Biotechnological Importance of Torulaspora delbrueckii

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Torulaspora delbrueckii has attracted interest in recent years, especially due to its biotechnological potential, arising from its flavor- and aroma-enhancing properties when used in wine, beer or bread dough fermentation, as well as from its remarkable resistance to osmotic and freezing stresses.

Torulaspora delbrueckii non-Saccharomyces wine bread biotechnology genomics

1. Introduction

Non-*Saccharomyces* yeasts were described for many years as sources of spoilage and contamination, and are also associated with a negative contribution to the organoleptic profile of wines. However, in recent decades, wines produced by some non-*Saccharomyces* yeasts revealed distinct and unique characteristics attracting the attention of many research groups [1]. Improved wines are obtained benefiting from their physiological and metabolic features, which have a positive effect on the wine's sensorial and chemical properties, namely in terms of sugar and acid consumption, alongside an enhanced aroma complexity through the release of important metabolites [2][3][4]. Within this group of yeasts, *Torulaspora delbrueckii* stands out as one of the most advantageous non-*Saccharomyces* species due to its potential to introduce diversity and multiplicity to the standard wine's market, currently established by the use of *Saccharomyces cerevisiae* [5]. The rising interest in *T. delbrueckii* is reflected by the number of scientific publications involving this species. According to the Web of Science™ database, between the years 1987 and 2013, an average of eight publications per year were related to the topic *T. delbrueckii* (search queries by title, abstracts and keywords), and this number is continuously growing with a 6-fold increase between 2013 and 2021.

2. Occurrence and General Characteristics

Yeasts from the genus *Torulaspora* have been reported in a wide variety of habitats, such as fruits [6], insects [7][8], soils [9], soil invertebrates [10], plants [11][12], seawater [13], spoiled food [6] and malt environments [6], where yeast from other genera like *Saccharomyces* and *Zygosaccharomyces* may also be present [14][15]. Although not considered a human pathogen, the species *T. delbrueckii* can also be found as a clinical isolate [16]. In addition to the diversified isolation substrates, *T. delbrueckii* also presents a worldwide geographical distribution, with reports describing its isolation in 37 countries from the five continents, as shown in **Figure 1**

Figure 1. Geographical distribution of *Torulaspora delbrueckii*. Countries in which *T. delbrueckii* isolation was reported are highlighted in blue. Data were collected from Albertin et al. [6], Drumonde-Neves et al. [1] and de Vuyst et al. [17].

Species belonging to the genus *Torulaspora* can reproduce asexually by cell division (budding division) or sexually through asci, containing one to four spherical ascospores, characteristic of ascomycetous yeasts [4][18][19]. Regarding its shape, *Torulaspora* yeasts are mainly discerned by spherical cells (hence the torulu terminology), but also ovoid and ellipsoidal forms, with dimensions of approximately 2–6×3–7 µm, which are smaller than those of *S. cerevisiae*. The sharing of multiple morphological and physiological characteristics between some species has led to a misclassification of some of them. Within the genus *Torulaspora*, four strains presumed to be *T. delbrueckii* were later reclassified into the genera *Debaryomyces* and *Saccharomyces*. Currently, this group includes at least six species: *T. delbrueckii* (anamorph *Candida colliculosa*), *T. franciscae*, *T. pretoriensis, T. microellipsoides, T. globosa* and *T. maleeae* [20]. Two other species — *T. indica* and *T. quercuum* — have also been proposed to be included in this genus, after the employment of molecular tools to discriminate them [21]. For many years, *T. delbrueckii* was described as a haploid yeast, essentially because of its small cell size and due to the rare detection of tetrads in sporulation media [20]. However, Albertin et al. [6] suggested that this species may be mainly diploid. The reduced size of this yeast is not, in this way, associated with the ploidy level, and may be explained by the fact that *T. delbrueckii* only possesses 16 chromosomes in the diploid phase, instead of the 32 chromosomes found in *S. cerevisiae* diploid yeasts [20]. Given the lack of deep knowledge about the life cycle of *T. delbrueckii*, it is still difficult to design strategies for the biotechnological improvement of *T. delbrueckii* using classical genetic techniques such as those commonly proposed for *S. cerevisiae* [22]. New techniques are, in this way, being explored, as will be detailed further.

The phylogenetic proximity between *T. delbrueckii* and *S. cerevisiae* may contribute to explain why *T. delbrueckii* is one of the non-*Saccharomyces* yeasts suggested to be most promising for use in biotechnological industries, especially the ones using fermentative processes such as wine- or bread making. *T. delbrueckii* was one of the first non-*Saccharomyces* species to be applied commercially in wines, even though only a few species are available in companies' catalogues: Prelude™, Biodiva™, Zymaflore® Alpha, Vinifer NS™, and Primaflora® VB BIO [4].

