UNIVERSITAT ROVIRA I VIRGILI

OPTIMIZACIÓN DEL USO DE LEVADURAS NO-SACCHAROMYCES EN FERMENTACIONES MIXTAS: REQUERIMIENTOS NUTRICIONALES E INTERACCIONES MICROBIANAS

Elena Roca Mesa

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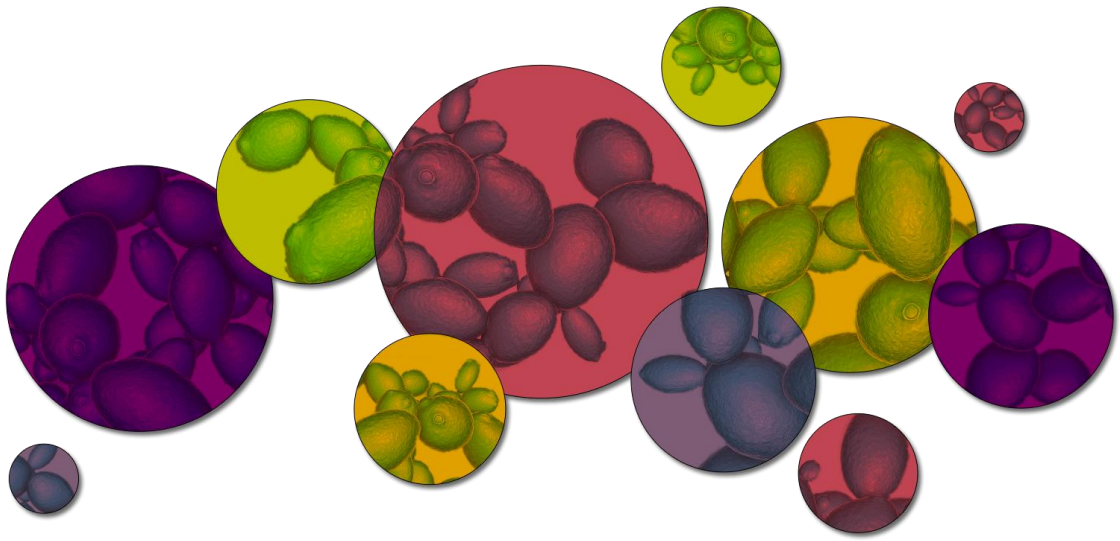

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TESIS DOCTORAL

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Tarragona, 2022

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La Dra. Maria Jesús Torija Martínez, profesora titular, y la Dra. Gemma Beltran Casellas, profesora agregada, ambas del Departamento de Bioquímica y Biotecnología de la Facultad de Enología de la Universitat Rovira i Virgili,

CERTIFICAMOS:

Que el presente trabajo titulado "Optimización del uso de levaduras no-*Saccharomyces* en fermentaciones mixtas: requerimientos nutricionales e interacciones microbianas", que presenta Helena Roca Mesa para la obtención del título de Doctor, ha sido realizado bajo nuestra dirección en el Departamento de Bioquímica y Biotecnología de la Universitat Rovira i Virgili.

Tarragona, 9 de Mayo del 2022.

Las directoras de la tesis doctoral.

Dra. Maria Jesús Torija Martínez

Dra. Gemma Beltran Casellas

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" Amb paciència es pot fer tot."

- Carme Cózar

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Al meu germà

Al Víctor

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Agraïments

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post-its motivadores… Te echaré mucho de menos (aunque ya lo hago). Nunca dejes de ser caóticamente buena.

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Hipótesis y Objetivos

UNIVERSITAT ROVIRA I VIRGILI
OPTIMIZACIÓN DEL USO DE LEVADURAS NO SACCHAROMYCES EN FERMENTACIONES MIXTAS: REQUERIMIENTOS NUTRICIONALES E INTERACCIONES MICROBIANAS
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El presente trabajo ha sido llevado a cabo desde 2017 hasta 2022 en el grupo de investigación de Biotecnología Enológica, en el Departamento de Bioquímica y Biotecnología en la Facultad de Enología de la Universitat Rovira i Virgili. Durante este período pude disfrutar de una beca del programa Martí i Franquès de ayudas a la investigación de la Universitat Rovira i Virgili (2018PMF-PIPF-10).

A lo largo del desarrollo de esta tesis doctoral he colaborado en dos proyectos de investigación. Uno de ellos es el proyecto "*Nutrición nitrogenada y su influencia en la liberación de aromas varietales tiólicos por levaduras*", que ha sido realizado con la empresa Agrovin S.A. y financiado a través del programa CDTI(IDI-20160102) del Ministerio de Ciencia e Innovación de España. El objetivo principal de este proyecto fue mejorar el conocimiento sobre las necesidades nutricionales que tienen las levaduras no-*Saccharomyces* y su relación con la síntesis de compuestos tiólicos. La liberación de estos aromas depende de la actividad beta liasa de las levaduras, la cual está regulada por el sistema de represión catabólico por nitrógeno (NCR). El metabolismo nitrogenado de las levaduras *Saccharomyces cerevisiae* durante la fermentación alcohólica ha sido ampliamente estudiado, pero no tanto el de otras especies de levaduras vínicas, que también se están utilizando actualmente en el sector enológico, principalmente en fermentaciones mixtas.

