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Importance of micronutrients and organic nitrogen in fermentations with *Torulaspora delbrueckii* and *Saccharomyces cerevisiae*

Helena Roca-Mesa, Ester Delgado-Yuste, Albert Mas, María-Jesús Torija *, Gemma Beltran

Universitat Rovira i Virgili, Departament de Bioquímica i Biotecnologia, Grup de Biotecnologia Enològica, Facultat d'Enologia, c/ Marcel·lí Domingo, 1, 43007 Tarragona, Catalonia, Spain

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A B S T R A C T

The current use of non-*Saccharomyces* yeasts in mixed fermentations increases the relevance of the interactions between yeast species. In this work, the interactions between *Saccharomyces cerevisiae* and *Torulaspora delbrueckii* were analyzed. For this purpose, fermentations with and without contact between strains of those yeast species were performed in synthetic must. Fermentation kinetics, yeast growth and dynamics were measured over time. Additionally, the effects of nitrogen and other nutrient supplementations on the mixed fermentations were determined. Our results showed that *S. cerevisiae* did not always dominate the sequential fermentations, and experiments without yeast contact (in which *T. delbrueckii* cells were removed from the medium before inoculating *S. cerevisiae* at 48 h) resulted in stuck fermentations except when the inoculum size was increased (from 2 $\times$ 10^6 to 10^8 cells/mL) or there was a supplementation of thiamine, zinc and amino acids at the same concentration as initially found in the synthetic must. Our findings highlight the importance of inoculum size and ensuring the availability of enough micronutrients for all yeast species, especially in sequential fermentations.

1. Introduction

Winemaking is a complex process that requires the alcoholic fermentation of grape must by indigenous or inoculated yeasts. In spontaneous fermentations, several yeast species are involved in the first stages of fermentation; however, in the middle and final stages of fermentation, when the alcohol content increases, *Saccharomyces* spp. species become predominant, making *Saccharomyces cerevisiae* the main yeast responsible for wine fermentation (Ribéreau-Gayon et al., 2006).

Winemakers must adapt wine production to consumer demands. For many years, one of the objectives of winemakers has been to obtain wines with predictable and reproducible quality and to place wines with similar characteristics on the market year after year. For this purpose, *S. cerevisiae* commercial yeasts have been widely used as single inocula in alcoholic fermentations, thus avoiding the presence and participation of non-*Saccharomyces* species during this process (Fleet and Heard, 1993). Currently, consumers and winemakers look for wines with special traits and characteristics, which can be obtained when several yeast species (also non-*Saccharomyces*) are involved in fermentation, e.g., in spontaneous or mixed fermentations. Although non-*Saccharomyces* yeasts have been considered spoilage microorganisms for many years, some of them are currently used in mixed fermentations with *S. cerevisiae* to improve certain wine characteristics (Jolly et al., 2014; Tufariello et al., 2021).

One of the non-*Saccharomyces* yeast most used in winemaking is *Torulaspora delbrueckii*. Strains of this species have exhibited some interesting oenological characteristics, such as lower production of acetic acid and ethanol, higher levels of glycerol, fruity esters, thiols and terpenes, and the ability to release mannoproteins and polysaccharides into wines (Azzolini et al., 2015; Bely et al., 2008; Benito, 2018; Renault et al., 2015). Previous studies showed that *T. delbrueckii* contributed to the sensory profile fingerprints in both white and red wines (Belda et al., 2015; van Breda et al., 2018). Moreover, this species has a high fermentative capacity, and some strains can undergo complete fermentation as a single inoculum, albeit more slowly than *S. cerevisiae* (Renault et al., 2015; Roca-Mesa et al., 2020; Taillandier et al., 2014; van Breda et al., 2018). In any case, the recommended strategy consists of using both species (*T. delbrueckii* and *S. cerevisiae*) in mixed fermentations, either in co- or sequential inoculation (Benito, 2018; Jolly et al., 2014; Tufariello et al., 2021). In this way, *T. delbrueckii* could produce fruity esters and higher alcohols, and *S. cerevisiae* could ensure the total consumption of sugars.

* Corresponding author.
E-mail address: mjesus.torija@urv.cat (M.-J. Torija).

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Wine is a competitive environment where different yeast species compete for nutrients to survive (Fleet, 2003). Indeed, nutrient deficiencies (especially nitrogen and vitamins) are associated with stuck and sluggish fermentations (Bataillon et al., 1996; Bisson, 1999; Ribéreau-Gayon et al., 2006; Wang et al., 2003). For these reasons, in some cases grape must are supplied with external nutrients, typically ammonium salts, to avoid fermentative problems (Bell and Henschke, 2005; Martínez-Moreno et al., 2014).

Previous studies showed the existence of different interspecific microbial interactions when cultivating different yeast species in the same medium. Transcriptomic analysis revealed a clear yeast response to the presence of others during mixed fermentations. For example, the presence of some non-*Saccharomyces* species, such as *T. delbrueckii, Hanseniaspora uvarum* and *Candida sake*, seemed to stimulate glucose and nitrogen metabolism in *S. cerevisiae* (Curiel et al., 2017; Ruiz et al., 2020; Tronchoni et al., 2017) and could affect the expression of genes linked to the competition for trace elements such as copper and iron (Alonso-del-Real et al., 2019; Ruiz et al., 2020; Shekhawat et al., 2019). These interactions can be direct or indirect (Zilelidou and Nisiotou, 2021). Direct microbial interactions, also known as cell-to-cell contact interactions, are due to physical contact between cells and often occur through the production of diffusible molecules that act as signals (Albergaria and Arneborg, 2016). This physical contact plays an important role in multispecies yeast ecosystems, where FLO genes were recently suggested to be a major factor in such interactions (Rossouw et al., 2018). It has been proven that in mixed fermentations, *Lachancea thermotolerans* and *T. delbrueckii* presented early arrested growth when they were in contact with *S. cerevisiae* (Nissen et al., 2003; Renault et al., 2013).

Other microbial interactions do not involve physical contact and are considered indirect interactions, such as competition for nutrients and the production of antimicrobial compounds (Wang et al., 2016). Regarding nutrient competition, several positive and negative interactions have been reported due to substrate limitation or depletion (Ivey et al., 2013; Oro et al., 2014). Moreover, several yeast species can also produce some inhibitory compounds, which could affect the growth or survival of other sensitive strains or species. This would be the case for killer toxins, which are not exclusively secreted by *S. cerevisiae* strains, as different non-*Saccharomyces* species, such as *T. delbrueckii*, can also produce them (Ramírez et al., 2015; Velázquez et al., 2015; Woods and Bevan, 1968; Yap et al., 2000). Some yeasts can produce other antimicrobial peptides that also compromise the growth of other species (Bely et al., 2008; Rollero et al., 2018). For example, some studies have shown that *S. cerevisiae* CCMI 885 can secrete a small protein fraction, corresponding to fragments of the glyceraldehyde 3-phosphate dehydrogenase protein, which inhibits the growth of several non-*Saccharomyces* yeasts (Albergaria et al., 2010; Branco et al., 2017, 2014). Recently, the possible involvement of extracellular vesicles of *T. delbrueckii* in fungal interactions during wine fermentation has been revealed (Mencher et al., 2020). Therefore, more research into *T. delbrueckii* and *S. cerevisiae* interactions is needed to improve their application and control in winemaking.

The aim of this work was to investigate the possible causes that can compromise *S. cerevisiae* domination in mixed alcoholic fermentation with *T. delbrueckii*. For this purpose, fermentations with two commercial strains of *T. delbrueckii* and *S. cerevisiae* were carried out in synthetic must with and without cell-to-cell contact. Fermentation kinetics, population dynamics and the synthesis of fermentative byproducts of pure cultures of each species were compared with sequential inoculations. The influence of different variables, such as species strain, inoculum size, nitrogen requirements and nutrient supplementations, were analyzed.

2. Materials and methods

2.1. Yeast strains and media

The strains used in this study were *T. delbrueckii* Viniferm NS-TD (Agrovin S.A., Spain) (Td5), *T. delbrueckii* Biodiva™ TD291 (Lallemand Inc., Canada) (TdB), *S. cerevisiae* Viniferm Revelación (Agrovin S.A., Spain) (ScR) and *S. cerevisiae* QA23® (Lallemand Inc., Canada) (ScQA).

