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## Article

# Physical Contact between *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* Alters Cell Growth and Molecular Interactions in Grape Must

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**Abstract:** The use of multi-starters in oenological conditions (*Saccharomyces cerevisiae* and non-*Saccharomyces* species) is becoming increasingly common. For the past ten years, the combination of *Torulaspora delbrueckii* and *S. cerevisiae* has been proposed to winemakers to improve the wine aromatic profile compared to pure inoculation with *Saccharomyces cerevisiae*. In this work, two commercial strains, *T. delbrueckii* Zymaflore<sup>®</sup> Alpha and *S. cerevisiae* Zymaflore<sup>®</sup> X5 (Laffort compagny, Floirac, France), were investigated in Sauvignon blanc must using a fermentor with a double compartment allowing for physical separation of the two yeast species. The physical separation of the two species resulted in significant differences in the growth, fermentation kinetics (maximum fermentation rate (+13%)), fermentation duration (−14%) and the production of 3SH (+35%) in comparison to mixed cultures with contact. Proteomic analysis confirmed cell–cell contact interactions, as strong differences were observed for both species between mixed cultures with and without physical contact. *T. delbrueckii* mortality in mixed cultures with physical contact may be explained by an oxidative stress. Indeed two proteins implicated in the oxidative stress response were found in significantly higher amounts: a cytosolic catalase T and a cytoplasmic thioredoxin isoenzyme. For *S. cerevisiae*, an increase in proteins involved in the respiratory chain and proton transport were found in higher amounts in pure cultures and mixed culture without physical contact. Our results confirmed that the two mixed inoculations increased certain minor esters (ethylpropanoate, ethyl dihydrocinnamate and ethyl isobutanoate) specifically produced by *T. delbrueckii*, 3.4-fold more compared to in the pure *S. cerevisiae* culture. In conclusion, these results provide new insights into the underlying mechanisms involved in cell–cell contact and confirm the benefits of using *T. delbrueckii* species under winemaking conditions.

**Keywords:** wine; fermentation; yeast association; cell–cell contact; proteomic; thiols; esters



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## 1. Introduction

The alcoholic fermentation of grape must is a complex microbial process. Sugars (fructose and glucose) are converted into ethanol and CO<sub>2</sub>. Many other by-products are also produced contributing to the wine's acidity and aroma (such as ester, higher alcohol, thiol, terpene, etc.). The sequential development of various yeast communities is involved. During the first few hours of fermentation, the predominant yeasts belong to the *Hanseniaspora* and *Candida* genera, which represent up to 90% of total yeast population.

Among them, *Hanseniaspora uvarum*, *Candida stellata* and *Starmerella bacillaris* (formerly called *Candida zemplinina*) are dominant species. Less frequently, *Metschnikowia pulcherrima* can also be present at fairly high populations. In addition, several minor species have been isolated, such as *Torulaspora delbrueckii*, *Pichia kluyveri*, *Hanseniaspora guilliermondi*, *Issatchenkia orientalis*, *Zygosaccharomyces bailii* and *Pichia anomala*. Then, due to their low ethanol tolerance and microbial competition, *Saccharomyces cerevisiae* develops rapidly and achieves alcoholic fermentation [1–7].

Among non-*Saccharomyces* yeasts of interest, *T. delbrueckii* is currently employed as an alternative yeast starter for winemaking, and several *T. delbrueckii* strains are now available as dry active yeast [8,9]. The contribution of this species to the analytical and sensory composition of wines has been the subject of numerous studies. *T. delbrueckii* produces very low levels of undesirable compounds (acetoin, acetic acid, ethyl acetate, acetaldehyde, etc.) [10] and improves the aromatic profiles of dry or sweet wines in correlation with the production of specific fruity esters, volatile thiols and lactones [7,11–18]. In addition, this species has the ability to release mannoproteins or polysaccharides, which improves the sensory perception of wine [17,19,20]. In order to achieve the alcoholic fermentation, this should be used in combination with a *S. cerevisiae* starter. The choice of *S. cerevisiae* strain is crucial to the success of alcoholic fermentation. There are different inoculation procedures: both species are inoculated at the same time (simultaneous inoculation) with a higher concentration for the non-*Saccharomyces* yeast, or the non-*Saccharomyces* species is inoculated before the *S. cerevisiae* (sequential inoculation) at equal concentrations. In the latter case, the timing of *S. cerevisiae* addition is very important. Late addition to a nutrient-poor must due to the development of the non-*Saccharomyces* yeast can lead to slow fermentations or even stuck fermentations. Moreover, it should also be noted that sequential cultures take longer to complete alcoholic fermentation than simultaneous cultures [21]. Despite numerous studies describing the impact of *T. delbrueckii* species according to the mode of inoculation with *S. cerevisiae* (simultaneous or sequential inoculation) on wine quality, the mechanisms of interaction involved between these two species remain unclear in winemaking, with the direct consequence of irregular wine quality.

There are different types of interactions: antagonism, commensalisms, synergism, etc. The inhibition of population growth of one species by another [22–24] has often been reported in oenological conditions, due to “competition” for essential nutrients and/or the impact of “toxic” compounds released in the medium (ethanol, short-chain fatty acids, proteins, glycoproteins, acetic acid, acetaldehyde and SO<sub>2</sub> [21,22,25,26]).

Another mechanism was described by Bisson in 1999, called “Quorum sensing”. This author showed that bacterial populations communicate with each other and adjust their growth [25]. This phenomenon could also play a role in yeast interactions. In 2003, Nissen and Arneborg, using dialysis tubes, suggested for the first time that interactions between *S. cerevisiae* and some non-*Saccharomyces* species could involve a cell–cell contact mechanism [27,28]. Indeed, mixed fermentations performed with *T. delbrueckii* or *Lachancea thermotolerans* in the presence of *S. cerevisiae* revealed a maximal population size lower than pure cultures for both the non-*Saccharomyces* species studied. An early death was also observed for *T. delbrueckii*. Mechanisms linked to nutrient limitation, toxic compounds or quorum-sensing were excluded. Indeed, in the presence of *S. cerevisiae*, *T. delbrueckii* and *L. thermotolerans* inhibition was also observed when yeasts were cultivated with the continued delivery of culture medium. Moreover, inhibition was observed only in the presence of viable *S. cerevisiae* cells and not when non-*Saccharomyces* were cultivated in the presence of supernatant from *S. cerevisiae* culture or when species were physically separated [27,28]. Then, in 2013, using semi-permeable membrane reactors instead of dialysis tubes, Renault et al., with a different couple of *S. cerevisiae* and *T. delbrueckii* strains confirmed the existence of a cell–cell contact/proximity mechanism [29]. In contrast, Taillandier et al. [30] discarded the mechanism of cell–cell contact between these two species, in their experimentations conditions.

A few studies have addressed microbial interactions in winemaking via transcriptomic approaches [31–33]. The authors found a transcriptional reprogramming for *S. cerevisiae* and *T. delbrueckii* species in the presence of each other, in the initial stages of fermentation. For both species, they suggested an activation of glucose, growth and nitrogen metabolism, as a consequence of the presence of competitors in the same medium.

Nevertheless, the majority of studies investigating the mechanisms of interaction between *T. delbrueckii* and *S. cerevisiae* are rarely carried out under oenological conditions, but with a YPD medium or synthetic must. Consequently, the impact of mixed cultures, with and without physical contact, on the aroma profile was not taken into account.

The aim of this work was to confirm the existence of cell–cell contact interaction between two industrial strains of *S. cerevisiae* and *T. delbrueckii* in Sauvignon grape must. Cell growth and the fermentation kinetics were monitored throughout alcoholic fermentation, and ester and thiol production was estimated in pure and mixed-culture wines (with and without physical contact). To gain more insights into the molecular interactions, a proteomic analysis was also performed for the first time.

## 2. Materials and Methods

### 2.1. Yeast Strains and Pre-Cultures

Two industrial strains were used: *S. cerevisiae* Zymaflore® X5 and *T. delbrueckii* Zymaflore® Alpha (Laffort, Bordeaux, France). *S. cerevisiae* Zymaflore® X5 is a high producer of thiols, and its association with *T. delbrueckii* Zymaflore® Alpha has already been described as beneficial [16]. They were streaked on Yeast extract Peptone Dextrose agar plates (2% (*w/v*) agar) and then grown in half diluted grape at 24 °C (300 rpm, 24 h). These precultures were used for inoculating the fermentations. Their cell concentration and viability were calculated using a flow cytometry with a Cell Lab Quanta™ (Beckman Coulter, Brea, CA, USA) equipped with a laser of 488 nm. Samples were diluted in Mc Ilvain buffer (sodium phosphate 0.2 M, citric acid 0.1 M, pH 4) with propidium iodide (0.3% *v/v*) added. Propidium iodide stains dead cells.