Por otro lado, también he colaborado en el proyecto CoolWine (PCI2018-092962), financiado por el Ministerio de Ciencia e Innovación de España y la Agencia Estatal de Investigación (MCIN/AEI/10.13039/501100011033) y cofinanciado por la Unión Europea. Este proyecto estudia utilizar diferentes aproximaciones microbiológicas para reducir el contenido de etanol en vinos, entre ellas, la utilización de levaduras no-*Saccharomyces* en fermentaciones mixtas. Por tanto, el estudio de las interacciones existentes entre las diferentes especies, así como sus requerimientos nutricionales, es crucial para asegurar el buen desarrollo de este tipo de fermentaciones.

Aunque durante muchos años las levaduras no-*Saccharomyces* se consideraron indeseables, durante los últimos años se ha popularizado el uso combinado de estas levaduras junto a *S. cerevisiae* para obtener vinos más complejos. Por ejemplo, *Torulaspora delbrueckii* y *Lachancea thermotolerans* son levaduras no-*Saccharomyces* capaces de aportar características especiales al vino final. Sin embargo, entre las diferentes especies de levaduras se pueden producir varios tipos de interacciones microbianas, no siempre positivas, como pueden ser la competencia por nutrientes y la secreción de compuestos antimicrobianos. Es por ello que es necesario conocer bien los requerimientos nutricionales de las levaduras no-*Saccharomcyes* de especial interés en enología, así como su relación/interacción con otras levaduras vínicas, y así poder mejorar el desarrollo de las fermentaciones mixtas y la calidad del vino.

Así pues, la hipótesis de esta tesis fue la siguiente: **El uso de levaduras no-*Saccharomyces* en fermentaciones mixtas afecta negativamente a las levaduras *S. cerevisiae*, debido a la competencia por los nutrientes del medio, así como a interacciones microbianas existentes entre ellas.**

Para comprobar esta hipótesis, el objetivo general consistió en analizar el potencial fermentativo y los requerimientos nutricionales de diferentes levaduras vínicas no-*Saccharomyces,* y evaluar su impacto en el desarrollo de fermentaciones mixtas con *S. cerevisiae*. Este objetivo general se dividió en los siguientes objetivos específicos:

Objetivo 1: Estudiar los requerimientos nitrogenados de levaduras no-*Saccharomyces* y su impacto sobre el crecimiento, el desarrollo de la fermentación y el vino final.

Para conseguir este objetivo, por un lado, en el Capítulo I.1 se analizó el consumo de nitrógeno y la capacidad fermentativa de cinco levaduras no-*Saccharomyces* vínicas (*Torulaspora delbrueckii*, *Lachancea thermotolerans, Starmerella bacillaris, Hanseniaspora uvarum* y *Metschnikowia pulcherrima*), en comparación con una cepa control de *S.*

cerevisiae. Se utilizaron tres tipos de mosto con distintas composiciones nitrogenadas: una mezcla de aminoácidos y amonio, sólo aminoácidos, y sólo amonio. Se hizo el seguimiento de las fermentaciones a lo largo del tiempo midiendo la densidad, la población viable (mediante siembra en placa) y el consumo de nitrógeno (mediante HPLC), analizando las preferencias nitrogenadas de cada especie, tanto a nivel de nitrógeno orgánico e inorgánico, como de cada aminoácido en particular. En los puntos finales se analizaron también los compuestos orgánicos por HPLC.

A continuación, en el Capítulo I.2 se seleccionaron dos cepas de *T. delbrueckii* y *L. thermotolerans* para hacer un estudio pormenorizado del efecto de la concentración y tipo de nitrógeno (mezcla de amonio y aminoácidos, sólo amonio y mezcla de amonio y levaduras inactivas autolisadas (IDY)) sobre el crecimiento de estas levaduras en microplacas. Posteriormente, se estudió el efecto de la suplementación con IDY en un mosto de uva Verdejo en fermentaciones secuenciales utilizando *T. delbrueckii* y *S. cerevisiae*. El seguimiento de la fermentación se realizó mediante la medida de la densidad, de la población y del consumo de nitrógeno. En los vinos finales se analizaron los compuestos orgánicos, así como la liberación de los aromas varietales tiólicos por parte de las levaduras.

