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Linking gene expression and oenological traits: Comparison between *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* strains

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ABSTRACT

Wine fermentations typically involve the yeast *Saccharomyces cerevisiae*. However, many other yeast species participate to the fermentation process, some with interesting oenological traits. In this study the species *Torulaspora delbrueckii*, used occasionally in mixed or sequential fermentation with *S. cerevisiae* to improve wine sensory profile, was investigated to understand the physiological differences between the two. Next generation sequencing was used to characterize the transcriptome of *T. delbrueckii* and highlight the different genomic response of these yeasts during growth under wine-like conditions. Of particular interest were the basic differences in the glucose fermentation pathway and the formation of aromatic and flavour compounds such as glycerol, esters and acetic acid. Paralog genes were missing in glycolysis and glycerol biosynthesis in *T. delbrueckii*. Results indicate the tendency of *T. delbrueckii* to produce less acetic acid relied on a higher expression of alcoholic fermentation related genes, whereas acetate esters were influenced by the absence of esterases, *ATF1–2*. Additionally, in the Δbap2 *S. cerevisiae* strain, the final concentration of short branched chain ethyl esters (SBCEEs) was related to branched chain amino acid (BCAA) uptake. In conclusion, different adaption strategies are apparent for *T. delbrueckii* and *S. cerevisiae* yeasts, an understanding of which will allow winemakers to make better use of such microbial tools to achieve a desired wine sensory outcome.

1. Introduction

Fermented foods have been produced for millennia, via process that was developed as a preservation method. Their traditional preparation relied on a spontaneous fermentation by ‘wild’ microorganisms. These practises promoted the adaptation and domestication of microorganisms to anthropic habitats. In wine, microorganisms had to adapt to harsh and dynamic environments through beneficial genomic variations that provided a fitness advantage. Yeast, along with lactic acid bacteria, were therefore selected in order to preserve food and beverages and also improve the sensory attributes, and addition of a pure selected yeast starter forms part of current fermentation practices (Mortimer, 2000).

Saccharomyces cerevisiae is undoubtedly the most well-known and broadly used of the yeasts. Its genome has a median length of 12 Mb and is divided into sixteen chromosomes (O'Leary et al., 2015). It encodes > 5400 genes and its selected oenological phenotype comprises several desired traits: tolerance to ethanol (10–14% v/v) and sulfur dioxide (SO_2), complete sugar catabolism (residual of < 5 g/L), low production of volatile acidity, low assimilable nitrogen consumption, high growth rate and fermentation reproducibility (Suárez-Lepe and Morata, 2012). Many of the non-*Saccharomyces* yeasts involved in wine fermentation are considered spoilage, due to their negative sensory traits, lower ethanol tolerance and low fermentation ability (Tataridis et al., 2013). However, more recently, the contribution of these to wine quality has been reconsidered (Jolly et al., 2014) with several investigations reporting many positive effects on wine sensory attributes amongst selected strains (Bely et al., 2008; Ciani and Picciotti, 1995). *Torulaspora delbrueckii* is a non-*Saccharomyces* yeast that can be found metabolically active on grape berries and juice of spontaneous or yeast inoculated fermentations (Albertin et al., 2014; Comitini et al., 2011). Previously, this species was identified as *Saccharomyces rosei*, which suggests a similar taxonomic lineage and function to that of *S. cerevisiae* (Bely et al., 2008). Evolutionarily, *Torulaspora* spp. and *Saccharomyces*

Abbreviations: Chemically Defined Grape Juice Medium, (CDGJM); Short chain ethyl esters, (SCEEs); Short branched chain ethyl esters, (SBCEEs); Branched chain amino acids, (BCAAs); Medium chain fatty acids, (MCFAs)

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spp. separated approximately 100–150 million years ago (Hagman et al., 2014). *Torulaspora delbrueckii* has a 9.7 Mb genome, with nearly 5000 open reading frames (ORFs) divided into 8 chromosomes (Gordon et al., 2011). Under typical winemaking conditions, *Torulaspora* sp. has a fermentative metabolism (Brandam et al., 2013) and can produce lower amounts of acetaldehyde, acetoin, acetate (approximately 0.2 g/L, as reported in the best scenarios) and ethyl acetate compared to *Saccharomyces cerevisiae* (Bely et al., 2008; Martinez et al., 1990; Taillandier et al., 2014), even if, to a certain degree, strain-dependent phenotypic variance have been observed (Velázquez et al., 2015; Van Breda et al., 2013). Its metabolism differs from that of *S. cerevisiae*, especially under hyperosmotic stress, where it produces lower levels of acetic acid (Blomberg and Adler, 1992). Furthermore, *T. delbrueckii* fermentation in combination with *S. cerevisiae* exhibits a different production of fusel alcohols, esters, terpenes and phenolic aldehydes (Azzolini et al., 2012; Azzolini et al., 2015; Renault et al., 2015; Velázquez et al., 2015). Empirically, it added sensory complexity described as “wild” or “natural”, which provides the final wine with desired regional characteristics, usually referred as an expression of terroir (Raynal et al., 2011; Tataridis et al., 2013).