All strains were preserved in YPD liquid medium (2 % (*w/v*) glucose, 2 % (w/v) bactopeptone, and 1 % (w/v) yeast extract; Cultimed, Barcelona, Spain) with 40 % (*v/v*) glycerol at −80 °C. They were streaked on YPD agar plates (YPD liquid with 2 % (*w/v*) agar) before their use. From these pure cultures, isolated colonies were grown in YPD at 28 °C and 120 rpm for 24 h. They were used as a preculture for inoculating the fermentations. Their cell concentration was calculated by microscopic counting using a Neubauer chamber.

2.2. Fermentation conditions and sampling

Single and sequential fermentations were performed in synthetic must as described in Beltran et al. (2004). The initial nitrogen content was 300 mg N/L in all cases, except for the high nitrogen condition (600 mg N/L) (Roca-Mesa et al., 2020). In some sequential fermentations, nutrient supplementation was performed before the inoculation of the second yeast strain. The concentrations of the supplemented compounds (vitamins, amino acids, oligoelements, anaerobic growth factors, ammonium, thiamine, or zinc) were the same as in the initial synthetic must. Actimax Natura (Agrovin S.A., Spain) was used as an inactivated dried yeast (IDY) supplement, added at 0.20 g/L. This concentration corresponds to 44 mg of yeast-assimilable nitrogen (YAN) per litre analyzed by HPLC.

Synthetic must was inoculated to a 2×10^6 cells/mL concentration. Fermentation was performed in triplicate at 120 rpm and at 16 °C in 250 mL borosilicate glass bottles containing 220 mL of medium and capped with closures that enabled carbon dioxide to escape and samples to be removed.

Inoculations were performed as described in Table 1. Coinoculated fermentations were performed with three different ratios of ScR and Td5 strains (1:1, 1:9 and 9:1) to study the impact of these inoculation ratios on yeast dynamics and fermentation performance. In sequential fermentations, the second species (*S. cerevisiae*) was inoculated when the density decreased by approximately 15 units (g/L). In the sequential

Table 1
Inoculation strategies performed with different *T. delbrueckii* (Td) and *S. cerevisiae* (Sc) strains.

Inoculum procedures	Name	Inoculum ratios (Td: Sc)	*T. delbrueckii* (Td) (CFU/mL)	*S. cerevisiae* (Sc) (CFU/mL)
Single inoculation	Td5 TdB	1:0	2×10^6	–
	ScR ScQA	0:1	–	2×10^6
Co-inoculation	ScR: Td5	1:1	1×10^6	1×10^6
		9:1	1.8×10^6	2×10^5
		1:9	2×10^5	1.8×10^6
Sequential inoculation	Td_x, Sc_x[b]	1:1	2×10^6	2×10^6
Sequential inoculation without cell contact[a]	Td_x/ Sc_x[b]	1:1	2×10^6	2×10^6

[a] In this procedure, the must fermented by *T. delbrueckii* species was centrifuged and filtered before the inoculation of *S. cerevisiae* species.

[b] Td_x and Sc_x refer to any strain of the species used in this study. Td_x: Td5 or TdB; Sc_x: ScR or ScQA.

fermentation with cell contact, both species coexisted in the medium, while in the sequential fermentations without cell-to-cell contact, the medium was centrifuged and filtered to discard the cells of the first species before the inoculation of the second species.

Fermentation kinetics were monitored by measuring the must density daily with an electronic densimeter (Densito 30PX Portable Density Metre (Mettler Toledo, Spain)). The viable yeast population was determined by plating serial dilutions of samples on YPD agar plates for total yeast and on lysine medium (lysine agar 66 % (*w/v*), potassium lactate 10 % (*v*/v) and lactic acid 4 % (v/v) (Thermo Fisher Scientific™, USA)) for *T. delbrueckii* yeasts. The endpoint of the fermentations was considered when the sugar concentration was <2 g/L or when the density remained stable for more than two days (in the case of stuck or sluggish fermentations). For the analysis of nitrogen compounds and organic metabolites, 1.5 mL of the supernatant was collected during fermentation and stored at −20 °C until analysis.

2.3. Microplate growth monitoring

To evaluate the effect of some nutrient supplementation on *S. cerevisiae* growth, different fermentation conditions were tested in 96-well microplates at 16 °C. In the first screening, the effect of vitamin and oligoelement supplementation was analyzed, and in the second screening, the effect of single amino acid supplementation was also determined. In all cases, the concentration of each tested compound was the same as in the initial synthetic must, and *S. cerevisiae* strains were inoculated at 2×10^6 cells/mL into synthetic must prefermented by *T. delbrueckii* for 48 h. Each well had 0.25 mL of total volume. Yeast growth was measured after preshaking the microplate for 30 s at 600 rpm by a SPECTROstar Nano microplate reader (BMG LABTECH, Germany) twice a day until it stabilized. For each growth curve, the generation time (GT), growth efficiency (OD max), lag phase (LP) and area under the curve (AUC) were measured.

2.4. Nitrogen analysis

The nitrogen content was analyzed by HPLC (high-performance liquid chromatography) according to the method of Gómez-Alonso et al. (2007). The HPLC (Agilent 1100, Agilent Technologies, Germany) was equipped with a DAD ultraviolet detector and a fluorescence detector (Agilent Technologies, Germany). Separation was performed on a Hypersil ODS C18 column (Agilent Technologies, Germany) with a particle size of 5 μm (250 mm × 4.6 mm) and thermostated at 20 °C. The mobile phase (A) consisted of 2.05 g/L sodium acetate anhydrous and 0.2 g/L sodium azide with Milli-Q water (Millipore Q-PODTM Advantage A10) adjusted to pH 5.8 with glacial acetic acid. Mobile phase (B) consisted of 80 % (*v*/v) acetonitrile and 20 % (v/v) methanol. Agilent ChemStation Plus software (Agilent Technologies, Germany) was used to analyze the chromatograms. Yeast-assimilable nitrogen (YAN, expressed as mg N/L) was calculated according to the nitrogen atoms of each amino acid and ammonium.

The total organic and inorganic nitrogen concentrations were also analyzed with a Y-15 multianalyzer (BioSystems, Barcelona, Spain) using enzymatic kits for ammonium and alpha-amino nitrogen (TDI, Barcelona, Spain).

2.5. Potential inhibitory compounds

The possible killer activities of Td5, TdB, ScR and ScQA strains were tested on low-pH (pH 4 and 4.7) methylene blue (MB) plates (Kaiser et al., 1994). Briefly, 100 μL of a 48 h grown culture of the killer-sensitive strain was seeded on MB plates. Then, the strains tested for killing activity were loaded as 4 μL drops of stationary phase cultures. The plates were incubated for 4–8 days at 20 °C (Maqueda et al., 2010). The *S. cerevisiae* reference strains used as controls were F166 ($K1^+$), EX73 ($K2^+$), and F182 ($K28^+$) as killer-producing strains and EX33 (K^-R^-) as a killer-sensitive strain (Velázquez et al., 2015). Moreover, the presence of virus dsRNA molecules in the tested strains was analyzed. The protocol for dsRNA extraction and agarose gel electrophoresis was performed as explained in Maqueda et al. (2010).

To determine the presence of peptides or proteins produced by *T. delbrueckii* with potential inhibitory activity against *S. cerevisiae*, the protein profile of the medium after 48 h of fermentation was studied by high-resolution size-exclusion chromatography (HRSEC) (Canals et al., 1998). Briefly, 15 mL of the must was lyophilized, resuspended in 0.6 μL of 300 mmol/L ammonium acetate and centrifuged (12,000 ×*g* for 10 min). The supernatant was filtered through a 0.22 μm pore size filter (Dominique Dutscher, Brumath, France), and 100 μL was injected into the HPLC Agilent 1200 Series system (Agilent Technologies, Barcelona, Spain) with a diode array detector (DAD) to monitor the output at 230, 280 and 320 nm. Separation was carried out at 20 °C using an S 165 Shodex gel permeation HPLC column (OHpak 166 SB-803 HQ, 300 mm × 8 mm i.d.; Showa Denko). The mobile phase consisted of an aqueous solution of 300 mmol/L ammonium acetate applied at a constant flow of 0.6 mL/min for 70 min. The proteins were quantified according to the peak area for each fraction using bovine serum albumin as an external standard (Sigma-Aldrich) in the range between 0 and 10 mg/mL (r2 > 0.99).