### 2.2. Fermentations in a Bioreactor with a Double Compartment

#### 2.2.1. Bioreactor

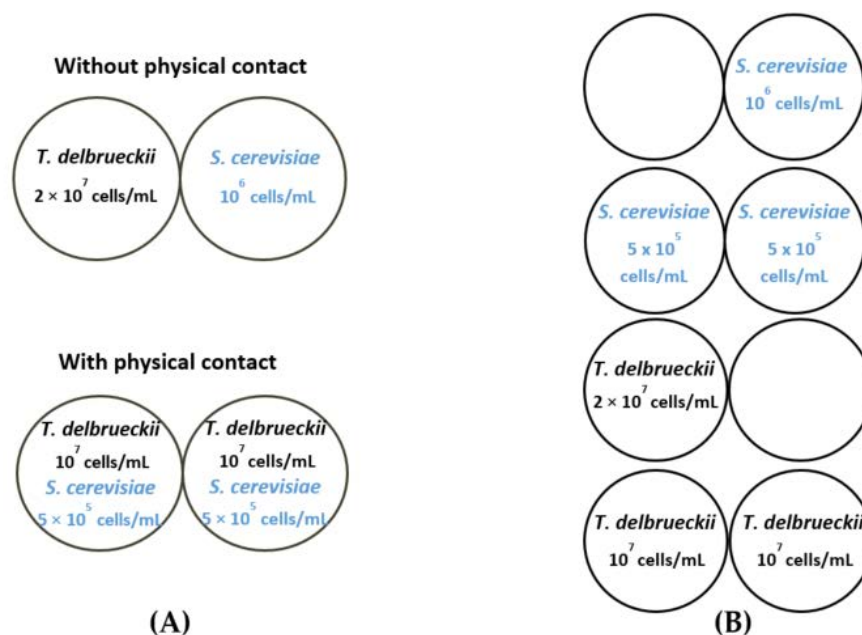
All fermentations were performed at 18 °C (usual temperature for white grape must fermentation) in a bioreactor with a double compartment as described by Renault et al. [29]. Two compartments of 1.1 L each were separated by a 0.45 µm magna ester cellulose membrane (Whatman, GE Healthcare Life Sciences, Chicago, IL, USA). Medium homogenization between the two compartments was performed with a peristaltic pump (Minipuls 3, Gilson, Middleton, WI, USA). To avoid microbial contamination from one compartment to another, two membrane filter capsules (Sartrobran 300 cellulose acetate, Sartorius, Dublin, Ireland) were placed above each compartment. The pump was computer-controlled (Alliance Automation, Talence, France). The bioreactor was placed on a balance (Signum 1 Supreme, Sartorius, Dublin, Ireland) and under magnetic stirring. Temperature and alcoholic fermentation kinetics were automatically monitored (Labview software 2012, National Instrument, Austin, TX, USA). Fermentation kinetics were monitored based on CO<sub>2</sub> release [34,35]. The amount of CO<sub>2</sub> release (g·L<sup>-1</sup>) was determined through the automatic measurement of bioreactor weight loss every 20 min. Weight loss due to evaporation was under 2%.

#### 2.2.2. Fermentation Medium

Alcoholic fermentations were carried out in Sauvignon blanc grape must from the Bordeaux region with a sugar concentration of 203 g·L<sup>-1</sup>, a total acidity of 4.19 g·L<sup>-1</sup> H<sub>2</sub>SO<sub>4</sub> and an assimilable nitrogen concentration of 202 mgN·L<sup>-1</sup>. Total and free sulfur dioxide concentrations were 73 mg·L<sup>-1</sup> and 18 mg·L<sup>-1</sup>, respectively.

### 2.2.3. Modalities

In total, six modalities were performed in triplicate in the bioreactor with double compartments (Figure 1). To study the interactions between the 2 species, two mixed cultures were carried out: without physical contact (each species is inoculated in one compartment) or with physical contact (physical contact, both species are inoculated in both compartments). Mixed cultures of *S. cerevisiae* were inoculated at  $5 \times 10^5$  viable cells·mL<sup>-1</sup> and *T. delbrueckii* at  $1 \times 10^7$  viable cells·mL<sup>-1</sup>, these concentrations were calculated in relation to global bioreactor volume (2.2 L) and not to one compartment (Figure 1A). These concentrations were selected based on preliminary tests as they made it possible to have both in mixed cultures for *T. delbrueckii* growth and not stuck fermentations. In order to determine if the inoculation of each species in one or both compartments (i.e.,  $1 \times 10^7$  vs.  $2 \times 10^7$  viable cells·mL<sup>-1</sup> for *T. delbrueckii*,  $5 \times 10^5$  vs.  $10^6$  viable cells·mL<sup>-1</sup> for *S. cerevisiae*) did not modify their behavior, we completed the experimental design with 4 pure cultures (Figure 1B). These last 4 modalities allowed us to distinguish a possible “density” effect in the compartment.



**Figure 1.** Experimental set up design in bioreactor with double compartment. (A) Mixed cultures of *S. cerevisiae*/*T. delbrueckii*. (B) Pure cultures in one or both compartments.

Yeast growth was monitored through plate counting on YPD medium (1% yeast extract (*w/v*), 1% bacto peptone (*w/v*), 2% glucose (*w/v*), and 2% agar (*w/v*)) and YPD with cycloheximide at  $1 \text{ mg} \cdot \text{mL}^{-1}$  (inhibition of *S. cerevisiae* growth). YPD medium was used for total yeast enumeration in mixed cultures with physical contact and for *S. cerevisiae* in pure and mixed cultures without physical contact. YPD with cycloheximide was used for *T. delbrueckii* enumeration in all conditions. In mixed cultures with contact, the *S. cerevisiae* population was calculated as the difference between the enumeration on YPD medium and YPD + cycloheximide medium. Plates were incubated at 24 °C.

### 2.3. Volatile Thiol Analysis

4-Methyl-4-sulfanylpentan-2-one (4MSP) and 3-sulfanyhexan-1-ol (3SH) were extracted through the reversible combination of the thiols with sodium-p-hydroxymercuribenzoate (p-HMB), from 50 mL of wine previously preserved from oxidation by adding  $50 \text{ mg} \cdot \text{L}^{-1}$  of  $\text{SO}_2$ , as described by Tominaga and Dubourdiou [36] and quantified via gas chromatography–mass spectrometry according to methods described by Tominaga et al. [37,38].

#### 2.4. Ester Analysis

Sample preparation and chromatography analysis were performed via headspace and solid phase micro-extraction (HS-SPME) as described by Antalick et al. [39].

#### 2.5. Classic Wine Analysis

Ethanol concentrations (% vol.) were measured via infrared reflectance (Spectra-Analyser from ZEUTECH, Rendsburg, Germany). Glycerol was analyzed via an enzymatic method (Rhoche-Biopharm, Darmstadt, Germany). Total and free SO<sub>2</sub> (mg·L<sup>-1</sup>) were measured through pararosaniline titration [40]. These analyses were performed by Excell laboratory (Bordeaux, France).

#### 2.6. Data Analysis

R software was used to determinate growth and alcoholic fermentation parameters for each species (R Development Core Team, version 4.0.5): maximum population size (CFU·mL<sup>-1</sup>), yeast mortality (dead cells/h), fermentation lag phase (time between yeast inoculation and the beginning of alcoholic fermentation in h), maximum fermentation rate (g·L<sup>-1</sup>·h<sup>-1</sup>) and maximum CO<sub>2</sub> release (g·L<sup>-1</sup>·h<sup>-1</sup>). Due to variance heteroscedasticity, a Kruskal–Wallis test was applied to determine the difference between growth and fermentation parameters, volatile thiols, esters, ethanol and the SO<sub>2</sub> concentration. The Kruskal–Wallis test was performed by means of the Kruskal function, package agricolae.

#### 2.7. Proteomics

Proteomic analysis was performed to compare the proteome of each species in the four modalities: mixed culture with physical contact, mixed culture without physical contact, pure culture with inoculation in one compartment and pure culture with inoculation in two compartments.

##### 2.7.1. Protein Extraction and Digestion

Cell samples were harvested at mid-growth for all modalities. One milliliter of culture was centrifuged. The pellets were rinsed twice with 1 mL of distilled water, frozen in liquid nitrogen and kept at −20 °C until protein extraction. Total proteins were extracted using acetone precipitation and solubilized, and their concentration was determined as described previously [41]. Following ten-fold dilution in ammonium bicarbonate 50 mM, proteins were alkylated for 1 h in 50 mM iodoacetamide and digested overnight at 37 °C with 1/50 (*w/w*) trypsin (Promega, Charbonniere, France). Trifluoroacetic acid (TFA) was added up to a 1% final concentration to stop the digestion. After washing with 0.06% acetic acid and 3% acetonitrile (ACN) on a polymeric C18 column (Phenomenex, Le Pecq, France), peptides were eluted in 0.06% acetic acid and 40% ACN, vacuum-dried and suspended in 2% ACN and 0.1% formic acid.

##### 2.7.2. LC-MS/MS Analysis

Protein digests (400 ng) were analyzed via LC-MS/MS using the nano2DUltra (Eksigent, Les Ulis, France) connected to a Q-Exactive (Thermo Electron, Waltham, MA, USA). LC buffers were 0.1% formic acid in water (A) and 0.1% formic acid and 100% ACN (B). Samples were first loaded at 7.5 µL·min<sup>-1</sup> on a Biosphere C18 pre-column (0.3 × 5 mm, 100 Å, 5 µm; Nanoseparation, Nieuwkoop, The Netherlands) and desalted (0.1% formic acid and 2% ACN). After 3 min, samples were loaded on a Biosphere C18 nanocolumn (0.075 × 150 mm, 100 Å, 3 µm, Nanoseparation) for separation using a linear gradient from 5 to 35% buffer B for 110 min at 300 nL·min<sup>-1</sup> (125 min run-to-run including regeneration and equilibration steps). Ionization was performed with a 1.4 kV spray voltage applied to an uncoated capillary probe (10 µm tip inner diameter; New Objective, Woburn, MA, USA). Data were acquired in a data-dependent mode using Xcalibur 2.2 (Thermo Electron). MS scan parameters were as follows: mass-to-charge ratio (*m/z*) 400 to 1400, 70,000 resolution, profile mode. MS/MS scan parameters were the following: isolation window of 3 *m/z*,

17,500 resolution, collision energy = 27%, profile mode, dynamic exclusion = 40 s, top8 on ions with  $z = 2$  or 3. Xcalibur raw datafiles were transformed to mzXML open source format using msconvert software in the ProteoWizard 3.0.3706 package [42]. During conversion, MS and MS/MS data were centroided.

### 2.7.3. Data Availability

LC-MS/MS data were deposited in PRIDE [43] with the dataset identifier PXD030835.