An enhancement of volatile compounds, such as esters, higher alcohols, and an acceptable low production of off-flavours, have been established as criteria for selecting new yeasts for use in winemaking (Pacheco et al., 2012). An increased influence of non-*Saccharomyces* species during fermentation may be a way to enhance the production of desirable compounds, in order to produce more complex and higher quality wines. Nevertheless, there is a need for a better understanding of the molecular basis behind the production of these compounds by non-*Saccharomyces* yeasts under different winemaking conditions. Variations in yeasts activity of aroma-active metabolic pathways will produce organoleptic difference in wine (Bisson and Karpel, 2010). Such pathways include those involved in the production of volatile acidity (as acetate off-flavours), the Ehrlich pathway (fusel alcohols and aldehydes) and the acetate and ethyl ester pathways (Bisson and Karpel, 2010; Chidi et al., 2016; Procopio et al., 2011). Yeast cells use these pathways in response to stress to maintain intracellular redox balance and to adjust the pool of essential cofactors and amino-acids (Hazelwood et al., 2008). To investigate these aspects, next generation RNA sequencing (using an ILLUMINA platform) has emerged as a powerful tool to provide a comprehensive analysis of the simultaneous expression of thousands of genes (Marks et al., 2008). Comparative transcriptomics investigation of *Saccharomyces* vs. non-*Saccharomyces* yeast species could increase our knowledge on genes associated with the production of aroma compounds characteristic of unique wine styles (Masneuf-Pomarede et al., 2015). Findings could aid in the identification of wine yeast strains with promising sensory characteristics and higher fermentation efficiencies (Erasmus et al., 2004).

The aim of this work was to identify the genes responsible for oenological differences between *S. cerevisiae* and *T. delbrueckii*. We also explored if the differences in metabolite production during fermentation between the two species were related to their different genetic backgrounds and/or the manner in which relevant genes and metabolic pathways were regulated.

2. Materials and methods

2.1. Yeast selection and fermentation procedure

Indigenous strains of *S. cerevisiae* YAL1 and *T. delbrueckii* COFT1 were originally isolated from spontaneous fermentations conducted at Yalumba Winery (Angaston, South Australia). Glycerol stocks were streaked onto YEPD agar plates (1% yeast extract, 2% bacto peptone, 2% glucose, 2% agar) and grown for 48 h at 28 °C. For each species, a single colony was isolated and grown overnight in YEPD medium (1% yeast extract, 2% bacto peptone, 2% glucose). A 20 μL cell suspension was then transferred into 150 μL phosphate buffer saline (PBS) solution, stained with 10 μL of 1 mg mL^{-1} propidium iodide (Deere et al., 1998) before 20 μL of Flow-Count fluorospheres (Beckman Coulter, USA) were added to three separate subsamples and a count made by a flow cytometer (FACSCalibur; Becton Dickinson, USA). Accordingly, the appropriate volume of starter culture was added to 200 mL Chemically Defined Grape Juice Medium (CDGJM, modified from McBryde et al., 2006, Supplementary File 2; 250 g/L equimolar glucose and fructose; 400 mg/L nitrogen) in 250 mL Erlenmeyer flasks to provide an initial inoculation rate of 1×10^6 cells/mL. Fermentations were conducted in triplicate with shaking at 120 rpm, to homogenise the samples and ensure common gene expression between all cells (Puig and Pérez-Ortín, 2000) and, at 22 °C, an average temperature between red and white wine fermentations. After initiating the fermentations and consequent establishment of anaerobic condition, 10 mL samples were taken every 24 h, from which 5 mL were divided into 5 aliquots and frozen immediately in liquid nitrogen for subsequent RNA extraction (Piper et al., 2002). The supernatant from the remaining 5 mL subsample was divided into 5 equal aliquots, which were frozen for subsequent metabolite analysis. Yeast growth during fermentation was quantified by measuring cell density at 660 nm with a Tecan Infinite 200 spectrophotometer (Tecan, Switzerland).

2.2. Yeast transformation

Genomic DNA was isolated from BY4741 bap2::KanMX4 yeast strain according to Amberg et al. (2005). PCR amplification of the *BAP*2 deletion cassette (bap2::KanMX4) was undertaken using VELOCITY DNA Polymerase (Bioline, UK) according to the manufacturer's instructions and primers BAP2 A (CTTCAACGGTAAATATGTCAGCAG) and BAP2 D (AATATCCTTTCCATTACCCAAAGAG; www-sequence.stanford.edu/group/yeast_deletion_project). PCR products were purified using Wizard SV Gel and PCR Clean-Up System (Promega, USA) following gel electrophoresis. The identified *BAP*2 deletion cassette was subsequently transformed into the haploid wine yeast AWRI 1631, according to Gietz and Schiestl (2007). Heat-shocked cells were incubated for 2.5 h in 1 mL YEPD, prior to plating on YEPD agar containing 200 mg/L G418. Plates were incubated at 28 °C for approximately 48 h. Individual colonies were selected and genomic DNA was isolated. Gene deletions were verified by PCR amplification using primers BAP2 A and Kan B (CTGCAGCGAGGAGCCGTAAT) primers.