3. Genomics and Taxonomy

In opposition to the extensive knowledge about *S. cerevisiae* genome, the most thoroughly annotated eukaryotic organism [23], there has been a hinder in progress regarding *T. delbrueckii* genomic characterization, also delaying the understanding of the genomics underlying the unique aptitudes showed by this species, in comparison with other yeasts. The genome of *T. delbrueckii* type strain CBS1146 is organized in eight chromosomes, it is 9.52 Mb long and has a GC content of 41.9% [24]. Recently, our in-depth study [25] analysed publicly available genomes of *T. delbrueckii* strains, improving their annotation and concluding about important intra-strain differences. In terms of genome size, variations between 9.22 Mb and 11.53 Mb were found. This variation corresponds also to a diverse number of protein-coding genes being annotated (between 464 and 503). Interestingly, the similarity obtained when analysing pairwise comparisons between the four tested strains' genomes was only as high as 99.63%, and in one case was as low as 97.62%. The improved genome annotation obtained in this work allowed to extend this diversity to a particular functional characterization, showing inter-strain differences in proteins related to ATP-synthesis, proton transports, biosynthesis of inositol and resistance to antiviral Brefeldin A. These differences highlight the importance of using different yeast strains in beverages production (and also in other biotechnological applications), improving their quality and diversity.

T. delbrueckii belongs to the phylum Ascomycota, subphylum Saccharomycotina, class Saccharomycetes, order Saccharomycetales, family Saccharomycetaceae. In our previous work [25] we detailed the *T. delbrueckii* phylogenetic placement in relation to 386 other fungal species/strains, concluding about the proximity between this species and the genera *Zygosaccharomyces* and *Zygotorulaspora*. Our results were in accordance with the work of Shen et al. [26], which showed the phylogenetic reconstruction of more than 300 budding yeasts, even though the *T. delbrueckii* branch was concluded as not being robustly supported. Aiming at elucidating the proximity between the three genera — *Torulaspora, Zygosaccharomyces* and *Zygotorulaspora* — we performed a robust phylogenetic reconstruction, filling this gap with the inclusion of additional genomes publicly available in NCBI. As can be depicted in **Figure 2**, all the 15 available genomes of *T. delbrueckii* were grouped together in a single isolated clade (highlighted in green in **Figure 2**), separated from the ones of *T. pretoriensis*, *T. franciscae*, *T. maleae*, *T. globosa* and *T. microellipsoides*. The large branch containing all genomes of the genus *Torulaspora* revealed to be isolated from *Zygotorulaspora* clade (containing species *Zygotorulaspora florentina* and *Zygotorulaspora mrakii*, and highlighted in red in **Figure 2**). In addition, both these genera — *Torulaspora* and *Zygotorulaspora*—formed an isolated group, separated from the one containing *Zygosaccharomyces* species (highlighted in blue in **Figure 2**).