### 2.7.4. Protein Identification

Protein Identification was performed using a custom database that contains 6352 ORFs of *S. cerevisiae* EC1118 (version 7/8/2011, SGD project, <http://www.yeastgenome.org/>, accessed on 1 March 2021), 4972 ORFs of *T. delbrueckii* CBS 1146 (SGD project, <http://www.ncbi.nlm.nih.gov/>, accessed on 1 March 2021) and a contaminant database containing the sequences of 55 standard proteomic contaminants (BSA, ovalbumin, keratin, trypsin). A decoy database was also used and contained the reverse protein sequences of the custom database. Databases were searched with X! tandem Sledgehammer (<http://www.thegpm.org/>, accessed on 7 September 2023). Unique labels were attributed to proteins encoded by orthologous genes. The following settings were used: trypsin digestion with a maximum of one possible misscleavage; carboxyamidomethylation of cysteine residues set as static modification; oxidation of methionine residues set as possible modification; 10 ppm for precursor tolerance, 0.02 Da for fragment tolerance. A refinement search was performed with the same settings, except that protein N-terminal acetylations with signal peptide excision were also searched. Identified proteins were filtered and sorted using X!TandemPipeline (version 3.3.4 [44]). The peptide E-value threshold was set to 0.01 and the protein E-value threshold was set to  $1 \times 10^{-5}$ . Only proteins identified based on two different valid peptides were reported. The false discovery rate was estimated using the decoy database at 0.03% per peptide.

### 2.7.5. Peptide and Protein Quantification

Peptides were quantified using MassChroQ software version 2.1.4 [45], by integrating precursor ion peak areas. The detection thresholds on min and max were set at 30,000 and 50,000, respectively. Raw data (containing intensity measurements of 32,849 peptides) were then filtered using the MCQR package (version 0.3.0; <http://pappso.inrae.fr/bioinfo/mcqr/>, accessed on 7 September 2023) in order to remove the following: (1) dubious peptides for which the standard deviation of retention time was superior to 20 s; (2) peptides shared by several proteins; (3) peptides shared by both *S. cerevisiae* and *T. delbrueckii*; (4) peptides quantified in only one replicate; (5) proteins quantified based on only one peptide after filtering. For each protein, quantification was performed on the basis of the mean peptide intensity, normalized by the median value of all peptides. Within each species, some proteins were not quantified in all samples (159 and 169 proteins with missing data for *S. cerevisiae* and *T. delbrueckii*, respectively). These missing data were distributed amongst the samples (no relationship between missing data and modalities) and corresponded to non-reproducible proteins, so we decided to discard proteins with missing data. Finally, two proteome subsets were analyzed, corresponding to *S. cerevisiae* (1186 proteins) and *T. delbrueckii* (1477 proteins) quantified in all 20 and 13 samples, respectively.

### 2.7.6. Statistical Analysis of Proteomic Data

Principal component analyses (PCA) were performed using the R program (version 4.0.5) and ade4 package [46] on the basis of 1186 and 1477 protein abundancies for *S. cerevisiae* and *T. delbrueckii*, respectively.

Analyses of variance (ANOVA) were performed with R to identify differentially expressed proteins. A Benjamini–Hochberg correction was applied to  $p$ -values to take into account multiple testing (aka false discovery rate) [47]. For proteins showing heteroscedasticity (Levene test) or non-normal residues (Shapiro–Wilks test), non-parametric tests

(Kruskal–Wallis) were performed as a substitute of ANOVA. However, no proteins were identified as differentially expressed based on the Kruskal–Wallis test.

For Gene Ontology (GO) analysis, the gene\_association.sgd file (<https://www.yeastgenome.org/>, accessed on 1 March 2021) was used to identify the GO terms associated with all quantified proteins. Hypergeometric tests were performed to identify over- or under-represented GO terms (functions, processes or components). Hypergeometric test *p*-values were corrected for multiple testing using FDR's correction. Since no GO annotations were available for *T. delbrueckii* species, we used *S. cerevisiae* paralogous genes as defined previously (see the Yeast Gene Order Browser, <http://ygob.ucd.ie/>, Homology data, Tdelbrueckii\_genome.tab, released the 24 August 2012).

For reactome pathway analysis, the ReactomePA R package was used [48]. Hypergeometric tests were performed to identify over- or under-represented biological pathways, and *p*-values were corrected for multiple testing using FDR's correction. For *T. delbrueckii* species, we used *S. cerevisiae* paralogous genes as described for GO analysis.

### 3. Results

Except for *T. delbrueckii* pure cultures, all fermentations were complete with the ethanol content between 11.36 and 11.58% vol. No significant differences were observed between mixed cultures and *S. cerevisiae* pure cultures. When fermentations were performed only with *T. delbrueckii*, 18 to 24 g·L<sup>-1</sup> residual sugars were found in wine, leading to a 10.2% vol. of ethanol. All parameter values and compounds analyzed are summarized in Supplementary Table S1.

#### 3.1. Analysis of Growth Parameters, Alcoholic Fermentation Kinetics and Wine Composition

##### 3.1.1. Density Effect

As the concentrations in mixed cultures of the two species differ in the compartments  $1 \times 10^7$  vs.  $2 \times 10^7$  viable cells·mL<sup>-1</sup> for *T. delbrueckii*,  $5 \times 10^5$  vs.  $1 \times 10^6$  viable cells·mL<sup>-1</sup> for *S. cerevisiae*, Figure 1A), we first checked whether or not this “density” effect existed. Pure cultures of each species were pairwise as follows: pure *S. cerevisiae* in one compartment vs. pure *S. cerevisiae* in both compartments on one hand and pure *T. delbrueckii* in one compartment vs. pure *T. delbrueckii* in both compartments on the other hand (Figure 1B). The Kruskal–Wallis test revealed that for both species, no significant differences were observed for all growth and fermentation parameters and compounds analyzed (ethanol, glycerol, thiols, esters, total SO<sub>2</sub>). This indicates that the differences further observed between mixed cultures with and without physical contact were due to species interactions and not to a “density” effect.

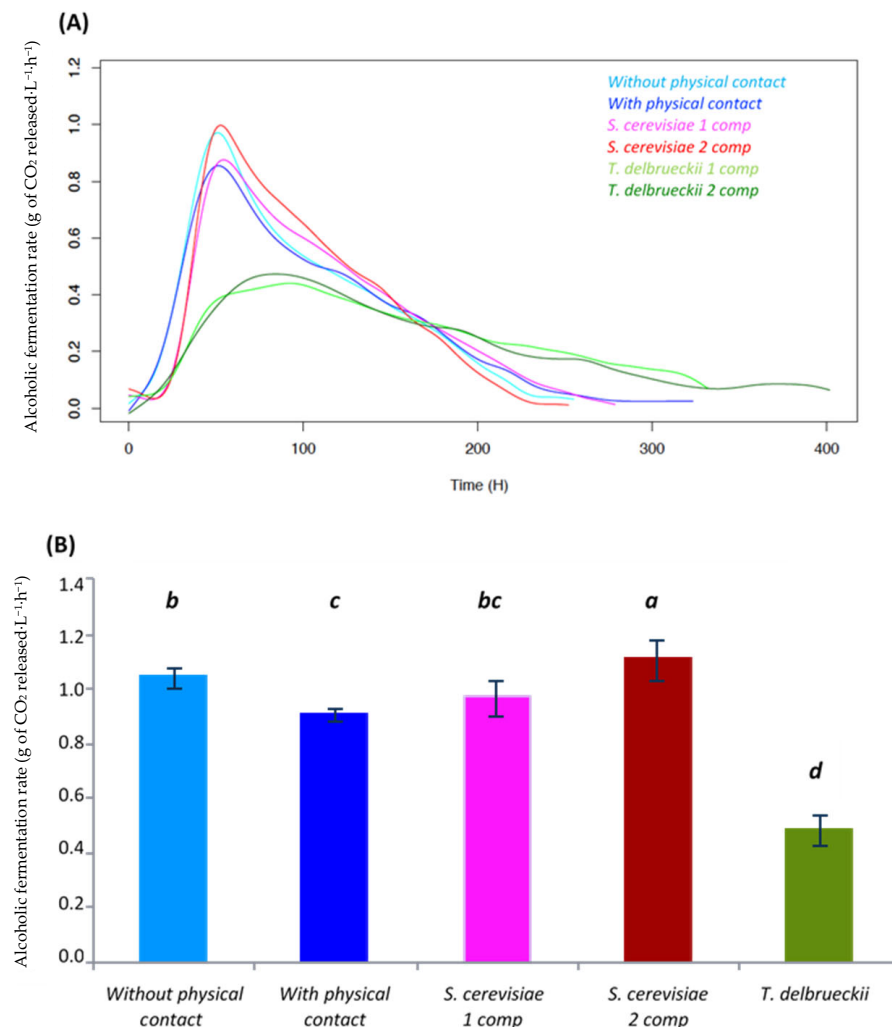
##### 3.1.2. Interactions between *T. delbrueckii* and *S. cerevisiae*

To study interactions effects, mixed cultures without physical contact were compared to pure cultures inoculated in only one compartment of the bioreactor, whereas mixed cultures with physical contact were compared to pure cultures inoculated in both compartments. The comparison between mixed cultures with and without physical contact allowed us to determine interaction mechanism.