2.3. Metabolite analysis

Sugars (glucose, fructose) and yeast metabolites (ethanol, malic acid, acetic acid, citric acid, glycerol, succinic acid) were measured by HPLC following the protocol of Liccioli et al. (2011). Profiling of volatile compounds was conducted by Metabolomics Australia (Adelaide, Australia). The quantitation of ethyl esters was carried out using the headspace–solid phase micro extraction – gas chromatography with mass spectrometry (HS-SPME-GC–MS) method. HS-SPME was performed using a Gerstel MPS autosampler fitted with a 1 cm preconditioned divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) SPME fibre (50/30 μm). An aliquot of wine (0.5 mL), 4.5 mL of Milli-Q water, 10 μL of internal standard 2-octanol solution, and 2 g of sodium chloride were added into a 20 mL clear, screw-cap GC–MS vial, which was then sealed with PTFE-lined cap. Samples were incubated under agitation (250 rpm) at 35 °C for 30 min followed by extraction at 35 °C for 30 min. Samples were analysed by an Agilent 7890 GC with 5897 mass selective detector (MSD) equipped with a DB-WAX column (60 m × 0.25 mm × 0.25 μm; Agilent Technologies, USA). Ultrapure helium was used as carrier gas with a constant flow rate of 2 mL/min. The oven program was 40 °C for 4 min and then increased to 220 °C at 5 °C/min, and held at 220 °C for 10 min. Temperatures for inlet, transfer line, MS source and MS quadrupole were set at 200, 250, 250 and 150 °C, respectively. Positive ion electron impact energy was 70 eV, and the mass range was 35–350 amu. Samples were analysed in triplicate.

2.4. RNA extraction and sequencing

Three different time points were chosen to investigate the transcriptional profile of each strain: 24 h (exponential growth phase), 140 h (early stationary phase) and 240 h (late stationary phase). RNA extraction was performed using the Trizol protocol described by Chomczynski and Sacchi (2006). To obtain high quality RNA, an RNeasy mini kit (QIAGEN, Melbourne, Australia) was used to purify and concentrate the samples in 2 mL microfuge tubes. The resulting extracts were treated with RNase-free DNase (Life Technologies, United States). Quality check, cDNA preparation, library preparation and Illumina sequencing were performed by the Australian Genomics Research Facility (AGRF, Melbourne, Australia) using a Hiseq 2000 platform. Only high quality RNA was sequenced, with an RNA integrity number (RIN) of > 8. Approximately 130 gigabytes of FASTQ files were obtained, which contained 100 bp paired-end reads.

2.5. Transcriptome assembly and analysis

Approximately 10 million reads/sample were obtained and a total of 30 million reads were used for assembly of the transcriptome for each species. Reads were quality trimmed (phred score > 30) using Trimmomatic (Bolger et al., 2014) to remove both low quality bases and Illumina adapter. The results were checked with FastQC (Andrews, 2010). Quality trimmed reads were mapped to the species reference genomes (*S. cerevisiae* and *T. delbrueckii* NCBI IDs 15 and 12254, respectively; O'Leary et al., 2015) using Tophat (Trapnell et al., 2009). The mapped reads were used to assemble the transcriptome using Trinity with the genome-guided option (Grabherr et al., 2011). Downstream analyses followed the Trinity protocol (Haas et al., 2013), and included the following steps: principal component analysis of the transcriptomes (Yeung and Ruzzo, 2001), abundance estimation of the transcripts using RSEM (Li and Dewey, 2011), normalization of expression values between samples of the same species with the trimmed mean of M-values normalization method (TMM; Robinson and Oshlack, 2010), and differential expression (DE) statistical analysis with the edgeR R package (Robinson et al., 2010). Quality of the assembly was evaluated using Ex50 stats (Haas, 2016), principal component analyses (Yeung and Ruzzo, 2001) and Benchmarking Universal Single Copy Orthologues of fungi (BUSCO) (Simão et al., 2015).

Functional annotation, functional enrichment and gene ontology of the transcriptome genes were made using Trinotate procedures (Finn et al., 2011; Kanehisa et al., 2011; Krogh et al., 2001; Lagesen et al., 2007; Petersen et al., 2011; Powell et al., 2012; Punta et al., 2011). Enzymatic pathways were modelled with Cytoscape (Shannon et al., 2003).

2.6. Statistical analysis

Statistical analysis was made with GraphPad Prism 7.02 (GraphPad Software, USA). Standard deviation and *p*-values were calculated on fermentation replicates. The statistical level of significance was set at p-value of ≤ 0.05 for metabolite concentrations.

3. Results

3.1. Fermentation

Differences were observed in the fermentation performance between *T. delbrueckii* and *S. cerevisiae*. *S. cerevisiae* consumed 25% more sugars and fermented faster than *T. delbrueckii*, consequently producing higher ethanol concentrations (Fig. 1A). After 10 days, neither strain was able to fully complete the fermentation (Fig. 1A).

The production of secondary metabolites such as acetic acid, glycerol, acetates, esters and higher alcohols highlighted differences in the metabolism of the two species. In *S. cerevisiae*, the production of glycerol and acetic acid followed similar trends to those described by Eglinton et al. (2002). Production increased during exponential growth before a modest decrease over the remainder of the experiment (Fig. 1B). Instead, for *T. delbrueckii*, only glycerol production was similar to that of *S. cerevisiae*. In the case of acetic acid, yields were ~10–20 times lower than *Saccharomyces* (Fig. 1B).

More differences between the species were apparent from volatile analyses. With the exception of ethyl propanoate, 2&3-methylbutanol, butanol and ethyl hexanoate, less ester and higher alcohol production was noted in *T. delbrueckii* fermentations (Table 1).