To evaluate the inhibitory effect of the *T. delbrueckii* protein extracts, *S. cerevisiae* ScR strain was cultivated in synthetic must with and without the addition of the protein fraction obtained from a medium fermented for 48 h with Td5 (1 and 10-fold concentrated). Fermentation kinetics of ScR was measured as weight loss normalized to the initial weight. The addition of PBS instead of the protein extract was used as a negative control.

2.6. Metabolite analysis

The concentrations of some organic metabolites were determined by HPLC at the end of fermentation, following the protocol described by Quirós et al. (2010). These analyses were performed using an Agilent 1100 HPLC (Agilent Technologies, Germany) equipped with a Hi-Plex H, 300 mm × 7.7 mm column inside a 1260 Multicolumn Thermostat (Infinity II MCT) and connected to two detectors, a multiwavelength detector (G1365B MWC) and a 1260 Infinity II refractive index detector (Agilent Technologies, Germany). Chromatograms were analyzed using Agilent ChemStation Plus software (Agilent Technologies, Germany). In some cases, residual sugars were also analyzed with a Y-15 multi analyzer using an enzymatic kit (BioSystems, Barcelona, Spain). Thiamine concentration was measured by a VitaFast® Vitamin B1 Microbiological Kit (R-Biopharm, AG, Germany).

2.7. Statistical analysis

Data are expressed as the mean and standard deviation (SD) of triplicate measurements. ANOVA and Tukey's test analyses were performed using XLSTAT 2019 software (Addinsoft, New York, New York, USA) to determine significant differences between fermentation conditions. The results were considered statistically significant at a p value of <0.05. Graphical data modelling was performed using the GraphPad Prism 7 program (GraphPad Software, San Diego, California, USA).

3. Results

3.1. Single, co-inoculated and sequential fermentations of T. delbrueckii and S. cerevisiae

The different fermentations performed (single, coinoculated and sequential fermentations) between Td5 and ScR strains are shown in Fig. 1a. As expected, the *S. cerevisiae* single fermentation was faster than the *T. delbrueckii* single fermentation. Td5 finished the fermentation in fourteen days, while ScR only required ten days. The sequential

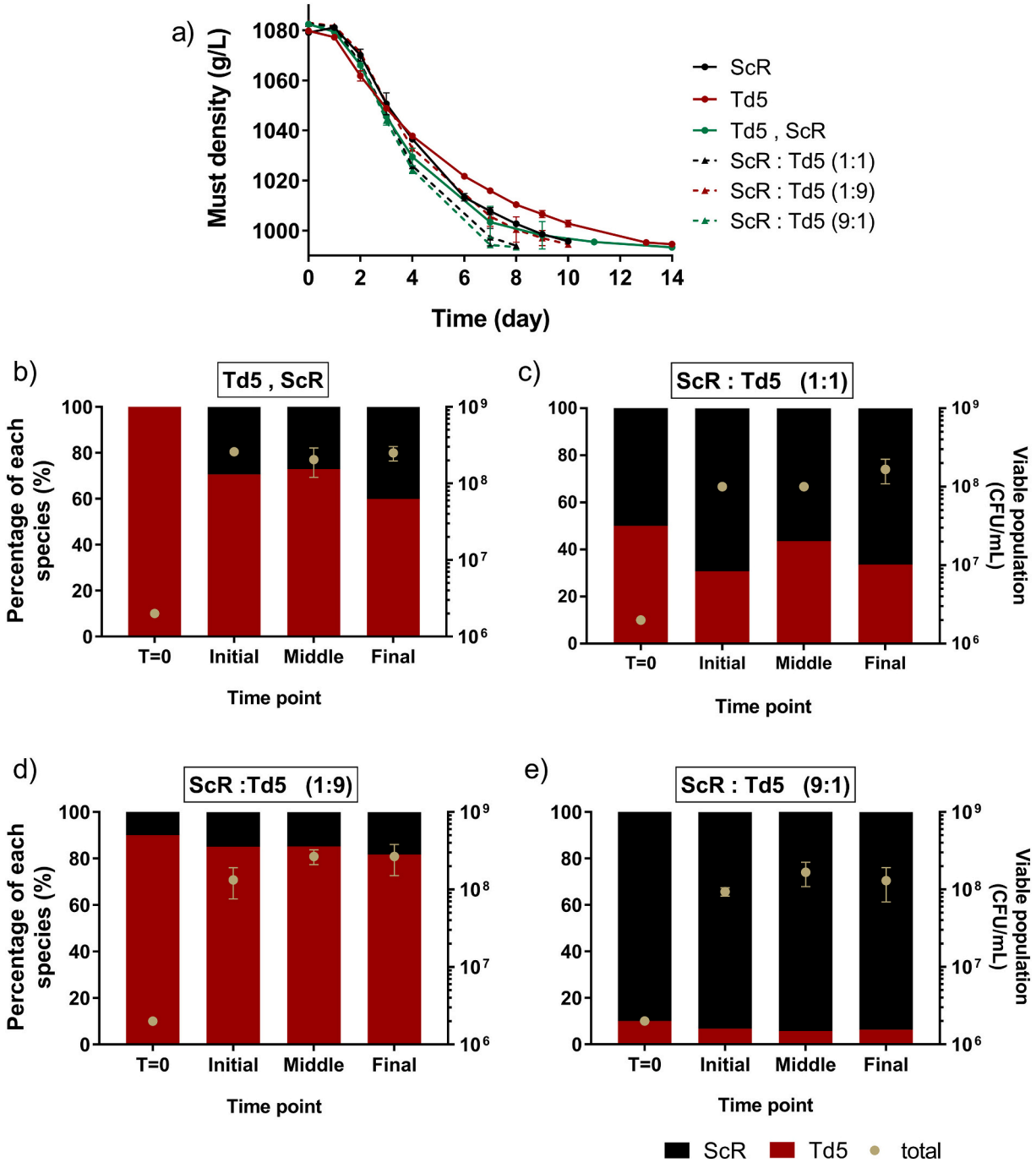

Fig. 1. (a) Must density of single inoculations with Td5 or ScR, sequential inoculation (Td5, ScR) and co-inoculation at different ratios (ScR:Td5, 1:1, 1:9, 9:1) in synthetic must fermented at 16 °C. Total yeast viability (CFU/mL) and percentage of each species through the sequential (b) and co-inoculated fermentations with different inoculation ratios of 1:1 (c), 1:9 (d) and 9:1 (e) in initial (Day 2), middle (Day 4) and final (Day 7) fermentations.

fermentation finished all the sugars in fourteen days, similar to Td5 single fermentation, but it showed a higher fermentation rate, mainly after *S. cerevisiae* inoculation. In the case of coinoculated fermentations, three different inoculation ratios of ScR and Td5 (1:1, 9:1 and 1:9) were used. Surprisingly, the coinoculations with a higher population of *S. cerevisiae* cells (9:1 or 1:1) finished faster than the ScR single fermentation, while the fermentation with a higher proportion of *T. delbrueckii* cells (1:9) required two more days, finishing at the same time as the ScR single fermentation (Fig. 1a).

We observed that in the sequential fermentation, even if ScR significantly increased its population, reaching 2×10^7 CFU/mL, this species was not able to become the dominant yeast species, as Td5 was present at higher population levels until the end of the fermentation (8.9×10^7 CFU/mL) (Fig. 1b). In the coinoculated fermentations, *S. cerevisiae* dominated the fermentation except when *T. delbrueckii* was inoculated at a higher proportion (9:1) (Fig. 1c–e). When the two species were inoculated with the same ratio (1:1), Td5 was still present at the end of fermentation and comprised 40 % of the total population.

To confirm if the dominance of *T. delbrueckii* over *S. cerevisiae* was strain dependent, we performed sequential fermentations using two strains of each species. We observed similar results in all tested combinations, with *T. delbrueckii* being the dominant species throughout all the fermentations (Supplementary Fig. 1). Indeed, ScR growth was impaired when TdB started the fermentation process, with no viable ScR

cells detected from mid-fermentation. Regardless, all sequential fermentations were able to deplete sugars (Supplementary Fig. 2), even though *T. delbrueckii* was the dominant species throughout the fermentation process.