- Alcoholic fermentation kinetics

No significant difference was found for the lag phase duration when comparing all modalities (Supplementary Table S1). In pure cultures, *T. delbrueckii* is characterized by a lower maximum fermentation rate lower than *S. cerevisiae* (0.46–0.50 vs. 0.9–1.1 g released CO<sub>2</sub>·L<sup>-1</sup>·h<sup>-1</sup>, Figure 2, Supplementary Table S1). No significant difference in the maximum fermentation rate was observed between the mixed culture without physical contact and *S. cerevisiae* pure culture in one compartment. However, in mixed cultures with physical contact, the maximum fermentation rate strongly decreased compared to mixed cultures without physical contact (–13%) and to *S. cerevisiae* pure culture in two compartments (–18%). This difference is consistent with the fermentation duration (324 h in mixed cultures with physical contact, 262 h without physical contact). These variations

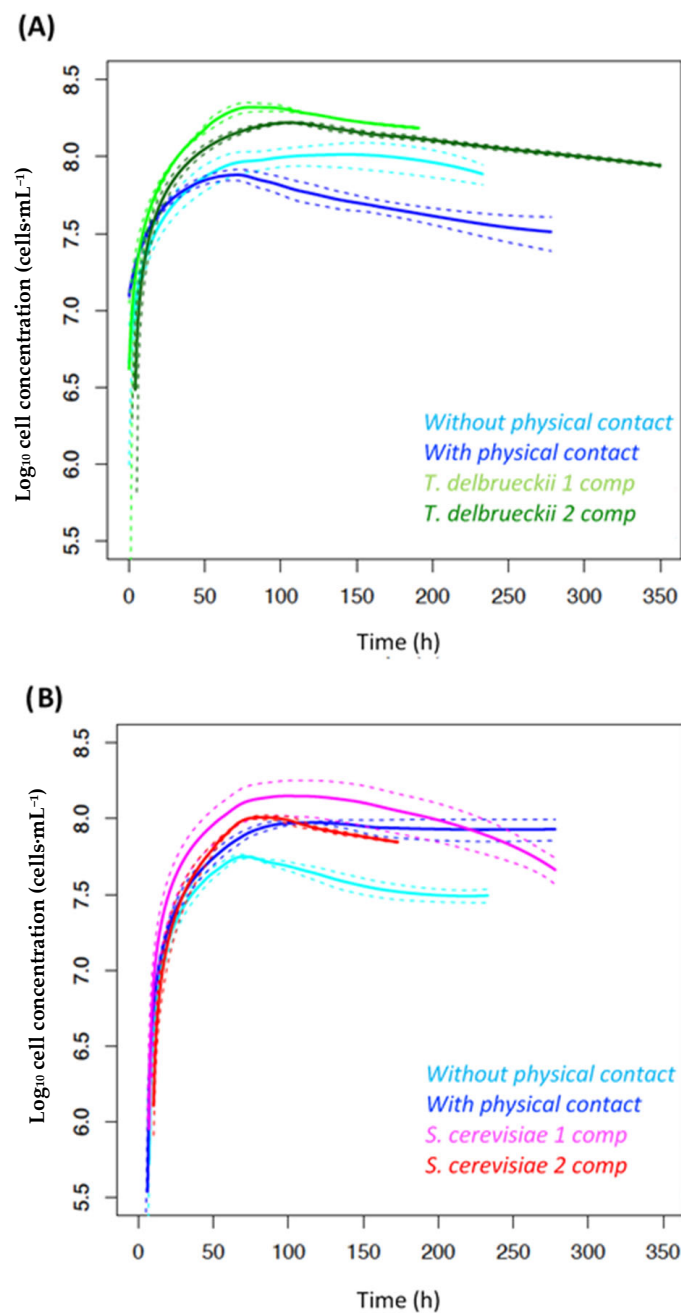
in the maximum growth rate were already observed in a previous work with another *T. delbrueckii*/*S. cerevisiae* couple [29].



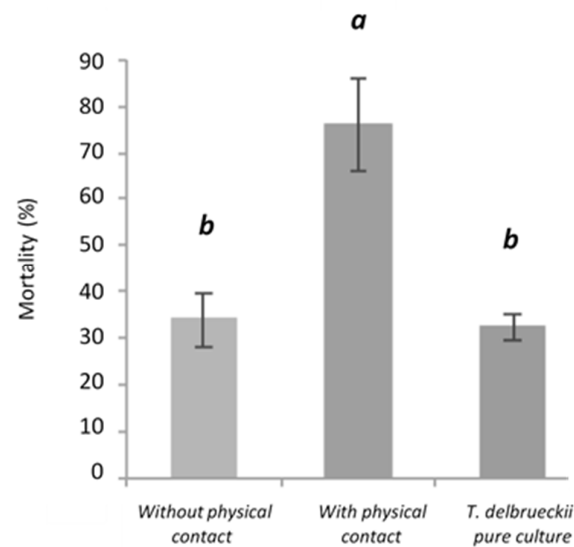
**Figure 2.** Alcoholic fermentation kinetics for mixed and pure cultures in a bioreactor with a double compartment (A) and maximum fermentation rate (B). Without physical contact: mixed culture with each species inoculated in one compartment; with physical contact: mixed culture with both species inoculated in both compartments; *S. cerevisiae* 1 comp: pure culture of *S. cerevisiae* inoculated in only one compartment; *S. cerevisiae* 2 comp: pure culture of *S. cerevisiae* inoculated in both compartments; *T. delbrueckii*: for this species, no significant difference was observed between pure cultures inoculated in one or both compartments. Kinetics smoothing was conducted with R software. The average of the triplicate is presented. a, b, c and d: result of Kruskal–Wallis test, 5%.

- Growth kinetics

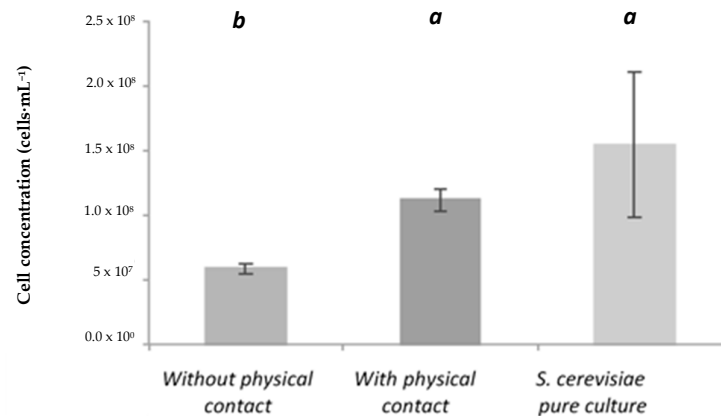
Results of plate counting are presented on Figure 3. No significant difference was observed in the growth lag phase, maximum growth rate and maximum population size for *T. delbrueckii*, in mixed culture with and without physical contact with *S. cerevisiae*. However, *T. delbrueckii* mortality was two-fold higher in mixed culture with physical contact with *S. cerevisiae* (76%) compared to with pure and mixed cultures without physical contact (34%, Figure 4). Only the *S. cerevisiae* maximum population size was impacted by separation. Indeed, in this case *S. cerevisiae* reached  $5.8 \times 10^7$  CFU·mL<sup>-1</sup> compared to  $1\text{--}1.5 \times 10^8$  CFU·mL<sup>-1</sup> in other modalities (Figure 5).



**Figure 3.** *T. delbrueckii* (A) and *S. cerevisiae* (B) growth kinetics in a bioreactor with a double compartment, in pure and mixed cultures. Without physical contact: mixed culture with each species inoculated in one compartment; with physical contact: mixed culture with both species inoculated in both compartments; *S. cerevisiae* 1 comp: pure culture of *S. cerevisiae* inoculated in only one compartment; *S. cerevisiae* 2 comp: pure culture of *S. cerevisiae* inoculated in both compartments; *T. delbrueckii* 1 comp: pure culture of *T. delbrueckii* inoculated in only one compartment; *T. delbrueckii* 2 comp: pure culture of *T. delbrueckii* inoculated in both compartments. Samples were taken each 24 h. Continuous lines: average kinetics of the three repetitions. Dotted lines: standard deviation. Kinetics smoothing was conducted with R concentrations calculated considering the global bioreactor volume (2.2 L).



**Figure 4.** *T. delbrueckii* mortality in a bioreactor with a double compartment, in pure and mixed cultures. Mortality (%) =  $1 - (N_{\min}/\text{Maximum population size}) \times 100$ , with  $N_{\min}$  = minimum population size at the end of alcoholic fermentation. Without physical contact: mixed culture with each species inoculated in one compartment; with physical contact: mixed culture with both species inoculated in both compartments; *T. delbrueckii* pure culture: mortality of this species in pure culture was the same when it was inoculated in both or only one compartment of the bioreactor. Average value is represented here. a, b: result of Kruskal–Wallis test, 5%.

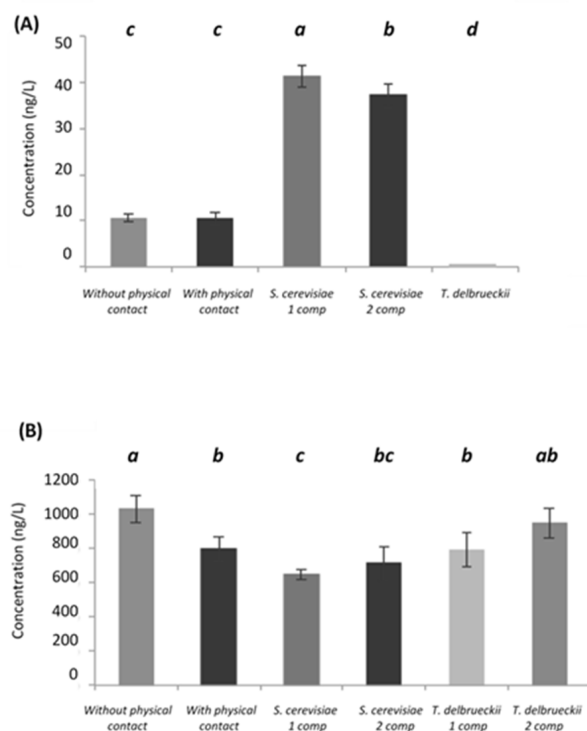


**Figure 5.** *S. cerevisiae* maximum population size in a bioreactor with a double compartment, in pure and mixed cultures. Without physical contact: mixed culture with each species inoculated in one compartment; with physical contact: mixed culture with both species inoculated in both compartments; *S. cerevisiae* pure culture: maximum population size of this species in pure culture was the same when it was inoculated in both or only one compartment of the bioreactor. Average value is represented here. a, b: result of Kruskal–Wallis test, 5%.