Los resultados de este objetivo se encuentran descritos en los siguientes capítulos:
Capítulo I. 1. Nitrogen preferences during alcoholic fermentation of different non-*Saccharomyces* yeasts of oenological interest. Resultados publicados en *Microorganisms*, 2020, 8, 157.
Capítulo I. 2. The effect of must nitrogen composition on yeast growth and volatile thiol release in sequential fermentations. Manuscrito en preparación.

Objetivo 2: Estudiar las interacciones entre levaduras vínicas en fermentaciones mixtas.

En este objetivo se estudiaron las interacciones entre *S. cerevisiae* y algunas levaduras no-*Saccharomyces* de interés enológico (*T. delbrueckii* y *L. thermotolerans*), así como las interacciones entre las dos especies no-*Saccharomyces*.

Primero, en el Capítulo II.1, se investigó el impacto de la presencia de *T. delbrueckii* en fermentaciones mixtas con *S. cerevisiae* en el desarrollo de la fermentación, utilizando diferentes estrategias de inoculación: co-inoculación (con diferentes ratios) e inoculación secuencial (inoculando la segunda levadura a las 48 horas). Debido a la falta de imposición de *S. cerevisiae* en algunas fermentaciones mixtas, se estudiaron posibles interacciones que pudieran existir entre ambas especies: inhibición por contacto celular, presencia de compuestos inhibitorios (toxinas killer u otros péptidos) o limitación de nutrientes.

- Para estudiar la posible inhibición por contacto celular entre las dos especies, el mosto se inoculó con *T. delbrueckii*, y pasadas 48 h, ésta se eliminó del medio (mediante centrifugación y filtración) y se inoculó *S. cerevisiae*, analizando su crecimiento y el desarrollo de la fermentación en estas condiciones.
- Para determinar si las cepas de *T. delbrueckii* sintetizaban toxinas killer se utilizaron tanto medios específicos como la detección de partículas dsRNA en el genoma. Por otro lado, se realizaron purificaciones de extractos proteicos a partir del mosto fermentado 48 h por *T. delbrueckii*, y éstos se añadieron a fermentaciones realizadas con *S. cerevisiae* para detectar posibles inhibiciones.
- Para detectar si, después del crecimiento de *T. delbrueckii,* había limitación de nutrientes en el mosto para el óptimo crecimiento y fermentación de *S. cerevisiae*, se analizó el efecto de suplementar dicho mosto a las 48 h con diferentes fuentes de nitrógeno y otros nutrientes (vitaminas, oligoelementos, factores de anaerobiosis, IDY).

Posteriormente, los resultados obtenidos con *T. delbrueckii* se comprobaron utilizando *L. thermotolerans* como levadura no-*Saccharomyces* para las fermentaciones secuenciales, con y sin contacto (Capítulo II.2).

Finalmente, dada la buena capacidad fermentativa de estas levaduras no-*Saccharomyces,* en el Capítulo II.3 se evaluó la posibilidad de utilizarlas cómo inóculo mixto sin la presencia de *S. cerevisiae*, analizando tanto su cinética fermentativa, como su potencial aromático. Para ello, se realizaron fermentaciones en mosto sintético con dos cepas de *T. delbrueckii* y *L. thermotolerans*, utilizando inoculación individual, co-inoculación e inoculación secuencial (alternando el orden de inoculación de las dos especies), con y sin contacto celular. Al final de la fermentación se analizaron los compuestos volátiles de los vinos obtenidos por cromatografía de gases.

En todas estas fermentaciones se hizo el seguimiento a lo largo del tiempo de la densidad, la población viable (mediante siembra en placa) y el consumo de nitrógeno (mediante HPLC). En los vinos finales se analizaron los compuestos orgánicos por HPLC.

Los resultados de este objetivo se encuentran descritos en los siguientes capítulos:
Capítulo II. 1. Micronutrients and organic nitrogen importance in fermentations with *Torulaspora delbrueckii* and *Saccharomyces cerevisiae*. Resultados enviados a International Journal of Food Microbiology.
Capítulo II. 2. The impact of nutrient supplementation and inoculum size in sequential fermentations with *Lachancea thermotolerans* and *Saccharomyces cerevisiae*. Manuscrito en preparación.
Capítulo II. 3. Effect of the combined use of *Torulaspora delbrueckii* and *Lachancea thermotolerans* as yeast starters on fermentation performance and wine aroma composition. Manuscrito en preparación.