3.2. Sequential fermentation without cell-to-cell contact between yeast species

To better understand the microbial interactions and growth impairment of *S. cerevisiae* cells in the presence of *T. delbrueckii*, we performed sequential fermentations without cell-to-cell contact between the two yeast species. For this purpose, *T. delbrueckii* was first inoculated, and after 48 h of fermentation, *T. delbrueckii* cells were removed from the medium by centrifugation and filtration before inoculation with *S. cerevisiae* (Td/Sc). After this point, fermentation was carried out only by *S. cerevisiae* cells.

Surprisingly, when *T. delbrueckii* cells were removed prior to *S. cerevisiae* inoculation, all fermentations were stuck in all strain combinations (Fig. 2a). Indeed, even if *S. cerevisiae* was able to grow up to 2.5×10^7 CFU/mL (Fig. 2b), this population might not have been sufficient to complete the fermentation.

For this reason, sequential fermentations without cell-to-cell contact but with a higher *S. cerevisiae* inoculum size were performed (10^8 cells/mL instead of 2×10^6 cells/mL). Our results showed that with this inoculum size, fermentations finished, although ScQA needed 12 days to consume all the sugars, while ScR required 24 days (Fig. 2a).

3.3. Analysis of potential inhibitory compounds

A possible explanation for the growth impairment of *S. cerevisiae* could be the presence of killer toxins or other inhibitory compounds released by the *T. delbrueckii* strains. Therefore, we first tested the killer phenotype of the *T. delbrueckii* strains used in this study. The results obtained by the MB plates were confirmed by analyzing the presence of virus dsRNA molecules, indicating the absence of the killer viruses in those strains (Supplementary Fig. 3) and thus discarding killer toxins as a possible cause for *T. delbrueckii* dominance. Then, the secretion of peptides or proteins with potential inhibitory activity against *S. cerevisiae* by *T. delbrueckii* was studied. For this purpose, the protein content in the medium after 48 h of fermentation with each strain was compared. A species-dependent profile was observed, with the media fermented with *S. cerevisiae* strains presenting the highest protein concentration compared to those fermented with *T. delbrueckii* strains. In both species, the most enriched protein fractions were those that contained the largest (the fraction of proteins with molecular weights of >75 kDa) and the smallest proteins (proteins of <25 kDa) (Fig. 3a). Finally, the protein extract was added to fresh synthetic must to determine if any substances present in this extract affected the ScR fermentation performance or even inhibited its growth (Fig. 3b). The addition of Td5 protein extracts did not have an effect on the fermentation kinetics of ScR at any concentration (1× and 10×), thus indicating that the proteins secreted by Td5 during fermentation might not be a cause of the fermentation problems of *S. cerevisiae* strains.

3.4. Effect of nitrogen supplementation

In sequential inoculations, the consumption of nitrogen and other nutrients by the first yeast species inoculated into the medium could limit the growth and fermentation of the second species, *S. cerevisiae*. Thus, we first analyzed the nitrogen consumption by *T. delbrueckii* cells, as well as the effect of nitrogen supplementation, to determine whether nitrogen limitation could explain the growth impairment of *S. cerevisiae*.

The analysis of the nitrogen in the medium revealed that *T. delbrueckii* strains consumed a large fraction of the nitrogen present in the synthetic must in 48 h, with the remaining nitrogen being rather limited for *S. cerevisiae* growth (44.94 mgN/L in Td5 fermented medium and 89.44 mgN/L in TdB fermented medium) (Table 2). Indeed, the patterns of nitrogen consumption were quite different between the two *T. delbrueckii* strains. While Td5 consumed amino acids and ammonia faster than *S. cerevisiae* strains, TdB consumption was similar to that of *S. cerevisiae* strains. As expected, when high-nitrogen must (600 N) was used, more nitrogen was left in the medium at 48 h (Fig. 4a, Table 2), with 176.55 mg N/L still remaining when *S. cerevisiae* was inoculated.

Then, we tested the effect of adding each amino acid individually to the medium (previously fermented by *T. delbrueckii* for 48 h) on *S. cerevisiae* growth (Supplementary Table 1). No large differences were observed among the different amino acid supplementations, and the best growth was obtained with the supplementation of a mixture of all amino acids.

After that, we analyzed whether supplementation of the medium with more complex nitrogen sources, such as a mixture of amino acids and ammonium, a mixture of only amino acids or commercial nutrients (IDY), was enough to prevent the growth impairment of these *S. cerevisiae* strains. Additionally, a sequential fermentation with high

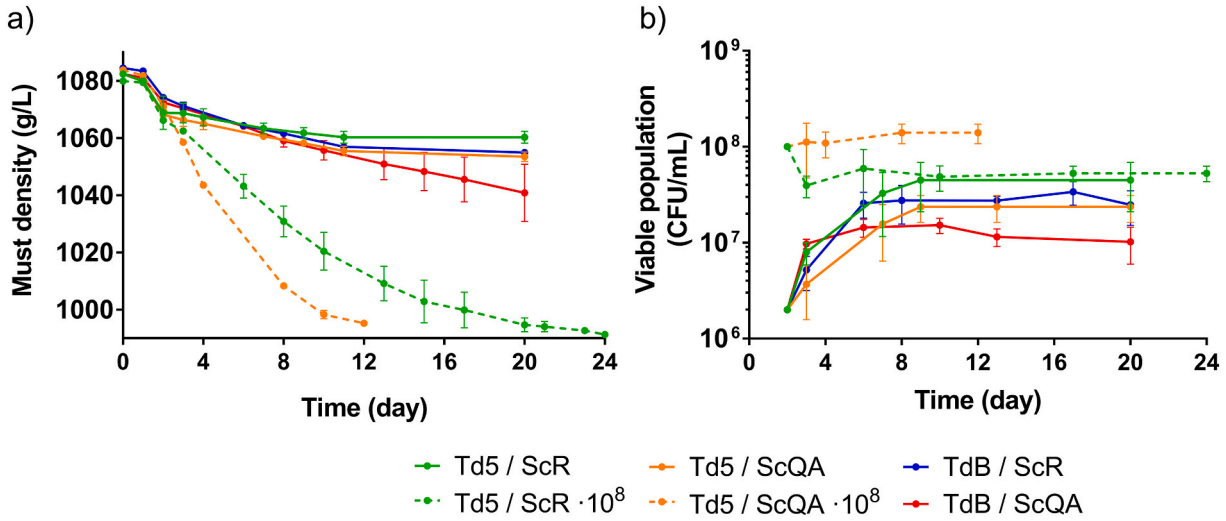

Fig. 2. (a) Must density and (b) *S. cerevisiae* cell growth (CFU/mL) through the sequential fermentations performed with *T. delbrueckii* (Td5 and TdB) and *S. cerevisiae* (ScR and ScQA) strains, without contact between the two yeast species (*T. delbrueckii* cells were removed 48 h after fermentation, just before *S. cerevisiae* inoculation). Dotted lines indicate *S. cerevisiae* inoculation with higher inoculum size.

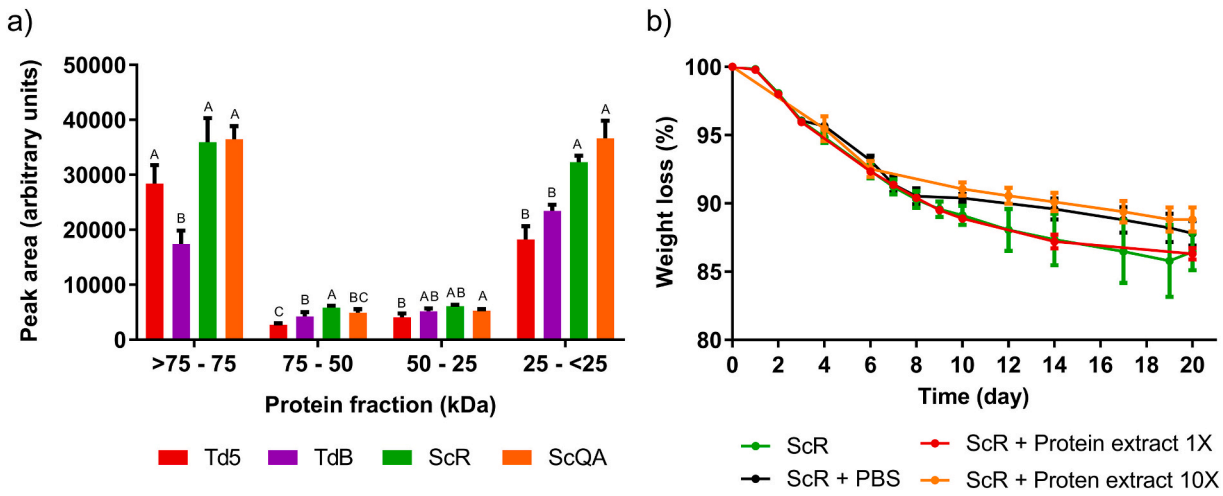

Fig. 3. (a) Protein fractions, expressed as peak areas (arbitrary units), obtained from the medium after 48 h of fermentation with different strains of *T. delbrueckii* (Td5 and TdB) and *S. cerevisiae* (ScR and ScQA). (b) Fermentation kinetics of ScR (measured as weight loss normalized to the initial weight), inoculated in a synthetic must, with and without the supplementation of protein extract obtained from a medium fermented for 48 h with Td5. The addition of PBS instead of the protein extract was used as a negative control.