- Volatile thiols

Volatile thiols are aromatic compounds with a decisive impact on the aroma of wines, particularly in those made with Sauvignon blanc. Among volatile thiols, 4-methyl-4-sulfanylpentan-2-one (4MSP, box tree, broom) and 3-sulfanylhexas-1-ol (3SH, grapefruit, passion fruit) are predominant in Sauvignon blanc. Results of GC-MS analysis are presented in Figure 6. In accordance with previous studies [14,49], *T. delbrueckii* released very small amounts of 4MSP compared to *S. cerevisiae*. Moreover, in *S. cerevisiae* pure cultures, 4MSP production was significantly higher when yeasts were cultivated in only one compartment

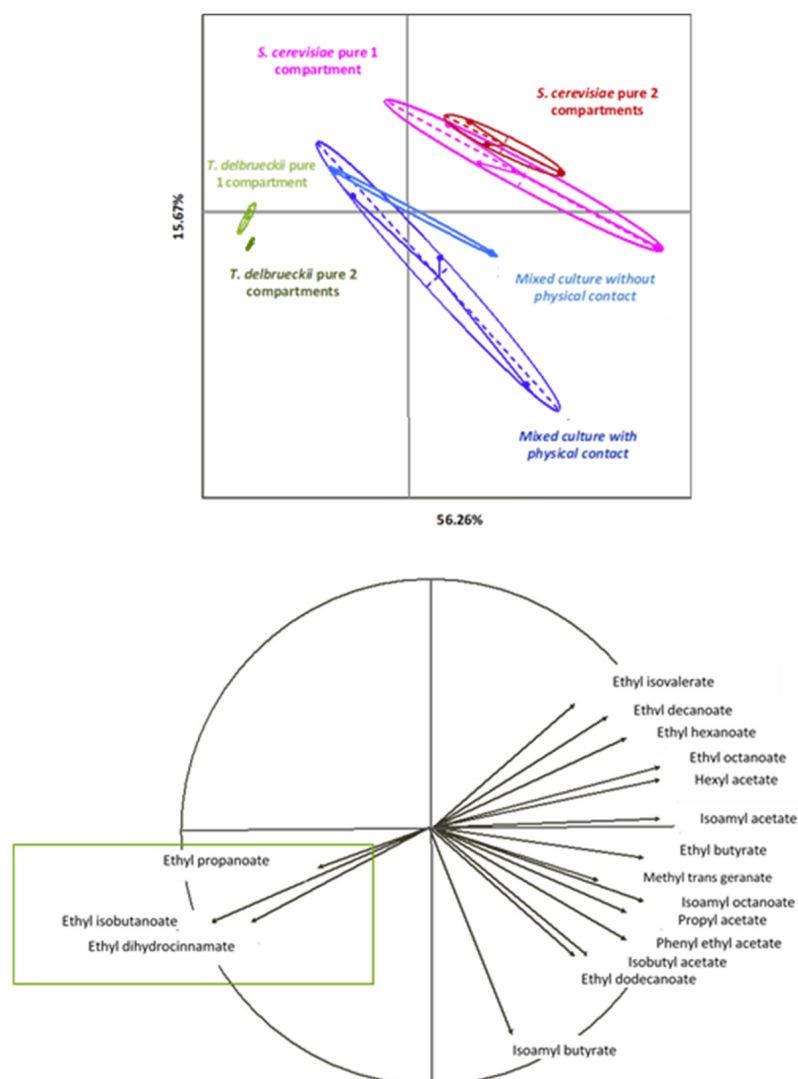
of the bioreactor (+9.5% in comparison with the pure culture in two compartments). In mixed culture, 4MSP concentrations were about four times lower than in *S. cerevisiae* pure cultures. 3SH concentrations were similar in the two types of pure cultures for both species. A synergistic effect was observed in mixed cultures when *T. delbrueckii* and *S. cerevisiae* were cultivated without physical contact (+61% compared to *S. cerevisiae* pure culture in one compartment) as already observed by Renault et al. [16]. With physical contact, 3SH concentrations were similar to *S. cerevisiae* pure culture in two compartments and *T. delbrueckii* pure cultures.



**Figure 6.** Volatile thiol concentrations in pure and mixed cultures. (A) 4-methyl-4-sulfanylpentan-2-one (4MSP); (B) 3-sulfanylhexas-1-ol (3SH). Without physical contact: mixed culture with each species inoculated in one compartment; with physical contact: mixed culture with both species inoculated in both compartments; *S. cerevisiae* 1 comp: pure culture of *S. cerevisiae* inoculated in only one compartment; *S. cerevisiae* 2 comp: pure culture of *S. cerevisiae* inoculated in both compartments. *T. delbrueckii* 1 comp: pure culture of *T. delbrueckii* inoculated in only one compartment; *T. delbrueckii* 2 comp: pure culture of *T. delbrueckii* inoculated in both compartments. For *T. delbrueckii*, the 4MSP concentration (A) was the same when the yeast was inoculated in both or only one compartment of the bioreactor. Average value is represented here. a, b, c, d: result of Kruskal–Wallis test, 5%.

- Esters

We particularly focused on esters known to impact the fruity aroma in wine: ethyl propanoate (strawberry), ethyl isobutanoate (strawberry, kiwi), propyl acetate (pear), isobutyl acetate (banana), ethyl butyrate (kiwi, strawberry), ethyl 2-methylbutyrate (kiwi), ethyl isovalerate (fruit), isoamyl acetate (banana), isoamyl butyrate (banana, strawberry, pineapple), hexyl acetate (pear), ethyl octanoate (fruit), isoamyl octanoate (pear), methyl-trans-geranate (pear), ethyl dihydrocinnamate (fruit, pineapple, almond), ethyl dodecanoate (fruit) and ethyl dodecanoate (fruit) [39]. Principal component analysis was performed to identify the impact of the different pure and mixed cultures on fruity ester compositions (Figure 7).



**Figure 7.** Principal Component Analysis of fruity esters in pure and mixed cultures. The average value is represented here. With physical contact: mixed culture with both species inoculated in both compartments; *S. cerevisiae* 1 comp: pure culture of *S. cerevisiae* inoculated in only one compartment; *S. cerevisiae* 2 comp: pure culture of *S. cerevisiae* inoculated in both compartments. *T. delbrueckii* 1 comp: pure culture of *T. delbrueckii* inoculated in only one compartment; *T. delbrueckii* 2 comp: pure culture of *T. delbrueckii* inoculated in both compartments. The 3 esters found in highest concentration in the presence of *T. delbrueckii* are shown in the green box. Solid lines represent the dispersion of our values; dashed lines represent the central axis of the ellipses.

The first two axes explained 72% of the total variance. Ester composition was specific to each type of culture. Indeed, *S. cerevisiae* pure cultures (one and two compartments) were similar, as with *T. delbrueckii*. Mixed cultures had a midway composition showing that each species participated in the aroma profile. The global ester concentration was similar in mixed cultures with and without physical contact. Fermentations performed with *T. delbrueckii* contained more ethyl dihydrocinnamate, ethyl isobutanoate and ethyl propanoate, whereas pure *S. cerevisiae* cultures contained more ethyl decanoate, ethyl hexanoate, ethyl octanoate, hexyl acetate and isoamyl acetate (Table 1).

**Table 1.** Ester compositions of pure and mixed cultures (only esters with different concentrations between modalities are shown here). a, b, c, d: result of Kruskal–Wallis test, 5%.

	Mixed Cultures with Physical Contact	Mixed Cultures without Physical Contact	<i>S. cerevisiae</i> One Compartment	<i>S. cerevisiae</i> Two Compartments	<i>T. delbrueckii</i> One Compartment	<i>T. delbrueckii</i> Two Compartments
Isoamyl acetate	1246.61 <sup>ab</sup> ± 79.1	1344.82 <sup>a</sup> ± 11.23	1273.52 <sup>ab</sup> ± 37	1156.10 <sup>a</sup> ± 18.1	1.57 <sup>c</sup> ± 0.7	5.08 <sup>c</sup> ± 2.33
Hexyl acetate	52.65 <sup>bc</sup> ± 5.05	62.65 <sup>ab</sup> ± 5.90	58.69 <sup>ab</sup> ± 4.94	66.85 <sup>a</sup> ± 2.70	0.22 <sup>d</sup> ± 0.01	2.31 <sup>d</sup> ± 0.74
Phenylethyl acetate	303.50 <sup>b</sup> ± 54.9	297.50 <sup>b</sup> ± 72.89	413.41 <sup>a</sup> ± 46.5	254.88 <sup>b</sup> ± 65.9	3.10 <sup>c</sup> ± 1.78	1.24 <sup>c</sup> ± 0.68
Isobutyl acetate	48.87 <sup>a</sup> ± 5.00	45.60 <sup>a</sup> ± 1.68	40.45 <sup>a</sup> ± 6.83	31.97 <sup>ab</sup> ± 7.06	26.86 <sup>b</sup> ± 0.56	18.5 <sup>b</sup> ± 2.94
Ethyl propanoate	173.41 <sup>a</sup> ± 53.0	199.19 <sup>a</sup> ± 3.69	60.52 <sup>b</sup> ± 5.75	56.52 <sup>b</sup> ± 1.34	134.55 <sup>a</sup> ± 41.36	108 <sup>a</sup> ± 11.39
Ethyl isobutanoate	48.35 <sup>bc</sup> ± 3.98	51.35 <sup>bc</sup> ± 11.42	8.94 <sup>c</sup> ± 2.58	11.93 <sup>c</sup> ± 1.83	134.55 <sup>a</sup> ± 1.21	82.9 <sup>b</sup> ± 14.02
Ethyl hexanoate	215.87 <sup>a</sup> ± 60.4	243.31 <sup>a</sup> ± 26.07	237.07 <sup>a</sup> ± 105	330.72 <sup>a</sup> ± 40.3	50.67 <sup>b</sup> ± 11.4	55.2 <sup>b</sup> ± 6.93
Ethyl octanoate	119.86 <sup>b</sup> ± 0.61	165.87 <sup>b</sup> ± 26.93	232.09 <sup>a</sup> ± 23.3	230.35 <sup>a</sup> ± 0.35	4.01 <sup>c</sup> ± 0.37	6.65 <sup>c</sup> ± 5.29
Ethyl decanoate	16.95 <sup>b</sup> ± 1.35	17.42 <sup>bc</sup> ± 5.06	42.38 <sup>a</sup> ± 6.36	48.94 <sup>a</sup> ± 0.34	24.17 <sup>d</sup> ± 3.30	7.4 <sup>d</sup> ± 1.43
Ethyl dihydrocinnamate	2.55 <sup>c</sup> ± 0.89	3.95 <sup>b</sup> ± 0.87	0.60 <sup>e</sup> ± 0.20	1.46 <sup>d</sup> ± 0.25	13.60 <sup>a</sup> ± 9.62	9.99 <sup>a</sup> ± 0.65

In mixed cultures (with and without physical contact), some esters were found at a similar concentration compared to *T. delbrueckii* pure cultures (ethyl dihydrocinnamate, ethyl isobutanoate and ethyl propanoate) or *S. cerevisiae* pure cultures (acetate isoamyl, ethyl hexanoate, hexyl acetate and isobutyl acetate). Ethyl octanoate and ethyl decanoate were found in lower concentrations in mixed cultures due to *T. delbrueckii*, which produced these esters in small amounts, highlighting a negative interaction between the two species for these compounds. This phenomenon was already observed with these species in a previous study [16].