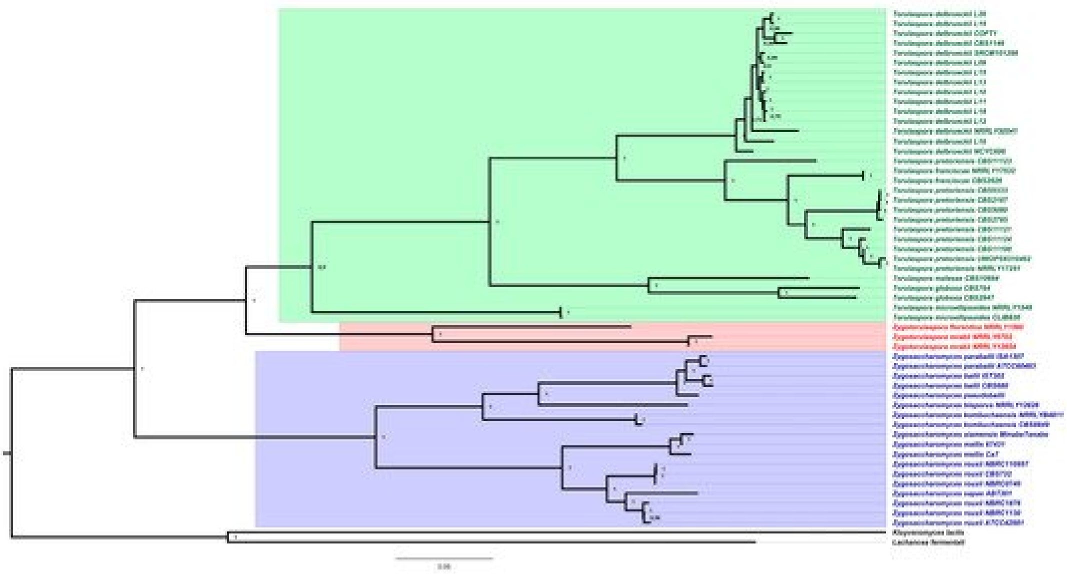

Figure 2. Phylogenetic placement of *Torulaspora delbrueckii,* showing the relationship of 15 strains with publicly available genomes, in relation to its closely related species, chosen from [25]. The phylogenetic reconstruction was obtained using the following parameters: maximum likelihood in IQ-TREE (http://www.iqtree.org, accessed on 28 July 2021), the model of amino acid evolution JTT (Jones–Taylor–Thornton), and four gamma-distributed rates. Homologues were detected for 3820 proteins across the proteome of the 55 fungal species/strains, collected from NCBI. The set of 3820 proteins was aligned and then concatenated for their use in the phylogenetic analysis. These proteins offer a clear high-resolution evolutionary view of the different species, as they are essential proteins beyond the specific biology of the different yeasts. Bootstrapping provided values of 100% for most nodes.

4. Metabolism

Concerning *T. delbrueckii* fermentative behaviour, no consensus has yet been gathered regarding its fermentative power. Some authors characterized this species as having a good fermentation performance in wines [3][4][14][20]. Bely et al. [14] even categorized *T. delbrueckii* as having a performance 9 to 10% higher when considering other non-*Saccharomyces* yeasts. On the contrary, Belda et al. [27] and Loira et al. [28] concluded that *Torulaspora* spp. have lower fermentative power. Still, Almeida and Pais [29] described similar fermentation ability for *T. delbrueckii* and *S. cerevisiae* strains in bread dough. These observations could support the idea of a strain dependent profile with respect to the fermentative capacity of this species, which is also supported by our unpublished data showing a heterogenous performance when analyzing a collection of *T. delbrueckii* strains.

T. delbrueckii presents poor fructose and glucose consumption under conditions of high ethanol and moderate acetic acid concentrations, that can be present in stuck wine fermentations, although it can survive in this environment. This behavior has been associated with the sensitivity of its hexose transport to the inhibitory effect of ethanol [30][31]. To address this limitation, a hybrid strain (F1-11) was constructed by Santos et al. [31] combining the advantageous characteristics of high tolerance to both ethanol and acetic acid of *T. delbrueckii,* and the high hexose consumption of *S. cerevisiae*. This hybrid exhibited a hexose consumption comparable to the one of the *S. cerevisiae* and revealed improved resistance to ethanol and acetic acid, presenting lower cell death rates.

Comparatively, both *T. delbrueckii* and *S. cerevisiae* species behave quite particularly regarding oxygen availability. As the oxygen feed rate decreases, *S. cerevisiae* is the first yeast to switch to a respiro-fermentative metabolism, thus exhibiting lower biomass yields at reduced amounts of oxygen, in comparison to *T. delbrueckii*, which is able to maintain full respiration under these conditions, translating into a lower fermentation strength and a slower growth rate [32]. This occurrence could be less favorable in a winemaking environment since wine production is usually performed under strictly anaerobic conditions (e.g., white, and sparkling wine), or in the presence of very low oxygen concentrations (e.g., red wines) [33].

Even though *T. delbrueckii* possesses lower tolerance to low-oxygen conditions [14][34], its metabolism is usually associated with several positive characteristics, mainly regarding the wine industry, related to high osmotic and sulphur dioxide resistance [23][24][25][26][27][28][35], enhanced capacity for biotransformation of terpenes [28][36][37], or high competence to produce lactic and succinic acids [28][36]. **Table 1** reviews experimental results obtained regarding the most relevant fermentation parameters towards wines' organoleptic profile, comparing *T. delbrueckii* and *S. cerevisiae*.

Table 1. Comparison between *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* concerning fermentation parameters quantified at the end of fermentation process with relevance in wine organoleptic profiles.