nitrogen content in the must was also performed (Td5/ScR 600N) (Fig. 4b). Surprisingly, this surplus of nitrogen did not result in higher *S. cerevisiae* populations or higher fermentation capacity, with this fermentation also stuck after *S. cerevisiae* inoculation and showing similar fermentation and growth profiles as the conditions with lower nitrogen. Indeed, *S. cerevisiae* reached similar cell populations with all nitrogen supplementations (Fig. 4b), and all of them resulted in stuck fermentations. In fact, the addition of IDY, which includes nutritional compounds other than nitrogen, seemed to be the most efficient supplement, both for growing and fermenting, although it was not sufficient to consume all sugars.

3.5. Effect of supplementation with other nutrients

To explore the impact of other nutrients on *S. cerevisiae* growth and sequential fermentations, the effects of vitamin, oligoelement and anaerobic growth factor supplementation were studied. The synergistic effect of adding vitamins, oligoelements, anaerobic growth factors and amino acids together was also tested (Fig. 5a).

The addition of only one of the tested nutrients at a time also resulted in stuck fermentations. In contrast, when all nutrients were added simultaneously (Td5/ScR + all nutrients), the fermentation process finished correctly in fifteen days. After these results, we performed fermentation in synthetic must with high nitrogen content (to ensure that nitrogen was not limiting) with the addition of two of the tested nutrients at a time. The results revealed that the joint addition of vitamins and oligoelements was the combination with the best results in the fermentation kinetics (Fig. 5b). Therefore, to decipher which vitamin and oligoelement were required for correct *S. cerevisiae* performance, screenings combining each vitamin and oligoelement at a time were performed in microfermentations. Our results showed that thiamine had the largest impact on ScR growth (Supplementary Table 2). Thus, thiamine consumption by the different strains of *T. delbrueckii* and *S. cerevisiae* was analyzed at 24 and 48 h. The initial thiamine concentration in the must was 0.29 mg/L, and after 24 h of fermentation, the remaining thiamine in the medium in all strains and conditions was <0.08 mg/L, the detection limit of the technique (Supplementary Table 3), indicating that thiamine was depleted and could be limiting in all conditions.

Subsequently, we tested the effect of thiamine on the growth of *S. cerevisiae* in sequential fermentation by a single thiamine addition, combined with all the oligoelements or combined with only zinc, as zinc was the oligoelement that resulted in better growth in the previous experiment (Supplementary Table 2). Our results showed that thiamine alone had the lowest impact (Fig. 6a). However, the addition of thiamine with the mixture of oligoelements or with zinc had a positive and similar effect on the fermentation process and yeast growth, although fermentations were still incomplete.

Finally, we tested the addition of thiamine, zinc and amino acids in synthetic must using different *T. delbrueckii* and *S. cerevisiae* strains without contact between species. All fermentations were finished, demonstrating the positive effect of this supplementation (Fig. 6b). ScQA was the fastest strain to ferment musts partly fermented by either TdB or Td5, depleting sugars in 13 days. In contrast, ScR needed between 22 and 24 days to consume all sugars in musts coming from Td5 or TdB fermentations, respectively. *S. cerevisiae* growth was similar in all fermentations performed without yeast species contact (Fig. 6b). This supplementation was also tested in sequential fermentations between Td5 and ScR but without removing Td5 to determine if the imposition of *S. cerevisiae* increased after this addition of nutrients (Fig. 7). Moreover, this addition of thiamine, zinc and amino acids was compared to IDY supplementation. In all cases, *S. cerevisiae* strains grew more than in the conditions without nutrient supplementation (Fig. 7, Fig. 2b), although still without becoming the dominant yeast species after (Fig. 7b–c). The supplementation that most increased the *S. cerevisiae* imposition in both strains was IDY, which was close to 50 % (Fig. 7d–e).

Compounds of oenological interest were analyzed at the end of fermentations (Supplementary Table 4). Single fermentations using *S. cerevisiae* strains presented higher glycerol and acetic acid contents and a lower lactic acid content than wines fermented with single *T. delbrueckii* strains. When the coinoculation strategy was applied, the acetic, lactic, citric and tartaric acid contents decreased, while the malic and succinic acid contents increased in comparison with *S. cerevisiae* single fermentations. Furthermore, coinoculated wines presented lower ethanol and glycerol contents. Under the sequential inoculation strategy, in general, wines presented a higher content of malic acid and a lower content of lactic acid. No significant effect on ethanol content was observed due to the inoculation strategy.

4. Discussion

The use of non-*Saccharomyces* species has become widespread in winemaking in recent years due to their desirable properties. Their use in simultaneous and sequential starter cultures has been shown to

Table 2
Nitrogen content (mg/L) present in the medium after 48 h of single fermentations with the *T. delbrueckii* (TdB and Td5) or *S. cerevisiae* (ScQA and ScR) strains in standard synthetic must (300 mg N/L), as well as after 48 h of Td5 fermentation in high nitrogen content must (600 mg N/L; Td5_600N).

YAN (mg/l)	ScQA				ScR				TdB				Td5				Td5_600N			
	24 h		48 h		24 h		48 h		24 h		48 h		24 h		48 h		24 h		48 h	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Inorg.	100.16^{bc}	7.52	44.53^{de}	5.23	94.64^{c}	16.44	49.62^{de}	10.86	98.52^{bc}	13.88	41.30^{de}	25.11	75.93^{cd}	15.60	28.55^{e}	1.62	227.20^{a}	2.68	134.16^{b}	7.45
Org.	132.13^{a}	5.36	33.90^{c}	20.12	111.50^{ab}	3.84	43.79^{c}	10.05	123.29^{ab}	17.45	48.13^{c}	13.80	95.68^{b}	6.26	16.39^{c}	9.47	112.54^{ab}	2.11	42.40^{c}	6.35
Total	232.29^{a}	2.16	78.43^{c}	25.35	206.14^{ab}	19.94	93.41^{c}	3.98	221.81^{ab}	31.21	89.44^{c}	38.82	171.61^{b}	14.11	44.94^{c}	9.48	227.20^{ab}	2.68	176.55^{ab}	12.44

Lowercase letters indicate significant differences between fermentations in each compound.

contribute positively to wine quality (Ciani et al., 2010; Jolly et al., 2014). *T. delbrueckii* is one of the most studied non-*Saccharomyces* species due to its interesting characteristics. This species has been reported to produce low amounts of acetic acid and higher glycerol levels, desirable characteristics in oenology, and to reduce ethanol content in wines (Belda et al., 2015; Bely et al., 2008; Contreras et al., 2015). Thus, in this study, we analyzed the interactions between *T. delbrueckii* and *S. cerevisiae* under oenological conditions to broaden the understanding of the behaviour of these species and improve the management of their implementation in mixed fermentations.

In accordance with previous studies, single inoculation of *T. delbrueckii* strains was able to finish the fermentation process, although more slowly than *S. cerevisiae* (Renault et al., 2016; Tronchoni et al., 2017). However, the *T. delbrueckii* fermentative capacity seems to be strain dependent and can also result in stuck fermentations (Bely et al., 2008; Renault et al., 2015, 2016). Nevertheless, to avoid arrested fermentations and ensure continuation of the process, *T. delbrueckii*, similar to most non-*Saccharomyces* strains, is mainly used in mixed fermentations with *S. cerevisiae* (Albergaria and Arneborg, 2016; Jolly et al., 2014). Thus, coinoculation and sequential fermentation strategies were investigated.