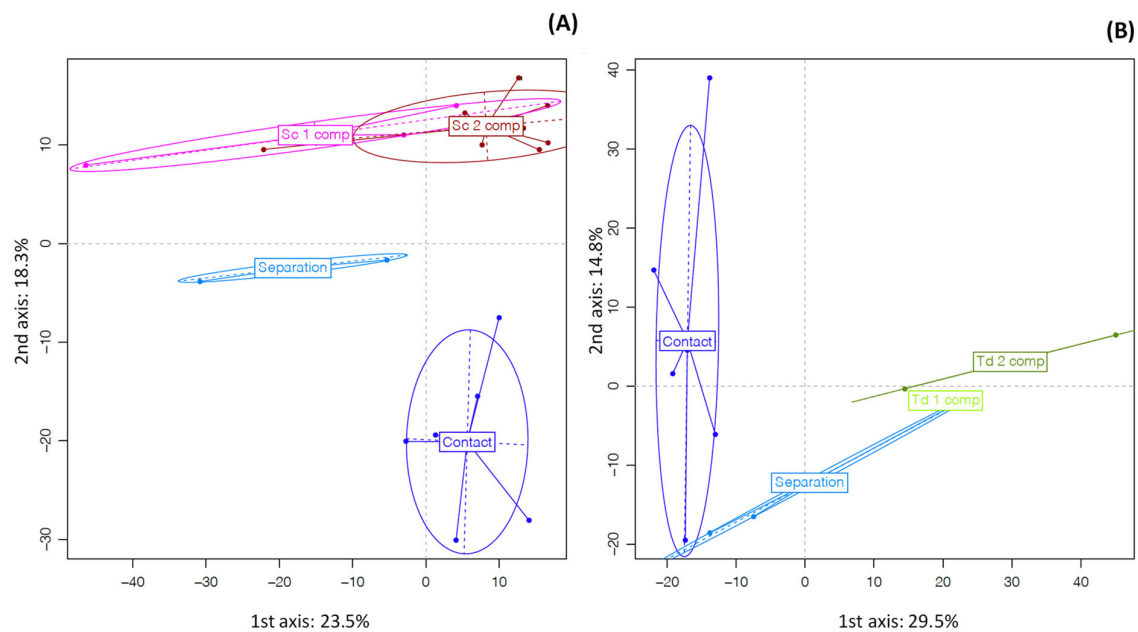
### 3.2. Proteomic Analysis

In order to identify the molecular mechanisms underlying cell–cell contact interactions, cells were sampled during exponential growth and proteins were extracted and analyzed using shotgun proteomics. Two proteome subsets were generated, containing 1186 and 1477 proteins for *S. cerevisiae* and *T. delbrueckii*, respectively. For each species, four modalities were included: mixed culture with physical contact, mixed culture without physical contact, pure culture with inoculation in one compartment and pure culture with inoculation in two compartments.

All subsequent analyses were performed in parallel for each species. In order to have the first insight into the proteome variations between the different modalities, a PCA was performed on both datasets (Figure 8).

For *S. cerevisiae*, the first two axes (Figure 8A) explained 41.8% of the total variation of the proteome with a good separation of the mixed culture with the physical contact modality from both pure cultures with one compartment and two compartments. The mixed culture without physical contact appeared intermediary to the one with contact and pure culture.

For *T. delbrueckii* (Figure 8B), the first two axes of the PCA explained 44.3% of the proteome variation and separated the mixed culture with physical contact modality from the three other modalities. For both species, pure cultures with one compartment and two compartments were co-located, indicating a low “density” effect. To formally test this hypothesis, a first ANOVA (analysis of variance) was performed to compare pure cultures with one compartment and two compartments (Table 2, ANOVA I), and *p*-values were adjusted for multiple tests (Benjamini–Hochberg correction).



**Figure 8.** Principal component analysis of *S. cerevisiae* (A) and *T. delbrueckii* (B) proteomes in pure and mixed cultures. The average value is represented here. Contact: mixed culture with both species inoculated in both compartment; Separation: one species is inoculated in each compartment; *S. cerevisiae* 1 comp: pure culture of *S. cerevisiae* inoculated in only one compartment; *S. cerevisiae* 2 comp: pure culture of *S. cerevisiae* inoculated in both compartments. *T. delbrueckii* 1 comp: pure culture of *T. delbrueckii* inoculated in only one compartment; *T. delbrueckii* 2 comp: pure culture of *T. delbrueckii* inoculated in both compartments. Solid lines represent the dispersion of our values; dashed lines represent the central axis of the ellipses.

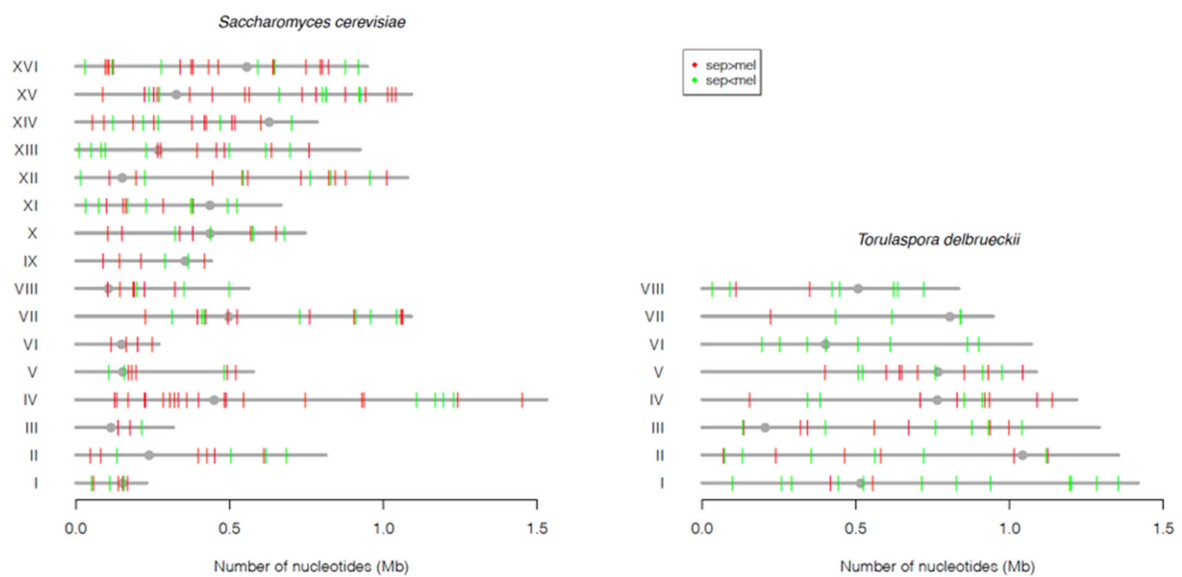
**Table 2.** ANOVA analysis.

ANOVA	Modalities Compared	Number of Significant Proteins	
		<i>S. cerevisiae</i> (1186 Proteins)	<i>T. delbrueckii</i> (1477 Proteins)
I	Pure culture with one compartment vs. pure culture with two compartments	0	0
II	Pure culture with one compartment vs. pure culture with two compartments vs. mixed culture without physical contact	8	0
III	“Pure culture” (one compartment; two compartments; mixed culture without physical contact) vs. mixed cultures with physical contact	209 (132 proteins more abundant in contact, 77 less abundant)	84 (33 proteins more abundant in contact, 51 less abundant)