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Introducción

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1. Las levaduras y el vino

Las levaduras son microorganismos unicelulares eucariotas del reino fungi. Están presentes en la producción de diferentes alimentos y bebidas como el pan, el queso, la cerveza y el vino, siendo la vinificación una de las prácticas más antiguas del mundo. Los historiadores han encontrado evidencias de la producción de vino que datan del Neolítico, de hace 7400-7000 años aproximadamente, así como en Egipto y Fenicia, datadas en el 5000 AC (Cantoral and Rodríguez, 2010). En el año 1680, Antonie van Leeuwenhoek observó por primera vez levaduras en el microscopio, aunque no fue hasta la segunda mitad del siglo XIX que Louis Pasteur descubrió que las levaduras intervenían en la fermentación del vino. Desde entonces, se ha estudiado el papel de la levadura en la fermentación alcohólica. En 1882, Hansen fue capaz de aislar levaduras de la cerveza, y en 1890, Müller introdujo el concepto de utilizar un cultivo puro de levadura para inocular mostos y llevar a cabo la vinificación. Con todo ello, la calidad y el volumen de producción de vino aumentaron notablemente. Todas estas investigaciones e innovaciones tecnológicas revolucionaron la industria del vino. Las investigaciones que se llevan a cabo actualmente pretenden profundizar en el conocimiento microbiológico, bioquímico y genético para continuar mejorando el uso y el control de las levaduras (Barnett, 2000; Barnett et al., 2001; Bauer and Pretorius, 2000; Ribéreau-Gayon et al., 2006).

1.1. Las levaduras vínicas

Existe una gran diversidad de levaduras autóctonas que colonizan la piel de la uva durante su maduración, principalmente de los géneros

Hanseniaspora, *Pichia, Candida, Metschnikowia, Kluyveromyces* y *Saccharomyces*, y ocasionalmente, *Zygosaccharomyces*, *Saccharomycodes*, *Torulaspora*, *Dekkera* y *Schizosaccharomyces* (Fleet, 2003). El número de estas levaduras dependerá de varios factores como el clima, la situación geográfica del viñedo, el estado sanitario de la uva y los pesticidas utilizados (Padilla et al., 2016). Durante la fermentación se da la sucesión de diferentes tipos de microorganismos debido a que las condiciones del medio (como la concentración de azúcares, oxígeno, sulfitos y etanol) van cambiando y los van seleccionando. A partir de la mitad de la fermentación domina principalmente la levadura *Saccharomyces cerevisiae*, la cual lleva a cabo la mayor parte del proceso (Ribéreau-Gayon et al., 2006). De esta manera, los vinos obtenidos mediante fermentación espontánea son impredecibles, pero resultan más complejos que los vinos inoculados. Estos últimos se basan en la inoculación de una levadura conocida y seleccionada, ya sea en forma de levadura seca activa (LSA) u otro formato. De esta manera, los enólogos utilizan una levadura seleccionada, que suele ser *S. cerevisiae*, para asegurar que la fermentación termine. Además, los vinos que se obtienen con este método, aunque no son tan complejos como los espontáneos, tendrán unas características conocidas y deseables, las cuales se podrán repetir en procesos posteriores de manera que esta técnica garantiza la producción de vinos con una calidad reproducible a escala industrial (Pretorius, 2000).

1.1.1. *Saccharomyces cerevisiae*

Saccharomyces cerevisiae se considera la levadura vínica por excelencia debido a su gran poder fermentativo y su alta tolerancia al etanol. Son

levaduras que tienen forma elipsoidal, y cuya presencia en los mostos tiene dos teorías, una que defiende que son parte de la microbiota que se encuentra en las uvas dañadas y la otra que apuesta porque su principal procedencia es el equipamiento de la bodega, aunque no descarta que una pequeña parte de *S. cerevisiae* esté en las uvas (Fleet and Heard, 1993). Su buena capacidad fermentativa, tanto en presencia como en ausencia de oxígeno, es la principal estrategia para competir con otras especies presentes en el mosto (Bisson, 1999). Debido al alto poder fermentativo de *S. cerevisiae*, ésta empieza a consumir los azúcares rápidamente, lo que aumenta las concentraciones de etanol y dióxido de carbono en el medio, resultando en un medio más restrictivo para otros microorganismos (Bisson, 1999). De esta manera, *S. cerevisiae* domina la fermentación alcohólica, ya que es la levadura que está mejor adaptada a las condiciones estresantes que genera la fermentación, como las altas concentraciones de azúcar o etanol, bajo pH y escasez de nutrientes (Albergaria and Arneborg, 2016). Actualmente en el mercado hay una amplia variedad de cepas de *S. cerevisiae* en diferentes formatos, las cuales pueden aportar diferentes propiedades al vino final (Vejarano and Gil-Calderón, 2021).