Although sequential fermentations proceeded normally and finished in the expected time, surprisingly, *S. cerevisiae* did not prevail over *T. delbrueckii* strains at the end of the fermentation. This fact was unusual because *S. cerevisiae* usually dominates the fermentation process in the middle and final stages of fermentation (Azzolini et al., 2012; Beltran et al., 2002; Lleixà et al., 2016; Renault et al., 2015, 2016), usually attributed to the different ethanol and sulfur dioxide (SO_2) sensitivities of the non-*Saccharomyces* yeasts (Ribéreau-Gayon et al., 2006). Similar results were obtained by Zhu et al. (2021), since in their study, *S. cerevisiae* failed to dominate sequential fermentations with a mixture of non-*Saccharomyces* species (*T. delbrueckii*, *Zygosaccharomyces bailii* and *Metschnikowia pulcherrima*), with the *T. delbrueckii* strain being dominant at the end of the process, despite SO_2 treatment of the natural must. However, the same authors also tested sequential fermentations with single non-*Saccharomyces* species, observing the imposition or not of *S. cerevisiae* depending on the non-*Saccharomyces* species used. The *S. cerevisiae* strains were able to clearly impose over the *M. pulcherrima* strain but not always over the *Lachancea thermotolerans* strain (Zhu et al., 2021). Yet, in the coinoculated fermentations, our results showed that *S. cerevisiae* was able to dominate the fermentation in all cases except when *T. delbrueckii* was inoculated at a higher ratio. In contrast, Zhu et al. (2021) showed that *S. cerevisiae* dominated at the end of the coinoculated fermentations regardless of the proportions of the non-*Saccharomyces* and *S. cerevisiae* used, and Taillandier et al. (2014) also observed *S. cerevisiae* dominance, even when inoculated in mixed fermentations with a population 20 times lower than that of *T. delbrueckii*.

Therefore, *S. cerevisiae* growth appeared to be more affected by the presence of non-*Saccharomyces* species in sequential inoculation than in simultaneous inoculation. This lack of imposition of *S. cerevisiae* on *T. delbrueckii* in sequential fermentations could be due to yeast species interactions. Yeast interactions have been reported to be able to influence the population variation and dominance throughout wine fermentations (Zilelidou and Nisiotou, 2021). In fact, yeast interactions could exert positive or negative effects on yeast populations. Among the latter, the efficient consumption of nutrients (especially nitrogen, vitamins or metal ions) by a particular species or the release of antimicrobial compounds such as killer toxins, ethanol, acetic acid, short-chain fatty acids or low-mass peptides could be responsible for the arrest or even death of yeast during wine fermentation (Zilelidou and Nisiotou, 2021). Moreover, physical contact between cells of different yeast species may also regulate the presence and persistence of particular species (Kemsawasd et al., 2015; Petitgonnet et al., 2019; Wang et al., 2016). A few years ago, a strain of *T. delbrueckii* with a killer phenotype (Kbarr-1) was described to be able to dominate fermentations in the presence of *S. cerevisiae* (Ramírez et al., 2015; Velázquez et al., 2015). However, in

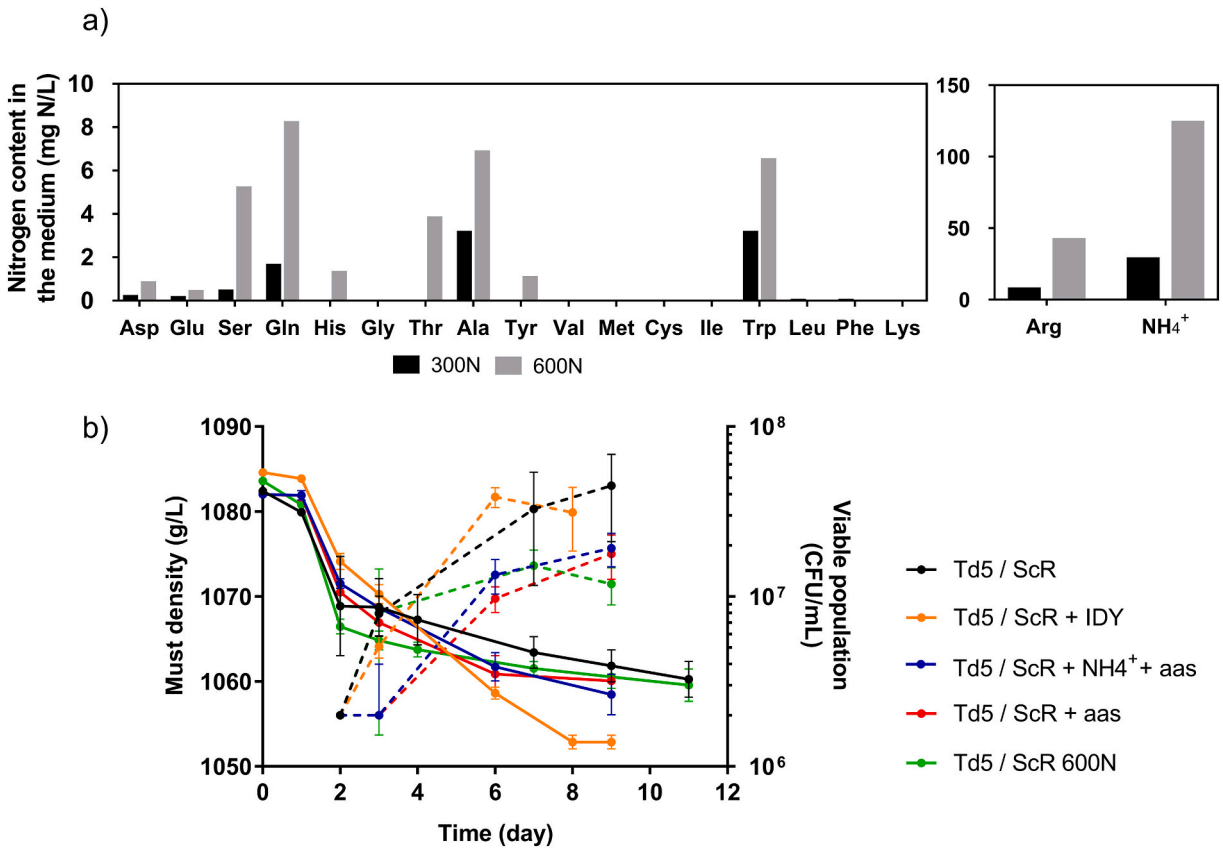

Fig. 4. (a) Nitrogen present in the medium at 48 h of Td5 fermentation in synthetic must with 300 and 600 mg N/L. (b) Must density (solid lines) and ScR viable population (dotted lines) throughout sequential fermentations without cell contact, with the addition of different nitrogen sources when ScR was inoculated. $NH4^+$: ammonium, aas: amino acids, IDY: inactive dried yeast.