3.2. Transcriptional landscape during fermentation

In order to characterize the different responses of the two yeast species we monitored the evolution of RNA during fermentation using Next Generation sequencing (Linde et al., 2015; Marks et al., 2008). Three different phases of growth (i.e. mid exponential growth, early stationary, late stationary), instead of equivalent fermentation stages, portrayed the transcriptional landscape during fermentation of each of the two species (Puig and Pérez-Ortín, 2000; Duc et al., 2017), to better characterize the metabolic differences. The assembly strategy generated 14,454 transcripts for the *S. cerevisiae* strain and 8853 for the *T. delbrueckii* strain. To assess the quality of the assemblies, the presence of Benchmarking Universal Single Copy Orthologues of fungi (BUSCO) in the transcriptome was checked. Completeness ranged from 74% for *S. cerevisiae* to 83% for *T. delbrueckii* with a moderate extent of fragmentation. Nevertheless, the missing genes were $< 5\%$ in each case (Supplementary File 1). The sequences of the unigenes were annotated and approximately 87% had a match in the Uniprot Database (Bairoch and Apweiler, 2000); specifically, a total of 12,846 transcripts for *S. cerevisiae* and 7691 transcripts for the *T. delbrueckii* strain (Supplementary File 1). Ninety percent of *Torulaspora* sp. sequences had significant homology matches in the SWISS-PROT database with an *E*-value cut-off of 10^{-5}. Species specific distribution analysis indicated that 89% of the *Torulaspora* sequences matched genes of *S. cerevisiae*, followed by yeasts belonging to the class *Saccharomycetes* (*Candida glabrata* 2.3%, *Zygosaccharomyces rouxii* 2%, *Kluyveromyces lactis* 1.6%, *Vanderwaltozyma polyspora* 1.3%, *Eremothecium gossypii* 1%), *Schizosaccharomycetes* (*Schizosaccharomyces pombe* 1.3%) and others (1.7%; Supplementary Fig. 2). Similarities between the species could be confirmed by analysis of the predicted proteins: the two species share 82.4% of the annotated proteins, while *Saccharomyces* has 14.6% and *Torulaspora* 3%, respectively, of unique annotated proteins (Supplementary Figs. 2, 3).

Both species indeed had a relatively strong and broad differential expression of genes during the progression of fermentation. In *S. cerevisiae* 11% of the unigenes (1746) were differentially expressed with at least a four-fold increase between the exponential phase and early stationary phase, whereas in *T. delbrueckii* 16% of its total unigenes (2333) were overexpressed with 110 transcripts increased at least by eight-fold. Response to stress, catabolic process, cellular protein modification process were all enriched functions, present in approximately 10% of these genes (data not shown). However no large differences were noted for *Torulaspora* between early and late stationary phases, whereas 177 *S. cerevisiae* genes were differentially expressed by more than an eight-fold change (Supplementary File 1, Supplementary Fig. 1).

3.3. Glucose fermentation pathway

S. cerevisiae and *T. delbrueckii* exhibited different fermentation rates (Fig. 1A). We compared the expression of genes involved in sugar catabolism to highlight differences between sugar consumption and ethanol production of *Saccharomyces* and the less active *Torulaspora*. Patterns of expression of low affinity glucose transporter (*HXT1*) and high affinity glucose transporters (*HXT2* for *Torulaspora* and *HXT6* for *Saccharomyces*) were similar. Both species initially transcribed *HXT1*

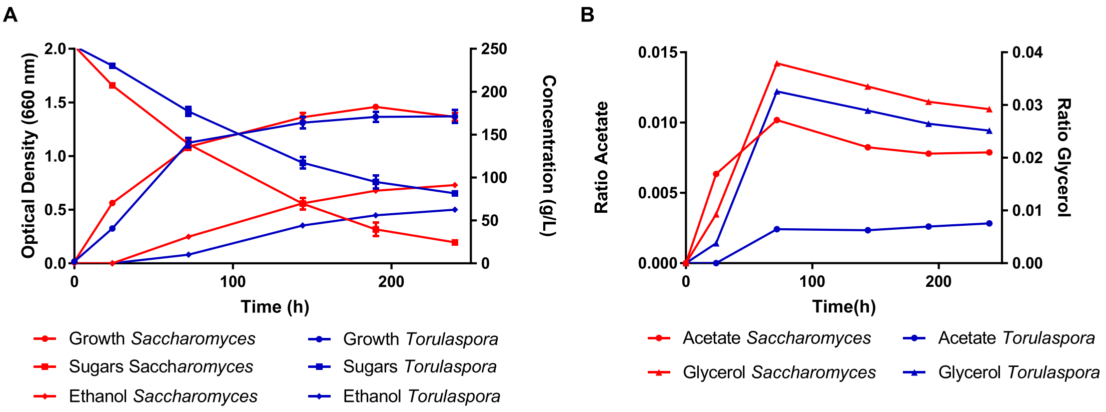

Fig. 1. Fermentation and metabolite production profiles. (A) *S. cerevisiae* and *T. delbrueckii* growth, sugar consumption and relative ethanol production. (B) Production of secondary metabolites during fermentation. Specific acetate and glycerol production (g per gram of sugar consumed) by *S. cerevisiae* and *T. delbrueckii*. (COLOR).

Table 1
Quantification (integrated peak area; relative to internal standards) of ethyl esters, acetate esters and higher alcohol in *S. cerevisiae* and *T. delbrueckii* fermentations.