1.1.2. Las levaduras no-*Saccharomyces*

El grupo de levaduras *no-Saccharomyces* es una clasificación no-taxonómica que incluye todas las levaduras que no pertenecen al género *Saccharomyces*. Estas levaduras se encuentran en la superficie de las uvas (Fleet y Heard, 1993). Hasta hace unos años se intentaba evitar su presencia durante la fermentación porque se creía que eran las responsables de obtener vinos con características poco deseables, como una elevada acidez

volátil y otros compuestos negativos (Jolly et al., 2014). Hoy en día se ha visto que pueden aportar características positivas al vino, incluyendo la capacidad de secretar enzimas que catalizan la formación de compuestos aromáticos volátiles a partir de precursores no volátiles (Hernández-Orte et al., 2008), de producir metabolitos secundarios de interés enológico, como glicerol, ésteres, alcoholes superiores (Padilla et al., 2016), de liberar manoproteínas o de contribuir a la estabilidad del color (Domizio et al., 2014), así como de obtener vinos con menos concentración de etanol (Quirós et al., 2014; Contreras et al., 2015; Morales et al., 2015; Canonico et al., 2019; Puškaš et al., 2020; Zhu et al., 2020), entre otras (Tabla 1). Todas estas contribuciones positivas, han hecho aumentar el interés por las levaduras no-*Saccharomyces* en el proceso de vinificación.

Como generalmente estas levaduras no son capaces de realizar la fermentación completa, se suelen inocular junto a *S. cerevisiae,* para asegurar que la fermentación termine (Ciani et al., 2010). Se utilizan dos estrategias de inoculación: coinoculación (inoculación simultánea de las dos o más especies de levaduras, en diferentes proporciones) o inoculación secuencial (se inocula primero la/s levadura/s no-*Saccharomyces*, y después de 24-72 horas se inocula la cepa de *S. cerevisiae).*

Tabla 1. Contribuciones de algunas levaduras no-*Saccharomyces* al vino.

Referencias 1: Cordero-Bueso et al. (2013); 2: Renault et al. (2016); 3: Cus and Jenko (2013); 4: Azzolini et al. (2015); 5: Renault et al. (2015); 6: Belda et al. (2016a); 7: Hu et al. (2016); 8: Garofalo et al. (2016); 9: Domizio et al. (2014); 10: Benito et al. (2015); 11: Englezos et al. (2016); 12: Tofalo et al. (2016); 13: Medina et al. (2013); 14: Lleixà et al. (2016); 15: Gobbi et al. (2013); 16: Comitini et al. (2011) (adaptada de Varela (2016)).

Especie no-*Saccharomyces*	Contribuciones	Referencias
Torulaspora delbrueckii	Incremento de ésteres de acetato y MCFAs	1
	Incremento de tioles volátiles	2
	Incremento de terpenos y linalool	3
	Incremento de la intensidad y complejidad aromática	4 , 5
	Incremento del color	6
Hanseniaspora uvarum	Incremento de terpenos y varios C13-norisoprenoides	7
	Incremento de ésteres de acetato	8
Metschnikowia pulcherrima	Incremento de manoproteínas	9
	Incremento de aromas cítricos, a pera	10
Starmerella bacillaris	Incremento de glicerol y complejidad volátil	11
	Disminución de caracteres reducidos	12
Hanseniaspora vineae	Incremento de aromas cítrico, a pera, a miel	13
	Incremento de feniletil acetato, etil lactato y α-terpineol (floral)	14
Lachancea thermotolerans	Incremento de ácido L-láctico	10
	Incremento de 2-phenylethanol y glicerol	15
	Disminución de la acidez volátil	16

Durante los últimos años ha ido creciendo el número de especies de levaduras no-*Saccharomyces* disponibles en el mercado en forma de levadura seca activa. Además, se ha visto que algunas de estas especies pueden acabar la fermentación por sí solas, como *T. delbrueckii* y *L. thermotolerans* (Vejarano and Gil-Calderón, 2021). Para conocerlas mejor y poder aplicarlas correctamente, durante los últimos años ha crecido el número de estudios con estas especies.

1.1.2.1. *Torulaspora delbrueckii*

Las células de *Torulaspora* son esféricas, aunque también son abundantes sus formas elipsoidales y ovoides, siendo su tamaño algo menor que el de la especie *S. cerevisiae* (Figura 1). Como comparten varias características morfológicas y fisiológicas, en el pasado hubo errores al clasificar algunas especies dentro de los tres géneros: *Torulaspora*, *Saccharomyces* y *Zygosaccharomyces*. El género *Torulaspora* incluye al menos seis especies, entre las cuales se incluye *Torulaspora delbrueckii* (anteriormente *Saccharomyces rosei*).

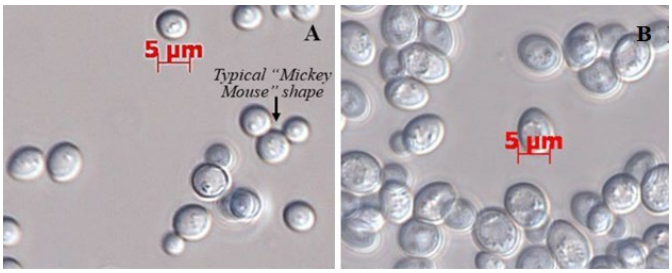

Figura 1. Levaduras *T. delbrueckii* (A) y *S. cerevisiae* (B) en microscopio óptico (Ramírez and Velázquez, 2018).