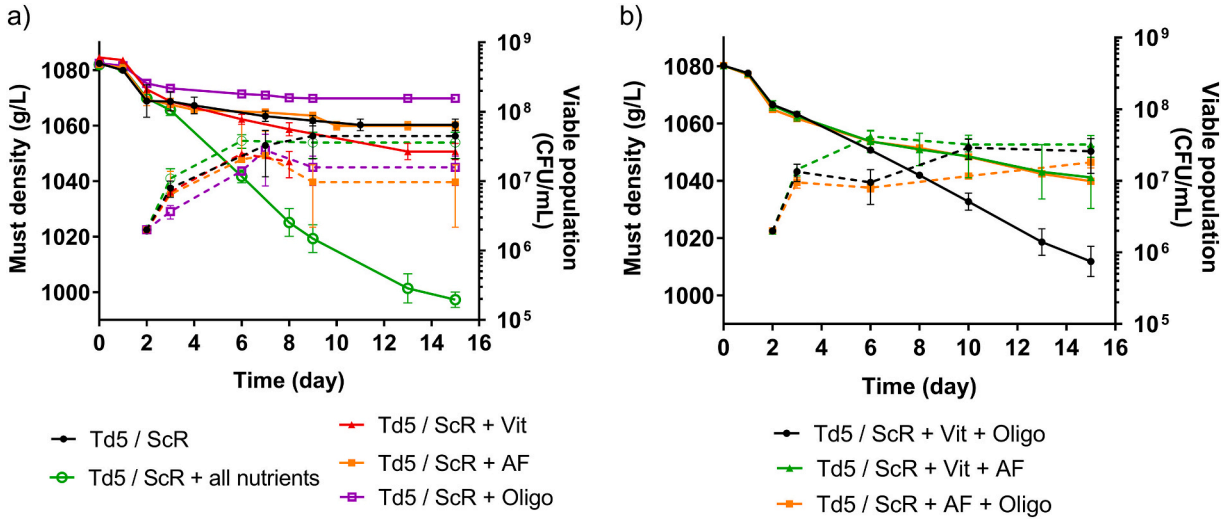

Fig. 5. Must density (solid lines) and ScR viable population (dotted lines) throughout sequential fermentations without yeast contact, with the addition of different nutrients when ScR was inoculated: (a) individual addition of different nutrients compared to adding a mixture of all these nutrients; (b) addition of combinations of two of these nutrients in a high-nitrogen must (600 mg N/L).
Vit: vitamins, AF: anaerobic growth factors, Oligo: oligoelements, all nutrients refers to the addition of vitamins, anaerobic growth factors, oligoelements and amino acids together.

our study, we disproved that the cause of the impaired growth and fermentation of *S. cerevisiae* strains was the secretion of killer toxins since the tested strains of *T. delbrueckii* did not have the genetic requirements. Moreover, the presence of other potential toxic compounds, such as some antimicrobial peptides, was also ruled out since supplementation with protein extracts from *T. delbrueckii*-fermented medium did not seem to affect *S. cerevisiae* growth in this case.

Finally, to study whether this lack of imposition was due to some cell-to-cell contact mechanism, sequential fermentations without contact between the *T. delbrueckii* and *S. cerevisiae* cells were performed, and

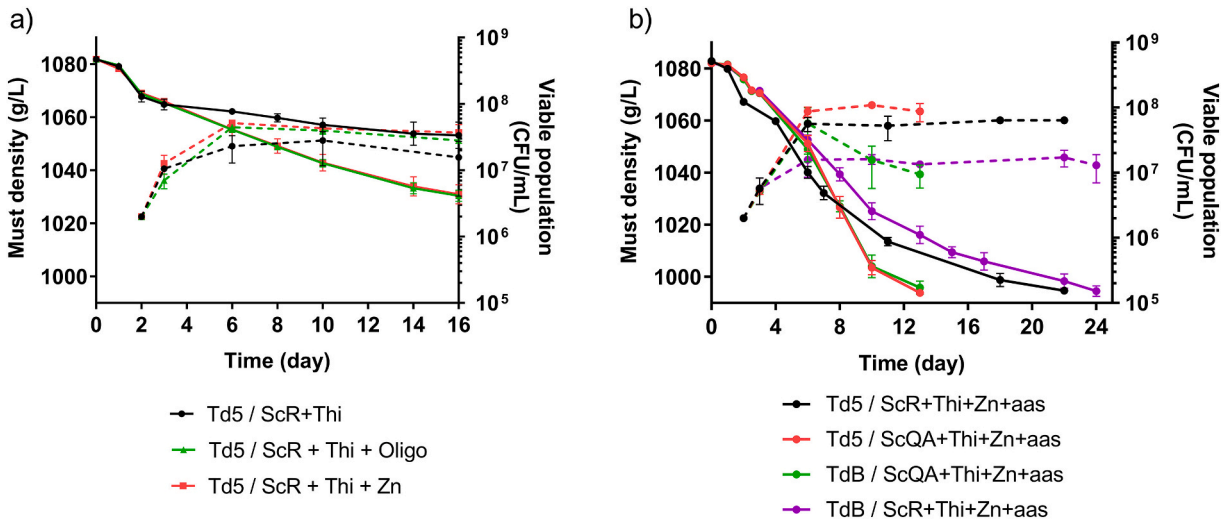

Fig. 6. (a) Must density (solid lines) and ScR viable population (dotted lines) in sequential fermentations without yeast contact, supplemented with thiamine (Thi) alone or combined with other nutrients: oligoelements (oligo) or zinc (Zn). (b) Must density (solid lines) and *S. cerevisiae* viable populations (dotted lines) of sequential fermentations combining different strains of *T. delbrueckii* and *S. cerevisiae*, without yeast contact and with the addition of a mixture of Thi, Zn and amino acids (aas).

surprisingly, the strains of *S. cerevisiae* were unable to complete these fermentations. However, when these noncontact sequential fermentations were carried out with a larger population of the *S. cerevisiae* strain (10^8 cells/mL instead of 10^6 cells/mL, the normal inoculum size), they were able to finish the fermentation. Therefore, these results suggested that these stuck fermentations were caused by the impaired growth of *S. cerevisiae*, probably due to a lack of some essential nutrients, since when a larger inoculum size was applied and consequently, *S. cerevisiae* hardly needed to double to reach its maximum population, it was able to deplete sugars. Indeed, some authors have described that the fast and efficient intake of nutrients could negatively affect the fitness of a particular yeast species (Rollero et al., 2018; Su et al., 2020). These results also correlated well with a greater effect in sequential fermentations since the previous growth of non-*Saccharomyces* strains reduced the nutrients available in the medium for *S. cerevisiae* strains, limiting their growth capacity. Instead, in coinoculations, as both species were inoculated simultaneously, this effect is reduced, and *Saccharomyces* can negatively affect the growth of non-*Saccharomyces* strains (Medina et al., 2012; Taillandier et al., 2014). Therefore, these results suggested a nutrient limitation or competition between *T. delbrueckii* and *S. cerevisiae* strains as a cause of *S. cerevisiae* growth impairment. Several studies supported this hypothesis, especially in relation to nitrogen limitation (Binati et al., 2020; Gobert et al., 2017; Lleixà et al., 2016; Medina et al., 2012; Renault et al., 2016; Rollero et al., 2018; Taillandier et al., 2014; Wang et al., 2016). This limitation could be especially relevant in the case of mixed fermentations performed with species considered stronger fermenters, such as *T. delbrueckii* and *L. thermotolerans*, because these species appear to compete more intensely with *S. cerevisiae* for nitrogen sources (de Koker, 2015; Medina et al., 2012). Indeed, Taillandier et al. (2014) observed growth arrest of *S. cerevisiae* due to nitrogen depletion by *T. delbrueckii* growth in a medium with low initial assimilable nitrogen but no effect on *S. cerevisiae* growth when the assimilable nitrogen content was higher. Similar results were obtained using other non-*Saccharomyces* species, such as *Hanseniaspora vineae* (Medina et al., 2012).

In our study, different nitrogen sources and concentrations were tested. However, supplementation with nitrogen alone was not enough to avoid stuck fermentations in sequential inoculations without cell contact. This was in agreement with other studies, such as Lage et al. (2014), where *H. guilliermondii* negatively interfered with the growth and fermentative performance of *S. cerevisiae*, regardless of the initial nitrogen concentrations, or Zhu et al. (2021), where a lack of dominance of *S. cerevisiae* in sequential fermentations was observed even after supplementing the medium with nitrogen before *S. cerevisiae* inoculation. Thus, the possibility of other limiting nutrients as the reason for *S. cerevisiae* growth impairment was analyzed. Several studies have associated vitamin limitation with sluggish fermentations (Bataillon et al., 1996; Guzzon et al., 2011; Wang et al., 2003). In fact, Medina et al. (2012) observed that the addition of diammonium phosphate with a vitamin mix increased the growth and fermentation rate of *S. cerevisiae* in sequential fermentations. Rollero et al. (2018) also suggested that there was competition for other kinds of nutrients, probably vitamins, between *Zygoascus meyerae* and *S. cerevisiae*, and it provoked stuck fermentations.