Product	*Torulaspora delbrueckii*	*Saccharomyces cerevisiae*	Notes	References
Acetic acid	0.27–0.56 g/L	1.0–1.17 g/L	Key signature in volatile acidity of wines	[14][20]
Malic acid	Consumption between 10.5–25%		Whether degradation or production is desirable depends on the must characteristics.	[23][38][39]
Citric acid	2.18–2.36 g/L	2.23 g/L	Citrus-like taste	[40]
Succinic acid	0.84–1.11 g/L	Maximum of 0.65 g/L	Minor acid in the overall wine acidity, although the combination with one	[41]

Product	*Torulaspora delbrueckii*	*Saccharomyces cerevisiae*	Notes	References
	-	Maximum of 1.13 g/L	molecule of ethanol creates the ester mono-ethyl succinate, responsible for a mild, fruity aroma	[42]
Mannoproteins	*T. delbrueckii* produces 25% more than *S. cerevisiae*		Released during fermentation or ageing processes	[23]
Polysaccharides	*T. delbrueckii* releases 50% more than *S. cerevisiae*		Increases the quality of mouthfeel properties	[43]
Glycerol	1–10.5 g/L	Maximum of 9.1 g/L	Smoothness and viscosity features	[44][45]
Ethanol	40.6–72.68 g/L	103–121 g/L		[39][46]

5. Biotechnological Importance of *T. delbrueckii*

5.1. Bread Industry

Bread making is a practice that has long been discovered and has been the subject of much progress. In more recent years, developments in bread making have been increasingly focused on the enhancement and diversification of the sensory pleasures of taste, texture, and appearance of the final product [19]. The degradation of the dough carbohydrates (namely fructose, glucose, sucrose and maltose) present in the flour, or even wittingly added, is carried out by yeasts, resulting in the release of carbon dioxide and ethanol, produced through glycolysis and posterior pyruvate decarboxylation and reduction [17][19][30]. Carbon dioxide is responsible for the dough leavening, while most of the ethanol evaporates during the baking process. However, the latter also plays an important role in the properties of the dough [17]. The choice of the appropriate yeast is usually based on (i) good fermentative power which could be translated into its ability to leaven the dough; (ii) capacity to use different carbon sources; and (iii) tolerance to stressful conditions, namely, osmotic, and freezing stresses [30][47][48]. *S. cerevisiae* strains have been domesticated and optimized for baking applications and are usually the manufacturer's required yeast for the baking industry. This species efficiently uses maltose as a source of energy, as opposed to *Candida humilis* and *Kazachstania exigua* which, according to de Vuyst et al. [17], are sourdough-specific maltose-negative yeasts. *S. cerevisiae* is commonly implemented as a leavening agent, becoming an

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alternative to sourdough (extensively used for years) particularly in rapid and industrial-scale bread productions [17]. However, *T. delbrueckii* is being pointed out as an alternative to *S. cerevisiae* in this industry, mainly due to its high osmotic and freeze-thawing resistance, showing improvement of the quality of the bakery products [29][30]. Experiments conducted by Almeida and Pais [29] demonstrated greater leavening activity in lean and frozen dough for *T. delbrueckii* strains, comparing to *S. cerevisiae*, as the traditional yeast was more prone to suffer from freeze damage during the storage of the doughs. Apart from this feature, *T. delbrueckii* strains displayed rapid growth, a more rapid response when exposed to hyperosmotic conditions, and high biomass production accompanied with sweet properties (associated with the release of aromatic compounds). These observations were later confirmed by Hernández-López, Prieto and Randez-Gil [49]. Due to its osmotolerant properties, *T. delbrueckii* has already been used in the bakery industry in Japan, for the production of sweet breads and pastries [50].

Co-cultures using *S. cerevisiae* and *T. delbrueckii* species enhanced bread quality with superior aroma and improved sensorial attributes, with 47 volatile compounds—predominately alcohols, aldehydes, and esters—being identified in the bread crumb leavened with both yeasts [19]. Wahyono et al. [19] highlighted some properties of the resulting mixed bread which, using a radar plot, rated within a range of 4.73–5.57 from a total of 7 points, such as acceptability, enhanced flavor, mouthfeel and color. In comparison with *S. cerevisiae* single cultures, which recorded within 4.07–5.71 range in the same radar plot.