T. delbrueckii es una de las levaduras que comparte más similitudes con *S. cerevisiae*, por lo que se considera que cumple con las características necesarias para desarrollar la fermentación alcohólica a nivel industrial. Esta es la razón principal por la cual ha sido la primera especie no-*Saccharomyces* propuesta para utilizarse en vinificación (Ramírez and Velázquez, 2018). El gran potencial enológico de *T. delbrueckii* se debe a que los vinos donde interviene resultan con menor cantidad de ácido acético y etanol (Bely et al., 2008; Contreras et al., 2015), mayor

concentración de glicerol (Belda et al., 2015) y compuestos aromáticos deseables (ésteres frutales, lactonas, tioles, terpenos…) (Azzolini et al., 2015; Belda et al., 2017a; Renault et al., 2016) mayor liberación de manoproteínas y polisacáridos (Belda et al., 2016a), y además promueve la fermentación maloláctica (Balmaseda et al., 2021). Todas estas contribuciones pueden mejorar la calidad y la complejidad del vino. (Benito, 2018b). En los últimos años, se ha visto que las fermentaciones con inoculación simultánea o secuencial de cepas seleccionadas de *T. delbrueckii* y *S. cerevisiae* dan lugar a vinos más complejos que aquellos que fueron inoculados únicamente con *S. cerevisiae* (Arslan et al., 2018; Azzolini et al., 2012; Belda et al., 2017a, 2015; Canonico et al., 2019; Contreras et al., 2015; Escribano-Viana et al., 2019; Renault et al., 2016, 2015) demostrado que existe un efecto sinérgico entre *T. delbrueckii* y *S. cerevisiae* en el vino (Figura 2).

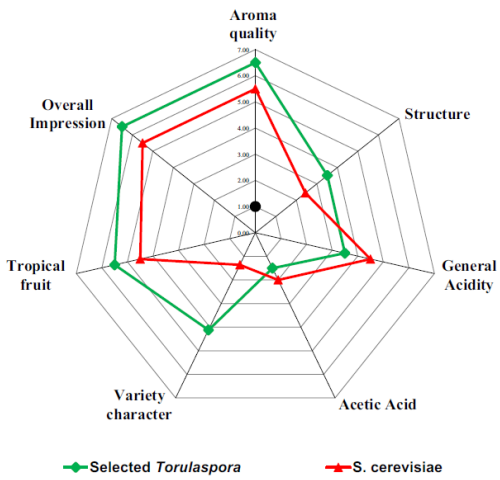

Figura 2. Influencia de *T. delbrueckii* en la percepción sensorial de un vino inoculado con *T. delbrueckii* y *S. cerevisiae* comparado con uno obtenido de la fermentación de *S. cerevisiae* (Benito, 2018b).

Aunque *S. cerevisiae* es la que especie que domina la fermentación, algunos estudios han demostrado que algunas cepas de *T. delbrueckii* pueden aguantar hasta el final de la fermentación. De hecho, se ha observado que dicha persistencia depende de varios factores como el tamaño del inóculo, la concentración de azúcar y de etanol, de la presencia de fenotipo killer, o la concentración de dióxido de azufre, entre otros (Taillandier et al., 2014; Ramírez et al., 2015; Velázquez et al. 2015; Lleixà et al., 2016; Escribano-Viana et al., 2021; Zhu et al., 2021), incluso hay cepas capaces de realizar la fermentación por sí solas (Renault et al., 2015; Taillandier et al., 2014; van Breda et al., 2018).

1.1.2.2. *Lachancea thermotolerans*

Lachancea thermotolerans (anteriormente *Kluyveromyces thermotolerans*) es una levadura del género *Lachancea*. Este género fue propuesto el año 2003 por Kurtzman con el propósito de unir diferentes géneros que presentaban similitudes a nivel de ARN ribosomal. Actualmente comprende doce especies (Kurtzman, 2003; Porter et al., 2019). *L. thermotolerans* son levaduras con forma elipsoidal (Figura 3) ligeramente más pequeñas que las levaduras *S. cerevisiae* (Benito, 2018a).

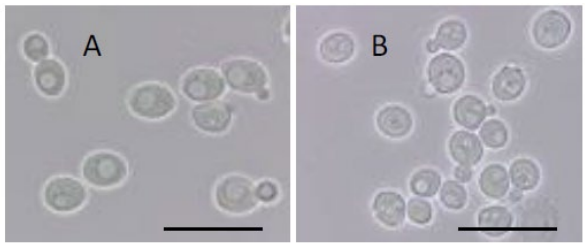

Figura 3. Levaduras *S. cerevisiae* (A) y *L. thermotolerans* (B) vistas con microscopio óptico a una escala de 10 µm (Morata et al., 2018).