Compound	Relative quantification		p-Value
	S. cerevisiae	*T. delbrueckii*	
Ethyl acetate	1.29 ± 0.06	1.02 ± 0.05	0.0049
Ethyl propanoate	2.45 ± 0.14	4.95 ± 0.55	0.0016
Ethyl 2-methylpropanoate	9.60 ± 0.24	1.02 ± 0.24	< 0.0001
2-Methylpropyl acetate	4.36 ± 0.50	1.90 ± 0.12	0.0004
Ethyl butanoate	2.20 ± 0.24	0.70 ± 0.87	0.0005
Ethyl 2-methylbutanoate	1.60 ± 0.60	< LOD	N/C
Ethyl 3-methylbutanoate	1.15 ± 0.10	< LOD	N/C
2-Methylpropanol	12.80 ± 0.06	9.40 ± 1.50	0.0234
2-Methylbutyl acetate	1.80 ± 0.24	< LOD	N/C
3-Methylbutyl acetate	1.42 ± 0.26	0.10 ± 0.01	0.0009
Butanol	8.70 ± 0.39	9.80 ± 1.00	0.1532
2&3-Methylbutanol	1.82 ± 0.10	1.88 ± 0.20	0.7266
Ethyl hexanoate	8.80 ± 1.50	8.48 ± 0.60	0.9000
Hexyl acetate	< LOD	< LOD	N/C
Hexanol	< LOD	< LOD	N/C
Ethyl octanoate	5.16 ± 1.40	3.00 ± 0.70	0.0039
Ethyl decanoate	1.00 ± 0.20	< LOD	N/C

LOD, limit of detection; N/C not calculable.

but with the progress of fermentation, switched to expressing high affinity transporters *HXT2/HXT6*. The glycolytic pathway of *Torulaspora* has all steps necessary to convert glucose in pyruvate, but compared to *S. cerevisiae* is missing paralog genes encoding glyceraldehyde-3-phospate dehydrogenase, enolase and pyruvate kinase. Furthermore the transcript of *PGI1*, *TPI1*, *ENO2* in *T. delbrueckii* were more similar to *Candida glabrata*, while *PYK2* was more similar to *Lachancea thermotolerans*. In the fermentation pathway, *T. delbrueckii* expressed only one pyruvate decarboxylase gene (*PDC1*, Fig. 2B), while *S. cerevisiae* expressed all three (*PDC 1*, *5* and *6*; Fig. 2A). Moreover, the sequence of the *T. delbrueckii PDC1* gene was more similar to that of *Kluyveromyces* sp. than *S. cerevisiae* (Supplementary File 1). All seven alcohol dehydrogenases (*ADH1–7*) responsible of converting acetaldehyde to ethanol, were found in *S. cerevisiae* but only four were detected in *Torulaspora* (*ADH1*, *ADH3*, *ADH4* and *ADH6*, Fig. 2). As expected, we observed a broad reduction of expression between exponential and stationary phase in both species, except for *ADH1* and *PDC1* in *T. delbrueckii*, which expression levels increased during fermentation.

3.4. Glycerol and acetic acid pathways

Glycerol is an important secondary metabolite of alcoholic fermentation: it is produced in wine at a concentration of 2–11 g/L and it is known to contribute positively to the perception of body and viscosity in wine (Gawel et al., 2007, Remize et al., 2003,). Both *T. delbrueckii* and *S. cerevisiae* produced similar amounts of glycerol during fermentation, proportional to the amount of sugar consumed, therefore *S. cerevisiae* ended up with 6.67 ± 0.29 g/L compared to 4.32 ± 0.12 for *T. delbrueckii*. Transcriptome expression analysis, however, showed differences between the two species for the genes involved (Fig. 3; Ansell et al., 1997). *T. delbrueckii* lacked *GPD2* and *GPP2*. Transcription of the glycerol pathway genes seemed to be higher in the exponential growth phase (24 h) in both yeast species (Fig. 3). *T. delbrueckii* expressed more *GPP1* compared to *GPD1*, contrary to *S. cerevisiae*.

Acetic acid is responsible for the negative attribute of volatile acidity in wine. In agreement with earlier reports (Bely et al., 2008; Blomberg and Adler, 1992), *T. delbrueckii* produces less acetic acid than *S. cerevisiae* (Fig. 1B). Overall production of acetic acid was 0.486 ± 0.07 g/L (2 mg/g of sugar consumed) for *T. delbrueckii* compared to 1.80 ± 0.12 g/L (8 mg/g of sugar consumed) for *S. cerevisiae*. Transcripts for aldehyde dehydrogenases *ALD2–6*, enzymes involved in acetic acid production, were all expressed in *S. cerevisiae* while *T. delbrueckii* lacked *ALD3* expression. Nevertheless, acetyl-CoA synthetases (*ACS1* and *ACS2*) and acetyl-CoA hydrolase (*ACH1*) transcripts were present in both transcriptomes. Expression analysis of these genes during fermentation, showed two different patterns in metabolic flow. Low acetate producer, *T. delbrueckii* consistently expressed *ADHs* transcripts higher than *ALDs* transcripts during fermentation. In contrast, *Saccharomyces ALD* transcript expression was dominant, with *Saccharomyces* producing 10-times more acetic acid that *Torulaspora* (Figs. 1B, 3). Also *T. delbrueckii* tended to express *ACSs* more than *ACH1*, while in *Saccharomyces* expression was similar.

3.5. Higher alcohols, ethyl and acetate esters

T. delbrueckii and *S. cerevisiae* differed in their production of acetate esters, ethyl esters and higher alcohols at the end of fermentation (Table 1). *Saccharomyces* produced a wider range of acetate and ethyl esters, while *Torulaspora* instead produced higher amounts only of ethyl propanoate, as previously reported (Ramírez et al., 2016; Renault et al., 2015; Velázquez et al., 2015). Transcriptome analysis revealed *T. delbrueckii* lacked the main genes responsible for acetate ester production, *ATF1–2* (Fig. 4B; Lilly et al., 2000). A minimal production of these esters was seen and might be explained by the expression of *YPL272C* (uncharacterized protein) and *SLI1* (*n*-acetyl-transferase; Supplementary File 1). The amino acid sequence of both of these genes includes an alcohol acetyltransferase domain.