Moreover, other nutrients, such as anaerobic growth factors, have been reported to also affect *S. cerevisiae* growth, although the results depended on the added dose. Low doses of anaerobic growth factors induced the dominance of *S. cerevisiae* over *T. delbrueckii*, though increasing concentrations upset this imposition (Brou et al., 2018). In our study, the joint addition of thiamine, zinc and amino acids showed a positive effect in the process, being the only combination of nutrients that allowed *S. cerevisiae* strains to complete the fermentation in sequential inoculation without contact. Thiamine, also referred to as vitamin B1, is involved in different metabolic processes for yeast growth, such as carbon assimilation and the production of lipids, amino acids, antioxidants, DNA and RNA (Labuschagne and Divol, 2021). In particular, thiamine pyrophosphate (TPP) is a cofactor of several essential enzymes involved in central metabolism and wine fermentation, such as pyruvate dehydrogenase (PDH) and pyruvate decarboxylase (PDC) (Hohmann and Meacock, 1998). It has been described that the overexpression of *THI4*, involved in thiamine biosynthesis, improves glucose consumption, and therefore, fermentation efficiency (Ruiz et al., 2020; Shi et al., 2018). Additionally, thiamine has been reported to be involved in protection against thermal, osmotic and oxidative stresses (Kartal et al., 2018; Kowalska et al., 2012; Li et al., 2019; Wolak et al., 2014). Although yeasts are able to synthesize thiamine de novo, this capacity is reduced under certain environmental conditions, such as those occurring during wine fermentations; therefore, yeast require sufficient exogenous thiamine to achieve maximum growth and fermentation rates (Bataillon et al., 1996; Hohmann and Meacock, 1998; Nosaka, 2006). Zinc is an essential microelement for yeasts that influences yeast physiology and fermentation performance (de Nicola

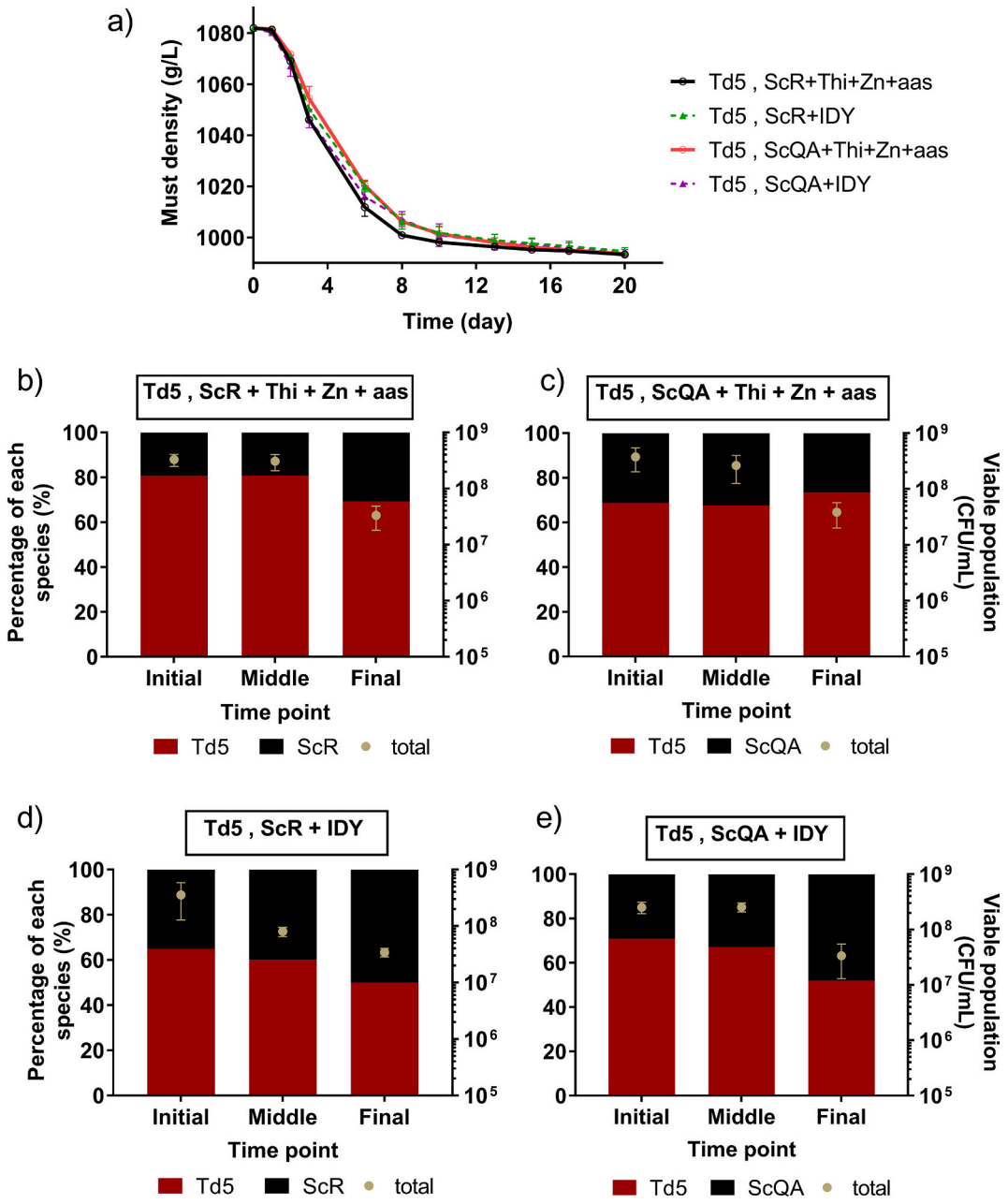

Fig. 7. (a) Must density of sequential fermentations performed with *T. delbrueckii* (Td5) and *S. cerevisiae* (ScR and ScQA) strains, supplemented with Thi, Zn and aas or with IDY when *S. cerevisiae* was inoculated (48 h). Population dynamics during sequential fermentations: (b) *Td5*, *ScR* + Thi + Zn + aas, (c) *Td5*, *ScQA* + Thi + Zn + aas, (d) *Td5*, *ScR* + IDY and (e) *Td5*, *ScQA* + IDY. Time points: initial (Day 3), middle (Day 8) and final (Day 17).
Thi: thiamine, Zn: zinc, aas: amino acids, IDY: inactive dried yeast.

et al., 2009). As a cofactor of many enzymes, such as alcohol dehydrogenase (ADH1), its deficiency can result in slow or incomplete fermentations (Walker, 2004). In addition to acting as a cofactor for many enzymes, zinc is also required for the structural stability of zinc finger proteins, and its supplementation has been shown to improve yeast stress and ethanol tolerance (Zhao and Bai, 2012).

Similar to our results, Maisonnave et al. (2013) showed that the joint addition of vitamins, minerals and nitrogen was required to restart stuck fermentations. Moreover, in this study, the importance of vitamins was highlighted since higher vitamin contents resulted in higher yeast growth and viability at the end of fermentation and better fermentation performance (Maisonnave et al., 2013). In contrast, Medina et al. (2012) suggested that thiamine and pantothenic acid were not key factors for fermentation performance since no significant differences were observed when thiamine and pantothenic acid were excluded from the vitamin mixture in comparison with the complete mixture. Regardless, in this case, the sequential fermentations were not performed with *T. delbrueckii* but with *H. vineae* or *M. pulcherrima*. In any case, it is expected that nutrient requirements and consumption are dependent on the species and even on the strain.

5. Conclusions

Our study confirmed clear nutrient competition between *T. delbrueckii* and *S. cerevisiae* during mixed fermentations, which may be especially relevant and problematic during sequential fermentations. In our hands, *S. cerevisiae* was able to complete fermentations without cell-to-cell contact between yeasts only if it was inoculated at higher

populations or if thiamine, zinc and amino acids were added when *S. cerevisiae* was inoculated. However, this nutrient addition had a lower effect on the imposition of *S. cerevisiae* over *T. delbrueckii* in mixed fermentations. Therefore, this work highlighted the importance of controlling nutrient availability and inoculum size in sequential fermentations to avoid problems, such as stuck or sluggish fermentations.

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CRediT authorship contribution statement

Conceptualization, A.M., M.J.T., and G.B.; Investigation, E.D.Y. and H.R.M.; Methodology, E.D.Y. and H.R.M.; Writing-Original Draft, H.R.M; Writing-Review and Editing, M.J.T. and G.B.; Funding Acquisition, A.M., M.J.T., and G.B.; Resources, A.M., M.J.T., and G.B.; Supervision, M.J.T. and G.B. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Data availability

Data will be made available on request.

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