Las levaduras *L. thermotolerans* pueden encontrarse en una amplia variedad de nichos ecológicos, incluidos insectos, plantas, suelo y cultivos hortícolas, así como principalmente en uvas y vino (Porter et al., 2019). En los últimos años han aumentado los estudios de fermentaciones donde se inocula *L. thermotolerans,* tanto de manera simultánea como secuencial, junto con *S. cerevisiae*. Los resultados obtenidos han demostrado que la presencia de *L. thermotolerans* en la fermentación produce vinos con mayores concentraciones de ácido láctico (Mora et al., 1990; Ciani et al., 2006; Kapsopoulou et al., 2007; Comitini et al., 2011; Gobbi et al., 2013; Balikci et al., 2016; Beckner Whitener et al., 2016; Benito et al., 2015, 2016, 2017), glicerol y 2-feniletanol (Kapsopoulou et al., 2007; Comitini et al., 2011; Gobbi et al., 2013); aunque estas características son dependientes de cepa (Vilela, 2018). De esta manera los vinos tienen más complejidad a nivel de aroma, sabor y sensación en boca (Figura 4) (Benito, 2018a).

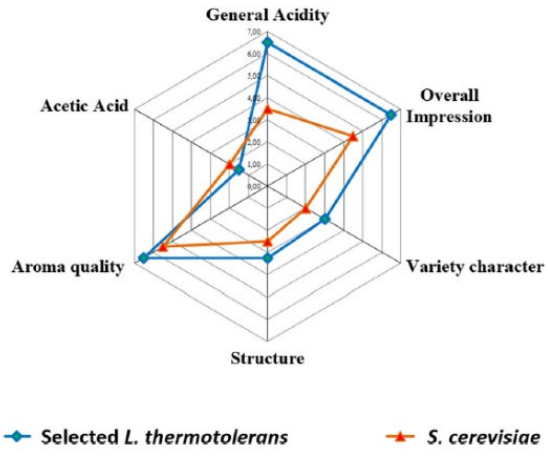

Figura 4. Influencia de *L. thermotolerans* en la percepción sensorial de un vino inoculado con *L. thermotolerans* y *S. cerevisiae* en comparación con un vino fermentado únicamente por *S. cerevisiae* (Benito, 2018a).

1.2. El metabolismo de las levaduras

1.2.1. El metabolismo del carbono

Las levaduras pueden degradar los azúcares mediante dos vías metabólicas diferentes, la fermentación alcohólica y la respiración, que dependen de la disponibilidad de oxígeno, y de la concentración de glucosa del medio. Ambas vías empiezan con la glucólisis como primer paso para degradar la glucosa (Figura 5). La glucólisis es una ruta metabólica en la que una molécula de glucosa se convierte en dos moléculas de piruvato, proceso que genera energía (en forma de moléculas de ATP y NADH). Una vez que se ha formado el piruvato, éste es oxidado por la vía de la respiración, que es una reacción dependiente de oxígeno, o mediante la fermentación alcohólica, que no requiere oxígeno. De estos dos procesos, la respiración es el más favorable energéticamente.

En la respiración, primero el piruvato es oxidado a acetil-CoA y dióxido de carbono por el complejo piruvato deshidrogenasa, generando una molécula de NADH y otra de dióxido de carbono. Después, el acetil-CoA es oxidado por el ciclo de Krebs (o del ácido cítrico o TCA) a dióxido de carbono y agua en presencia de oxígeno. En general, en los eucariotas esta vía produce hasta 38 moléculas de ATP a partir de una molécula de glucosa, incluyendo los dos ATP producidos por la glucólisis.

En la fermentación alcohólica se dan dos reacciones después de la glucólisis. En la primera, el piruvato se descarboxila a acetaldehído liberando una molécula de dióxido de carbono. En la segunda, el acetaldehído es reducido a etanol por la alcohol deshidrogenasa. En este proceso la ganancia neta es de dos ATP por cada molécula de glucosa degradada.

S. cerevisiae tiene un crecimiento facultativo en respuesta al oxígeno, de manera que puede crecer tanto en condiciones aeróbicas como anaeróbicas. Además, puede adaptar su metabolismo según la disponibilidad de glucosa en el medio. De hecho, *S. cerevisiae* se clasifica como Carbtree positiva, es decir, en presencia de altas concentraciones de azúcar siempre fermenta, teniendo reprimida la via respiratoria, independientemente de la presencia o no de oxígeno. Otras levaduras Carbtree positivas son, por ejemplo, *Hanseniaspora guilliermondii* y *Starmerella bacillaris*. Las levaduras que no muestran este efecto se conocen como Crabtree-negativas, entre ellas encontramos *Hanseniaspora uvarum* y *Metschnikowia pulcherrima* (Albergaria and Arneborg, 2016; Ribéreau-Gayon et al., 2006). En una fermentación estándar, la levadura crece y produce otros metabolitos, de manera que el 95% de los azúcares se convierten en etanol y dióxido de carbono, un 1% en material celular, y el 4% restante en otros productos como glicerol, ácido acético, ácido láctico y alcoholes superiores, entre otros (Ribéreau-Gayon et al., 2006). Estos productos afectan al aroma y al gusto, por lo que tienen un gran impacto en la calidad y en la complejidad del vino final (Fleet, 2003).

