± 1.451 ^{ab}	3.237 ± 0.001 ^b	9.409 ± 0.001 ^{bc}	0.007 ± 5.51 × 10 ^{−4} ab
NPCC1608	2.634 ± 0.176	0.008 ± 0.001	11.357 ± 2.086 ^b	3.215 ± 0.074 ^b	9.354 ± 0.031 ^{bc}	0.006 ± 5.51 × 10 ^{−4} cde
NPCC1609	2.383 ± 0.075	0.009 ± 0.001	13.456 ± 2.256 ^{ab}	3.248 ± 0.089 ^b	9.374 ± 0.089 ^{bc}	0.006 ± 2.24 × 10 ^{−4} cde
NPCC1629	2.470 ± 0.176	0.009 ± 0.001	16.211 ± 9.435 ^{ab}	3.046 ± 0.023 ^b	9.645 ± 0.014 ^{bc}	0.006 ± 1.18 × 10 ^{−4} cde
NPCC1844	2.421 ± 0.204	0.011 ± 0.001	13.520 ± 0.562 ^{ab}	3.335 ± 0.144 ^{ab}	9.292 ± 0.161 ^{bc}	0.007 ± 5.15 × 10 ^{−4} ab
NPCC1845	2.497 ± 0.165	0.009 ± 0.001	16.764 ± 2.992 ^{ab}	2.983 ± 0.100 ^b	9.630 ± 0.096 ^{bc}	0.005 ± 9.84 × 10 ^{−5} cde
Commercial <i>S. cerevisiae</i>	2.505 ± 0.193	0.012 ± 0.001	24.003 ± 2.061	3.727 ± 0.038	8.814 ± 0.036	0.012 ± 1.08 × 10 ^{−4}

CO₂ production kinetics were modeled using the modified Gompertz equation [31], and °Brix decrease kinetics were modeled using the equation described by [32]. Different superscript letters within the same column indicate significant differences among *T. delbrueckii* strains (Tukey’s test, *p* < 0.05). The control commercial *S. cerevisiae* strain was excluded from the ANOVA.

Table A2. Antagonistic activity of *T. delbrueckii* strains against the commercial *S. cerevisiae* strain.

Strains Grown on Plates	<i>S. cerevisiae</i> K2 (Killer Type)	<i>Td</i> NPCC1029	<i>Td</i> NPCC1031	<i>Td</i> NPCC1340	<i>Td</i> NPCC1341	<i>Td</i> NPCC1342	<i>Td</i> NPCC1534	<i>Td</i> NPCC1608	<i>Td</i> NPCC1609	<i>Td</i> NPCC1629	<i>Td</i> NPCC1844	<i>Td</i> NPCC1845
<i>Candida glabrata</i> NPCC106 (sensitive strain)	+	−	−	−	−	−	−	−	−	−	−	−
<i>S. cerevisiae</i> commercial strain	+	−	−	−	−	−	−	−	−	−	−	−

Antagonistic activity results between the *T. delbrueckii* strains and the commercial *S. cerevisiae* strain. The (+) and (−) signs indicate the presence and absence of an inhibition halo, respectively. *S. cerevisiae* K2 and *Candida glabrata* were used as the killer and sensitive control strains, respectively.

Table A3. Kinetic parameters of pure and mixed fermentations with *T. delbrueckii* and commercial *S. cerevisiae* strain.

	NPCC1608	NPCC1340	<i>S. cerevisiae</i>	NPCC1608 + <i>Sc</i>	NPCC1340 + <i>Sc</i>
D	4.344 ± 0.011 ^a	4.177 ± 0.060 ^{ab}	3.857 ± 0.011 ^c	3.974 ± 0.102 ^{bc}	4.031 ± 0.057 ^{bc}
S	9.257 ± 0.004 ^d	9.506 ± 0.026 ^c	10.240 ± 0.028 ^a	9.880 ± 0.056 ^b	9.832 ± 0.053 ^b
K	0.007 ± 7.64 × 10 ^{−5} ^b	0.007 ± 7.64 × 10 ^{−5} ^b	0.010 ± 8.34 × 10 ^{−5} ^a	0.006 ± 2.35 × 10 ^{−4} ^b	0.007 ± 7.94 × 10 ^{−6} ^b

The kinetics of °Brix decrease was evaluated with the [32] equation. Superscript letters per row indicate significant differences between strains. Tukey test *p*-value 0.05.

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