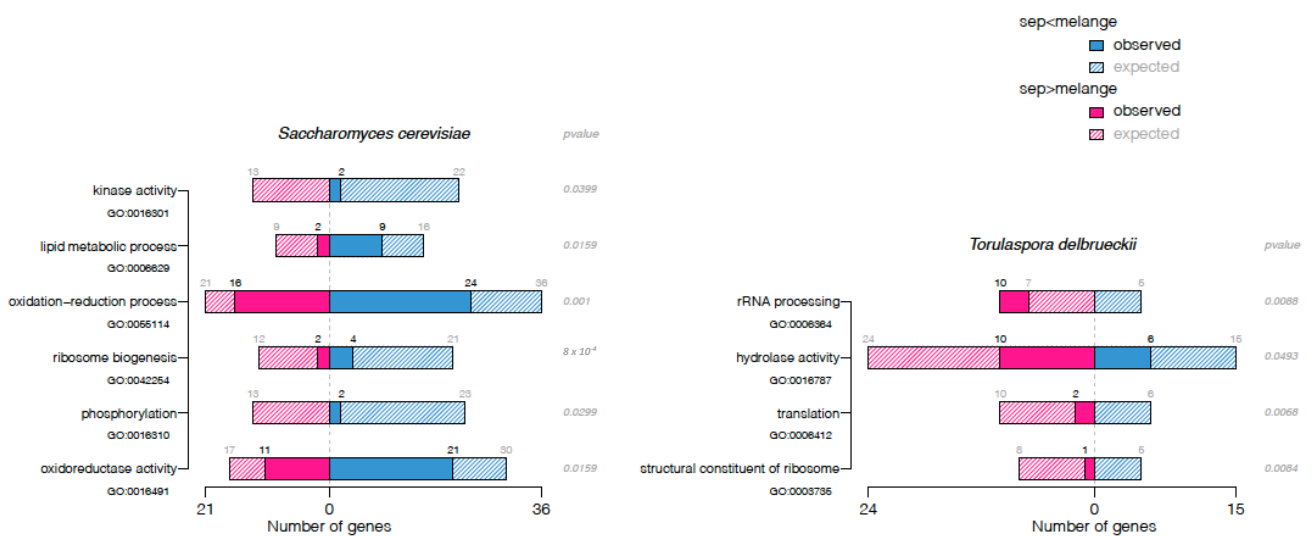
No protein had a significant density effect for *S. cerevisiae* and for *T. delbrueckii*, indicating that the variation in density had no effect on both yeast proteomes. A second ANOVA (ANOVA II) was performed among the three modalities encompassing “pure” cultures (pure cultures with one compartment and two compartments and mixed cultures without physical contact), which appeared to be quite close together based on PCA, particularly for *T. delbrueckii* species. In that case, a few proteins were found to be significant between pure cultures with one compartment and two compartments and mixed cultures without physical contact for *S. cerevisiae* (8/1186 proteins: YBL039C, YDR341C, YGL256W, YHR128W, YKL216W, YMR131C, YPL273W, YPR110C), with none for *T. delbrueckii*. Since very few differences were detected amongst either *S. cerevisiae* or *T. delbrueckii* pure cultures or separated cultures, we considered these modalities as one (*S. cerevisiae* alone or *T. delbrueckii* alone) and compared them to *S. cerevisiae* or *T. delbrueckii* when mixed together, in contact.

Thus, the third analysis compared “pure” versus mixed culture with physical contact modalities (Table 2, ANOVA III). It allowed for the identification of 202 differentially expressed proteins for *S. cerevisiae*, none of them including the eight proteins identified using ANOVA II. This result indicates that these 202 proteins are actually affected by the contact with *T. delbrueckii* cells, 128 of them being more abundant when *S. cerevisiae* is in contact with *T. delbrueckii*, while the remaining 74 were less abundant (Supplementary Table S2). Identically, for the *T. delbrueckii* proteome, 85 significant proteins were identified, of which 33 were more abundant when the species was in contact with *S. cerevisiae*, and 52 were less abundant (Supplementary Table S2).

The chromosomal localization of the genes encoding these differentially expressed proteins was examined (Figure 9). No gene cluster was evidenced either for *S. cerevisiae* or for *T. delbrueckii*, indicating that more specific regulatory pathways are involved in the contact response for both species (Figure 10).



**Figure 9.** Chromosomal localization of the genes encoding differentially expressed proteins in pure and mixed cultures for *S. cerevisiae* and *T. delbrueckii*.



**Figure 10.** Gene ontology of proteins differentially expressed in pure and mixed cultures. Expected: number of genes involved in gene ontology. Observed: number of genes encoding expected proteins differentially expressed.

Nevertheless, as presented in Figure 10, in *T. delbrueckii*, we could notice that most proteins found in higher amounts in “pure” cultures are linked to ribosome constitution, rRNA processing and protein biosynthesis, transformation, transport, targeting and degradation. In mixed cultures with physical contact, some proteins involved in the respiratory chain (YHR051W, YOR065W, YLR201C), proton transport (YBR127C, YPR036W, JR121W), TCA cycle (YGR240C, YCR012W, YNL037C) and the response to oxidative stress (YGR088W, YLR043C) were more abundant.

We observed an increase in proteins involved in fatty acid biosynthesis and transport and in protein metabolism for *S. cerevisiae* in mixed cultures with physical contact. In the case of mixed cultures with physical separation, proteins involved in the respiratory chain and proton transport were found in higher amounts.

#### 4. Discussion

Non-*Saccharomyces* yeasts are essentially found in grape must. If they begin alcoholic fermentation, they are quickly replaced by *S. cerevisiae*, the main agent of this bioprocess. However, it is now well-known that non-*Saccharomyces* can positively or negatively affect wine quality [1,4,22,50–53]. The interesting ones are proposed by several industrial companies to be used in association with *S. cerevisiae* [19].

*T. delbrueckii* is one of non-*Saccharomyces* yeasts of interest in winemaking as it is a low producer of undesirable compounds, like acetoin, acetic acid or ethylacetate [11,12,50,54–59]. Its low production of acetic acid is specifically requested in hyperosmotic conditions (sugars > 350 g·L<sup>-1</sup> like in sweet wines) [12,13]. *T. delbrueckii* is also able to release aromatic compounds, such as norisoprenoids, terpenols, lactones and volatile thiols [15,60–63]. Nevertheless, the lack of knowledge about the mechanisms of interaction between *T. delbrueckii* and *S. cerevisiae* make it difficult to ensure wine quality when these species are used together in industrial starters. The study of interaction mechanisms between *T. delbrueckii* and *S. cerevisiae* is required to take full advantage of their metabolism.

The presence or absence of physical contact between populations is one criteria to classify interactions between microorganisms [64,65]. Interaction mechanisms frequently described in winemaking to explain the inhibition of non-*Saccharomyces* yeast by *S. cerevisiae* are as follows: inhibition by ethanol, the production of peptides or short-chain fatty acids, killer toxins or nutrient and oxygen competition [12,66,67]. Moreover, in the case of *T. delbrueckii*/*S. cerevisiae* interactions, some authors suggested the existence of a “cell-cell contact” mechanism that could explain the inhibition of *T. delbrueckii* by *S. cerevisiae* [27–29].

##### 4.1. Interaction Mechanisms between *T. delbrueckii* and *S. cerevisiae*

All modalities were performed in a bioreactor with double compartments [29], which allowed us to carry out two types of mixed cultures: with (*T. delbrueckii* and *S. cerevisiae* were both inoculated in the two compartments) or without physical contact (only one species in each compartment). These mixed cultures were compared with pure cultures for each species. As expected, only the cultures with *S. cerevisiae* reached the expected alcohol content in wine. The amount of glycerol was similar in all modalities (5.45–6.07 g·L<sup>-1</sup>). In contrast, total SO<sub>2</sub> was strongly lower when fermentations were performed in the presence of *T. delbrueckii* (6.73–7.33 mg·L<sup>-1</sup> in pure cultures, 8.00–11.20 mg·L<sup>-1</sup> in mixed cultures) compared to in *S. cerevisiae* pure cultures (20.40–21.67 mg·L<sup>-1</sup>). These observations underline the importance of combining these two species to reduce sulfite contents in wines.

##### 4.1.1. Density Effect

First of all, we focused on pure cultures to determine if the inoculation in one or both compartments affected the behavior of the two species. Pure cultures were pairwise compared to determine if the “density” effect could impact yeast behavior: pure *S. cerevisiae* in one compartment vs. pure *S. cerevisiae* in both compartments on one hand and pure *T. delbrueckii* in one compartment vs pure *T. delbrueckii* in both compartments on the other hand. Either for *S. cerevisiae* or *T. delbrueckii*, the Kruskal–Wallis test revealed no significant

differences regarding alcoholic fermentation and growth kinetics, biochemical and wine aroma composition and the proteome composition (Supplementary Tables S1 and S2). Consequently, no significant density effect was observed for both species. This indicates that the differences further observed between mixed cultures with and without physical contact were due to species interactions and not to the “density” effect.

#### 4.1.2. Interaction by Cell–Cell Contact

In mixed cultures with and without physical contact, our kinetics showed that *S. cerevisiae* and *T. delbrueckii* growth was modified from pure cultures. Moreover, for both species, growth behavior was also different between mixed cultures with and without physical contact. Remembering that the medium culture was always the same between the two compartments of bioreactors, this last point allowed us to exclude the interactions being only linked to the production of toxic metabolites.

Regarding *T. delbrueckii*, an increase in mortality was observed only when the two species were cultivated in mixed cultures with physical contact. This was already observed in other studies with different couples of strains and suggest cell–cell contact interactions [22,27–29,68]. Cell–cell contact could be explained by two ways: either this type of interaction involves direct physical contact between cells and receptor–ligand molecules in membranes, or *S. cerevisiae* releases some inhibitory compounds at fairly high concentrations near the cells to be lethal for *T. delbrueckii*. Kemsawasd et al. [69], in their study about interactions between *L. thermotolerans* and *S. cerevisiae*, seemed to prefer this second hypothesis. They highlighted that some antimicrobial peptides (already described by Albergaria et al. [66]) at high concentrations were implicated in cell–cell contact interactions between *L. thermotolerans* and *S. cerevisiae*. The fungistatic effect of antimicrobial peptides was also found in laboratory conditions against *T. delbrueckii* [66]. Nevertheless, their real implication in oenological conditions during alcoholic fermentation has not yet been demonstrated.