Regarding ethyl esters, genes *EEB1* and *EHT1* were expressed in

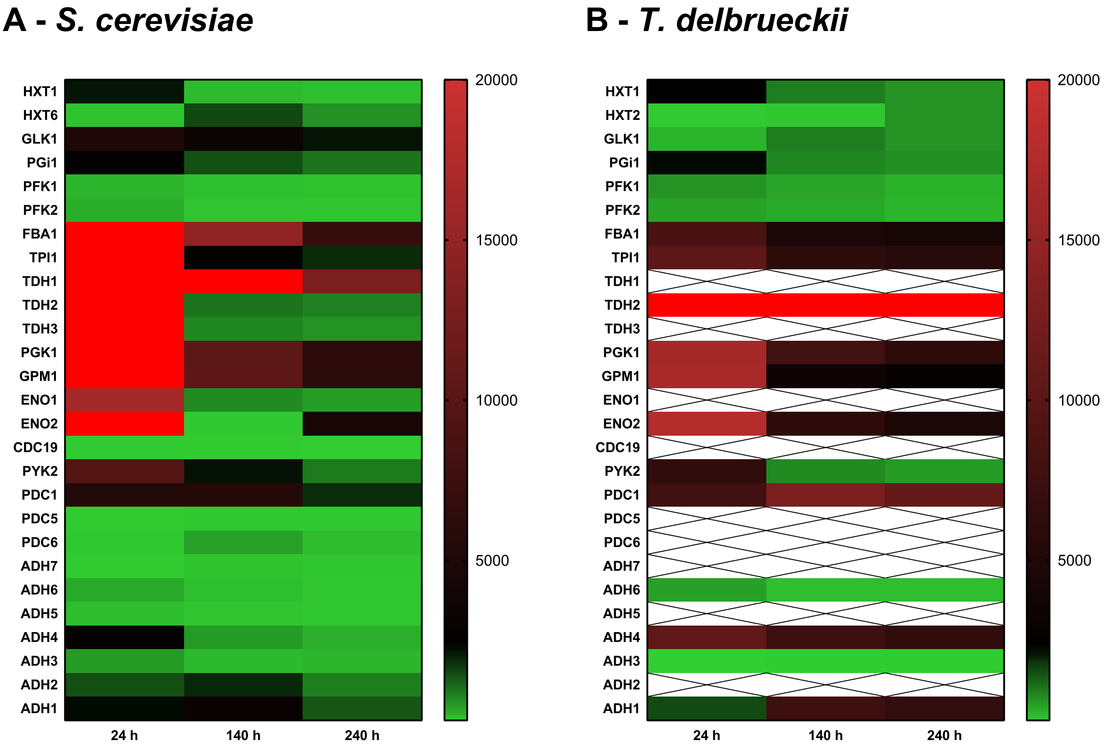

Fig. 2. Expression of genes of the glucose fermentation pathway. Expression values (FPKM) of the genes involved in the glucose fermentative pathway of (A) *S. cerevisiae* and (B) *T. delbrueckii*. Expression is showed from low (green) to high (red). Crossed squares indicate no expression. (COLOR). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

both transcriptomes (Fig. 4; Saerens et al., 2010, Tehlivets et al., 2007). *T. delbrueckii* mainly expressed *ETH1*, while *S. cerevisiae* expressed *EEB1* to a greater extent. However ethyl ester production has also been related to the availability of their precursors, medium chain fatty acids (MCFAs; Saerens et al., 2006) and therefore to enzymes acetyl–CoA carboxylase (*ACC1*) and the fatty acid synthase (FAS) complex (*FAS1/FAS2*; Bardi et al., 1998, Dufour et al., 2008). Interestingly *S. cerevisiae* expressed *ACC1* highly and the *FAS* complex during all stages of fermentation, which might result in a higher accumulation of MCFAs. Contrarily *T. delbrueckii*, in which *FAS2* transcript is more closely related to *L. thermotolerans* (Supplementary File 1), mainly expressed the pathway at the beginning of fermentation.

T. delbrueckii produced a lower concentration of higher alcohols. Formation of higher alcohols can be directly related to the catabolism of branched chain amino-acids (BCAAs; leucine, valine, and isoleucine) through the Ehrlich pathway (Hazelwood et al., 2008). *BAT1*, *BAT2* and *BAP2* have already been identified as being involved in this pathway (Colón et al., 2011; Procopio et al., 2011). We found all three of these genes expressed in *Saccharomyces*, but no transcripts for the BCAAs permease *BAP2* and for the BCCAs transaminase *BAT2* in *T. delbrueckii* (Fig. 4B). This could indicate an impaired amino acid uptake and Ehrlich pathway in *Torulaspora*.