5.2. Production of Fermented Beverages

In recent years, researchers worldwide have been paying particular attention to *T. delbrueckii* exploitation to improve wines organoleptic final profile and quality. As referred above, its physiological and metabolic properties revealed positive effects in wines characteristics towards acids and sugar consumption, but also an enhancement of the aroma complexity through the production of important metabolites [2][3][4][23][51][52][53][54]. During wine fermentation, higher alcohols (also termed fused alcohols) and esters contribute 30 to 80% to the aroma profiles of wine, being the two most relevant groups of metabolites [54]. Isobutanol, phenyl ethanol and isoamyl alcohol are the main fusel alcohols reported to contribute to the wine's scent in concentrations ranging from 1.41 mg/L to 9.2 mg/L [55]. According to Ebeler [56], yields of this type of metabolites can achieve 140–420 mg/L, but concentrations over 300 mg/L contribute negatively to the aroma quality. Besides fusel alcohols, the aromatic matrix of wine is composed of esters, which are by-products of yeasts metabolism during malolactic fermentation, ageing and, most relevant in this context, alcoholic fermentation. These molecules reach maximum values when yeasts achieve the stationary growth phase [57], as its production by *T. delbrueckii* is a strain-dependent feature [55]. Two main esters classes are present in wine: the ethyl esters and the acetate esters. The contribution of the latter encompasses desirable floral and fruity sensory properties in wine, contributing about 75% to the flavor profile [55][56][57]. However, as stated in Belda et al. [57], wines holding concentrations of ethyl acetate higher than 90 mg/L are considered to be faulty. Other important metabolites are fatty acids, which are detected in alcoholic beverages as mainly straight-chain and saturated molecules, with palmitoleic acid considered the most relevant unsaturated fatty acid. Besides these, fatty acids with different chain lengths are part of the wine's matrix but prevail in small amounts, which makes them not so significant as the previous ones [56]. The main fermented beverages in which *T. delbrueckii* is employed are reviewed in **Table 2**.

Fermentation 2018, 4, 22.

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Table 2. *Torulaspora delbrueckii* applications in fermented beverages.

Beverages Applications	Used Substrate	Advantages	Disadvantages	References
Beer	Wort	High tolerance to hop compounds; good flavor-forming properties	Low sugar utilization	[35][58][59][60]
Mezcal	Agave juice †	Rich in volatile compounds; acceptable in sensory tests	Low performance	[61][62][63]
Tequila	Agave juice *	Positive influence on the final sensory profile	–	[64]
Cider	Apple juice †	Great production of ethyl decanoate and ethyl hexanoate	Low performance; negligible amounts of acetate esters	[65][66]
Mead	Honey sugar	Good fermentation ability; Good sensory features	Grassy flavor	[7]
Soy alcoholic beverage	Soy whey	Enrich aroma profiles: high levels of ethyl decanoate and ethyl hexanoate; metabolize hexanal;	–	[67]

* Specially from *Agave tequilana*; † sterile.

5.3. Other Food Applications

The reported versatility of *T. delbrueckii* makes it a remarkable asset to be explored, not only for bread and fermented beverages purposes, but also in other diverse food products (**Table 3**). One example is the production of chocolate in which yeasts play a key role in flavour development, as the quality of chocolate is reduced if the cocoa fermentation process is conducted without these microorganisms [68]. This importance is reinforced by Visitin et al. [69] by showing the involvement of *T. delbrueckii* in the fermentation of cocoa beans (*Theobroma cacao* [68]) to

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produce chocolate, despite not yet being standard in this industry. Authors showed that through a combination with *S. cerevisiae*, modifications in the analytical profile of the chocolate are obtained. Moreover, differences in the samples obtained from *S. cerevisiae* and *T. delbrueckii* inoculated chocolate had a significant impact on the consumers' perception of the final product, mentioned by some as fruitier. Therefore, the use of this unconventional yeast resulted in a positive contribution to the development of the chocolate's final aroma. In addition, *T. delbrueckii* can also be explored in the cheese industry, benefiting from its tolerance to low temperatures, low pH, high salt concentrations and low water activity [70]. Andrade et al. [71] produced cheese from fermented milk, with the aim of evaluating the impact of *T. delbrueckii* (in mixed or pure inocula) on cheese production, detecting a slow consumption of lactose which can be translated into a reduced β-galactosidase activity, as stated by the authors.

Table 3. *T. delbrueckii* industrial food applications.

Food Applications	Used Substrate	Advantages	Disadvantages	References
Chocolate	Cocoa beans	Good flavor quality of cocoa and, therefore, the chocolate	Expedite in mixed fermentations with *S. cerevisiae*	[69]
Cheese	Cheese	Varied aromatic properties	Unable to inhibit pathogenic bacteria; low β-glucosidase activity	[71][72]
Honey	Honey sugar	Rapidly ferment sugar	Large-scale productions only in combination with *S. cerevisiae*	[7]
Olive oil	Black olives	Easy hydrolyzation of olive oil	Growth inhibition at concentrations higher than 0.5% (*w*/*v*) of oleuropein	[73]
Coffee	Coffee cherries	Improve coffee's sensorial quality	Pronounced astringency depending on the coffee variety	[74][75]
Bio-protection	–	Reduction in the use of chemical preservatives to control food spoilage	–	[76][77]

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Santamaria, P.; Gutiérrez, A.R. Wine aromatic compound production and fermentative behaviour

Food Applications	Used Substrate	Advantages	Disadvantages	References

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