In our experiments, *S. cerevisiae* reached a lower maximum population size in mixed cultures without physical contact compared to in other modalities. This was already observed in other non-*Saccharomyces/S. cerevisiae* mixed cultures. Beaufort et al. [70] noted the same behavior of *S. cerevisiae* in *S. cerevisiae*–*L. thermotolerans* mixed cultures without physical contact. In our study, the *S. cerevisiae* maximum population size in the two types of pure cultures (inoculation in only one or in the two compartments of the bioreactor) were similar regarding the global bioreactor volume. In this case, a higher *S. cerevisiae* cell density in pure culture inoculated in only one compartment did not result in the negative regulation of the maximum population size. Considering this, the drop in the *S. cerevisiae* maximum population size in the case of mixed cultures without physical contact could not be explained by a quorum-sensing mechanism defined as “the regulation of gene expression in response to fluctuations in cell-population density” [71]. This phenomenon did not appear to be linked to the production of metabolites from *T. delbrueckii* since no differences in *S. cerevisiae* populations were noticed between pure cultures and mixed cultures with physical contact. To explain the drop in the *S. cerevisiae* population, we speculated that the separation allowed *T. delbrueckii* to start its growth earlier than in mixed culture with physical contact, resulting in faster consumption of the medium nutrients. The other hypothesis, which would support the cell–cell contact mechanism, is that, due to the absence of physical contact, *S. cerevisiae* did not “detect” *T. delbrueckii* and did not start its growth soon enough.

#### 4.1.3. Volatile Thiol Composition

Volatile thiols, particularly 4MSP (4-méthyl-4-sulfanylpentan-2-one) and 3SH (3-sulfanylhéxan-1-ol), were identified as responsible for the “grapefruit”, “passion fruit” and “box tree” aroma in Sauvignon blanc wine [37,72,73]. These aromas are revealed by yeasts owing to  $\beta$ -lyases from two types of non-volatile and non-aromatic precursors: Glut-3SH (glutathionylated conjugate precursor) and Cys-3SH (cysteinylated conjugate

precursor). The Glut-3SH precursor is first transformed in the Cys-3SH form and then in volatile thiols [37,72,74–78]. Several factors can impact volatile thiol release, including the yeast strain and the large variability within *S. cerevisiae* species [36,79]. Among non-*Saccharomyces* yeasts, some species were previously described as greatly revealing volatile thiols [14,80]. *T. delbrueckii* species are known to release 3SH but low 4MSP [14,49].

In our study we confirmed the low capacity of *T. delbrueckii* Alpha to release low amounts of 4MSP compared to *S. cerevisiae* Zymaflore X5 (strain with high potential for thiol release). In the two types of mixed cultures (with and without physical contact), 4MSP concentrations were similar and about four times lower than in *S. cerevisiae* pure cultures. This was already described by Renault et al. [81] and suggest an interaction effect for this compound.

For both species, 3SH concentrations were similar in the two types of pure cultures (inoculation in one or two compartments). 3SH release was higher in *T. delbrueckii* pure cultures than in *S. cerevisiae*. In mixed cultures with physical contact, 3SH concentrations were comparable to *S. cerevisiae* pure cultures as described before [14,81]. However, physical separation in the other type of mixed cultures led to an increase of 61% in the 3SH concentration compared to in the *S. cerevisiae* pure culture. As presented by Renault et al. [81], *S. cerevisiae* is able to release 3SH from both Glut-3SH and Cys-3SH precursors, whereas *T. delbrueckii* can only metabolize the Glut-3SH precursor. Moreover, in a mixed culture, the presence of *T. delbrueckii* was also linked to higher degradation of the Glut-3SH precursor compared to in *S. cerevisiae* pure cultures, leading to higher amounts of Cys-3SH precursors in the extracellular medium.

In our study, the maximum population sizes of *T. delbrueckii* were similar in the two types of mixed cultures (without physical contact,  $1.1 \times 10^8$  viable cells·mL<sup>-1</sup>; with physical contact,  $9 \times 10^7$  viable cells·L<sup>-1</sup>), whereas for *S. cerevisiae*, it was lower in mixed culture without physical contact ( $5.8 \times 10^7$  viable cells·mL<sup>-1</sup>) than in other modalities ( $1.1 \times 10^8$  viable cells·mL<sup>-1</sup>). Therefore, the ratio of the maximum population of *T. delbrueckii*/*S. cerevisiae* was higher in the mixed culture without physical contact (1.89 vs. 0.9). Moreover, in the mixed culture without physical contact, *T. delbrueckii* maintained a high viable population longer than with physical contact. This suggests that in mixed cultures without physical contact, more Glut-3SH precursor was degraded into Cys-3SH, which could be transformed by *S. cerevisiae*.

#### 4.1.4. Ester Composition

*T. delbrueckii* was often described as a low producer of major volatile compounds (higher alcohols, esters). Only phenyl-2-ethanol seems to be produced at higher concentrations of the perception threshold [10,58,61,82]. In this study, we confirmed the increase in the ester composition when the two species are combined, underlining the benefits for the winemaker of using *T. delbrueckii* species. Nevertheless, upon co-inoculation with *S. cerevisiae*, aromatic complexity and fruity qualities were generally higher compared to in *S. cerevisiae* pure cultures [16]. An analysis of ester compositions showed that three minor esters were always found in higher amounts in the presence of *T. delbrueckii*: ethyl-isobutanoate (strawberry), ethyl-dihydrocinnamate (fruity, pineapple, almond) and ethyl-propanoate (strawberry).

We analyzed 18 esters implicated in the fruity quality in wine for our different modalities. Ethyl-isobutanoate (strawberry), ethyl-dihydrocinnamate (fruity, pineapple, almond) and ethyl-propanoate (strawberry) were always higher in cultures with *T. delbrueckii*. This highlights a positive interaction for these esters between *T. delbrueckii* and *S. cerevisiae* and confirms previous results. However, a negative interaction was observed for ethyl-octanoate and ethyl-decanoate as they were found in lower quantities in mixed cultures (with and without physical contact) compared to in *S. cerevisiae* pure culture.

#### 4.2. Proteomic Analysis

The proteomic analysis revealed a different behavior for *S. cerevisiae* and *T. delbrueckii* in mixed culture with physical contact compared to in other modalities. The two species were impacted by co-inoculation, as it was previously observed in a transcriptomic analysis of mixed cultures with these two species [31–33]. For each species, no significant difference was observed between the proteomes of pure cultures and mixed cultures without physical contact. This suggests interactions involving cell–cell contact between *T. delbrueckii* and *S. cerevisiae*.

For *T. delbrueckii*, in pure cultures and mixed cultures without physical contact, most proteins found in higher amounts are linked to the ribosome constitution, rRNA processing and protein biosynthesis, transformation, transport, targeting and degradation, reflecting an active growth metabolism. Among them, YGR152C, a Ras-type GTPase required for bud site selection during axial and bipolar budding, is directly linked to cell growth [83,84]. However, in the case of mixed cultures with physical contact, we can notice that *T. delbrueckii* activated some cell defense mechanisms. Indeed, two proteins implicated in the oxidative stress response were found in significantly higher amounts in these conditions: YGR088W and YLR043C. YGR088W is a cytosolic catalase T with a role in protection from oxidative damage by hydrogen peroxide [85,86], whereas YLR043C is a cytoplasmic thioredoxin isoenzyme [87,88]. Six other proteins implicated in the oxidative stress response showed higher, although not significant, amounts in the mixed cultures with physical contact, which supports this hypothesis: YCL035C (glutathione-dependent disulfide oxidoreductase), YJR104C (cytosolic copper-zinc superoxide dismutase), YHR008C (mitochondrial manganese superoxide dismutase), YDR453C, (stress inducible cytoplasmic thioredoxin peroxidase), YJL101C (gamma glutamylcysteine synthetase, for which expression is induced by oxidants) and YBR244W (phospholipid hydroperoxide glutathione peroxidase) [89–97]. Oxidative stress is due to the formation and/or accumulation of reactive oxygen species (ROS) in the cell. One of the main sources of ROS is the leakage of electrons from the respiratory chain during the reduction of molecular oxygen to water to generate the superoxide anion  $O_2^-$  [98,99]. In mixed culture with physical contact, we observed for *T. delbrueckii* an increase of some proteins implicated in the respiratory chain (YHR051W, YOR065W, YLR201C) and proton transport (YBR127C, YPR036W, JR121W) which suggests respiratory activity that is more intense than in other modalities, leading to more ROS formation in cell and oxidative stress. Oxidative stress could explain the mortality of *T. delbrueckii* at the end of alcoholic fermentation in mixed cultures with physical contact.

Interestingly, for *S. cerevisiae*, an increase in proteins involved in the respiratory chain and proton transport were found in higher amounts in “pure” modalities (pure cultures and mixed culture without physical contact), but contrary to *T. delbrueckii*, this is not linked to an oxidative stress response for this species. If Conacher et al. (2022) [33] also noticed metabolic changes in *S. cerevisiae* in co-culture, they described an increase in respiratory genes involved in the mitochondrial electron transport chain in mixed culture.

#### 5. Conclusions

Cell–cell contact interactions between *S. cerevisiae* and *T. delbrueckii* was suggested by different behaviors between pure and mixed cultures with and without physical contact. Proteomic analysis revealed strong differences between pure and mixed cultures without physical contact from one part and mixed cultures with physical contact from the other part, confirming cell–cell contact interactions. The increase in *T. delbrueckii* mortality in mixed cultures with physical contact may be explained by oxidative stress, as several proteins involved in ROS defense were found in higher amounts. Moreover, the aromatic composition of mixed cultures showed a decrease in the 4MSP concentration, whereas 3H increased (without physical contact). *T. delbrueckii* in mixed cultures also led to the production of some minor esters (ethylpropanoate, ethyl dihydrocinnamate and ethyl isobutanoate), increasing the fruity quality of wines. The amount of total  $SO_2$  in the two mixed culture wines was reduced by half, compared with that of *S. cerevisiae*.

In conclusion, these results provide new insights into the underlying mechanisms involved in cell–cell contact and confirm the benefits of using *T. delbrueckii* species under winemaking conditions.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/beverages9030081/s1>: Table S1: Growth, fermentation parameters and analyzed compounds for pure and mixed cultures of *S. cerevisiae* and *T. delbrueckii*; Table S2: Protein abundances in pure and mixed cultures of *S. cerevisiae* and *T. delbrueckii*.

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**Data Availability Statement:** All relevant data are within the article and the Supplementary Materials. LC-MS/MS data were deposited in PRIDE [43] with the dataset identifier PXD030835.

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