3.6. *Effect of BAP2 deletion on short chain ethyl esters production in S. cerevisiae*

Contrary to acetate esters and higher alcohols, ethyl esters production is related to branched chain amino acid uptake (Saerens et al., 2008). Branched chain amino acid uptake is mainly achieved in *S. cerevisiae* by the amino acid permease *BAP2*. Deletion and overexpression, respectively, show decreases or increases in BCCA uptake (Grauslund et al., 1995). To test the effect of an impaired uptake of BCAA in producing the patter of esters seen in *T. delbrueckii*, CDGJM was fermented with a wild type haploid *S. cerevisiae* AWRI 1631 and the corresponding AWRI 1631 Δ*bap2* deletant. The effect of *BAP2* deletion on ethyl esters was determined via five compounds: ethyl propanoate, ethyl 2–methylpropanoate, ethyl butanoate, ethyl 2-methylbutanoate and ethyl 3-methyl butanoate. No differences between the strains were seen in extent of sugar consumption after 10 days of fermentation (approx. 14 g/L residual sugar, *p*-value 0.33). Three SBCEEs were significantly lower, whereas the not branched short ethyl esters, ethyl propanoate and ethyl butanoate, weren't significantly reduced (Table 2). AWRI 1631 Δ*bap2* strain related SBCEEs final concentration with BCAAs uptake.

4. Discussion

Torulaspora delbrueckii shows great potential as an alternative to an *S. cerevisiae* inoculum during wine fermentation due to its different production of secondary metabolites. Its reduced production of volatile acidity and its sensory characteristics have not been previously investigated at the transcriptome and metabolome level. In this study, we have shown how transcriptional differences reflect the different metabolic characteristics of those yeasts and help highlight the genetic differences between a well-annotated species (*S. cerevisiae*) and a non-reference species with a sparsely annotated genome (*T. delbrueckii*).

The transcript analysis showed *T. delbrueckii* is missing multiple genes, results of the complex program of whole genome duplication in *S. cerevisiae* (Wolfe, 2015). Paralog genes are missing in the glycolic and fermentation pathways, in amino acid uptake and in production of secondary metabolites such as acetate and glycerol biosynthesis. Glycerol production is similar in both yeast species, in contrast to acetic acid; the results confirm *T. delbrueckii* produces less volatile acidity, under wine-like fermentation conditions, instead redirecting carbon flux through alcoholic fermentation.

Comparative analysis between metabolite profiles and transcriptomes also highlighted the link between the limited production of higher alcohols and esters to the lack or lower expression of related

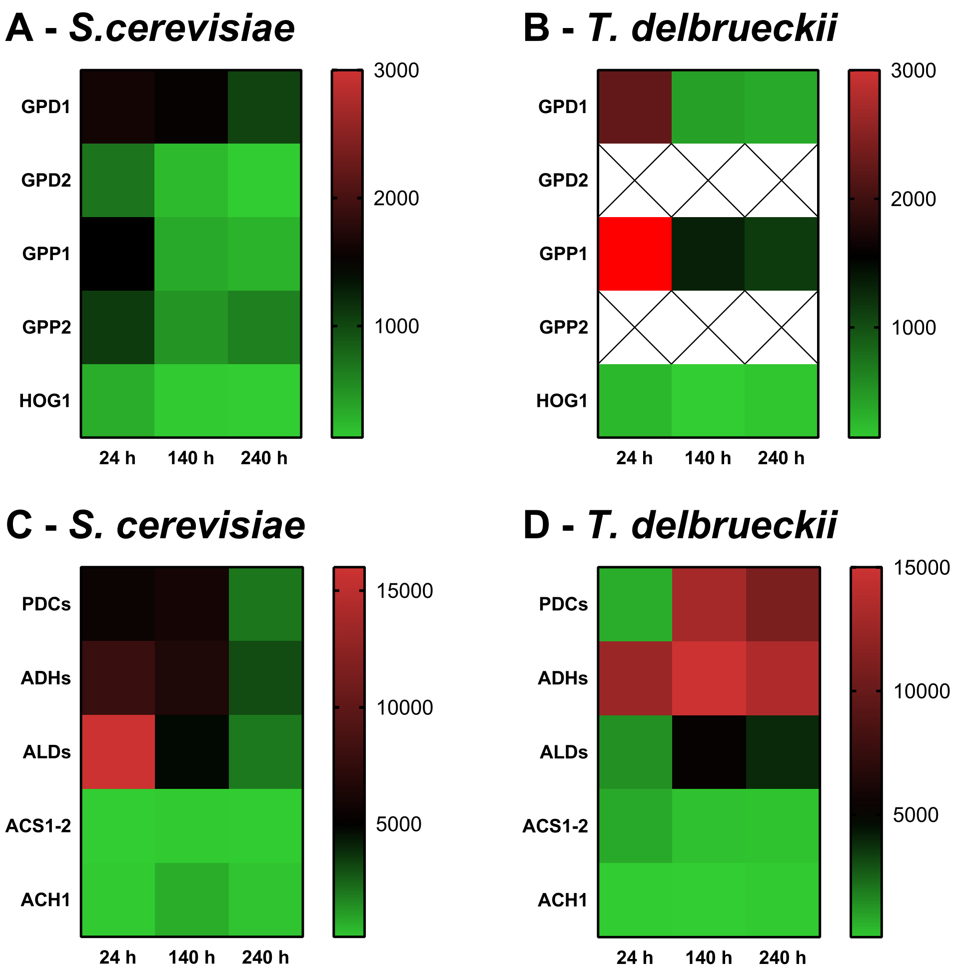

Fig. 3. Expression of genes of the glycerol and acetate pathways. Expression values (FPKM) of the genes involved in the glycerol pathway for (A) *S. cerevisiae* and (B) *T. delbrueckii*. (D) Normalised expression (see Material and methods) of the enzymes involved in the Acetate/Acetyl-CoA pathway for (C) *S. cerevisiae* and (D) *T. delbrueckii*. Expression is showed from low (green) to high (red). Crossed squares indicate no expression. (COLOR). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

genes. In the case of *T. delbrueckii*, which showed reduced production of higher alcohols and acetate esters, an absence of transcripts of key enzymes in these pathways was revealed. Instead, the lower production of ethyl esters by *Torulaspora* seemed to be related to the down-regulation of fatty acids biosynthesis. Based on (i) differences in expression of amino acid permeases between the two species and their influence on aromas (Supplementary File 1; Stahl and James, 2014), (ii) lower SBCEEs production by *T. delbrueckii* (Table 1) and *S. cerevisiae* Δ*bap2* (Table 2) and (iii) analysis of the structure and biosynthesis pathway of SBCEEs (KEGG compound, KEGG pathway; Kanehisa et al., 2011) we suggest the formation of SBCEEs depends on metabolism of BCAAs through a modified Ehrlich pathway, under these oenological conditions. Intracellular accumulation of α-ketoacids is a consequence of amino acid metabolism (Procopio et al., 2011). In the cytosol excess

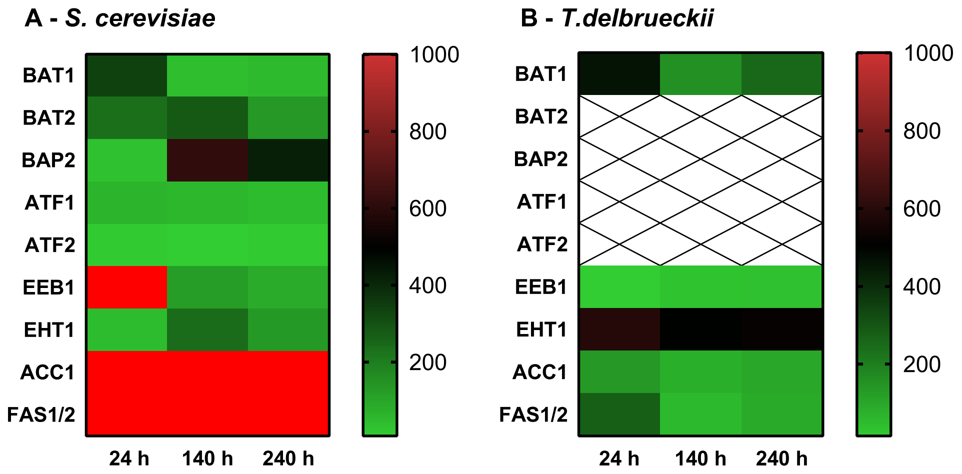

Fig. 4. Expression of genes involved in formation of higher alcohols, acetates and ethyl esters. Expression values (FPKM) of the genes involved in higher alcohols, acetates and ethyl esters pathways of (A) *S. cerevisiae* and (B) *T. delbrueckii*. Expression is showed from low (green) to high (red). Crossed squares indicate no expression. (COLOR). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Quantification (integrated peak area; relative to internal standards) of ethyl esters, in *S. cerevisiae* AWRI 1631 and *S. cerevisiae* AWRI 1631 Δ*bap2*.

Compounds	Relative quantification		p-Value
	AWRI 1631	AWRI 1631 Δbap2	
Ethyl propanoate	5.7 ± 0.6	5.1 ± 0.3	0.2694
Ethyl 2 – methylpropanoate	9.1 ± 0.3	6.7 ± 0.4	0.0038
Ethyl butanoate	1.2 ± 0.9	1.1 ± 0.35	0.1237
Ethyl 2 – methylbutanoate	9.3 ± 2.5	3.1 ± 0.2	0.0259
Ethyl 3 – methylbutanoate	8.4 ± 0.7	6.6 ± 0.3	0.0255

of α-ketoacid is processed through the Ehrlich pathway (Avalos et al., 2013; Hazelwood et al., 2008). However, in the mitochondria, an excess of these substrates may be processed by other enzymes. For example, an enzyme complex with branched-chain α-ketoacid dehydrogenase activity (similar to 2-oxoacid dehydrogenase; EC 1.2.4.4) has already been reported in *Saccharomyces*. This metabolic route was previously detected by ^{13}C-labeled intermediate products (Dickinson et al., 1997; Dickinson, 1999) but its physiological role is still unknown (Dickinson and Dawes, 1992; Sinclair et al., 1993). The catalytic subunit E3 of this complex, the protein encoded by *LPD1* gene, has been found to be expressed in both transcriptomes (Supplementary File 1). The reaction would produce the related acyl–CoA, and finally esterases would form an SBCEE. Further characterisation of *T. delbrueckii* amino acid metabolism is however necessary to understand the genetic basis for its distinct profile to provide winemakers a useful tool to predict spontaneous and co-inoculated fermentations.

In conclusion the comparative transcriptomic approach described here can not only be used to search for specific oenological traits in non-conventional yeast (Rossignol et al., 2003), but further used to explore the natural biodiversity of yeast communities and their interaction. New sequencing technology based on long DNA fragments may help differentiate transcriptomes and therefore further analyse the effects of mixed population and their effects on the wine sensory output, closely relating laboratory outputs to the wine industry conditions.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary 1, Supplementary Figs. 1–3 can be found online: http://dx.doi.org/10.17632/v8g3y2f35f.1

Supplementary File 2 (CDGJM composition) can be found online: http://dx.doi.org/10.17632/j9g3mxsh23.2

Appendix B. Raw sequences

Raw sequences can be found on SRA database, accession: **SRP136972**.

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