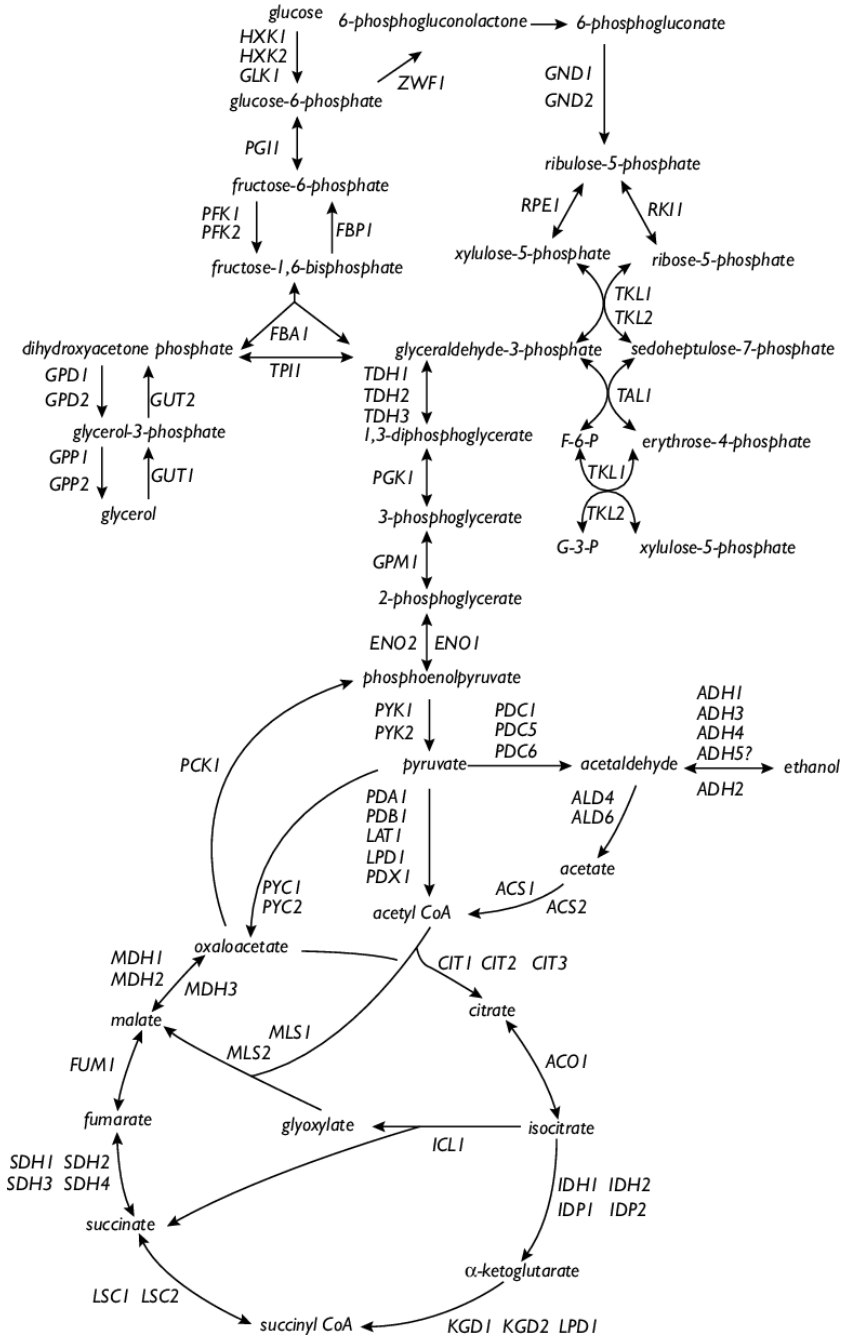

Figura 5. Metabolismo del carbono en *S. cerevisiae* (Dickinson and Schweizer, 2004).

1.2.2. El metabolismo del nitrógeno

Las levaduras pueden utilizar una gran variedad de compuestos nitrogenados, como aminoácidos, amonio y péptidos pequeños, que es lo que se conoce como nitrógeno asimilable (YAN). Por un lado, los aminoácidos entran en *S. cerevisiae* a través de un sistema de 19 permeasas de las cuales sólo tres (*AGP1, GAP1* y *PUT4*) son de alta capacidad y están reguladas por el nitrógeno. Una vez dentro, pueden utilizarse en rutas de biosíntesis (para generar purinas, pirimidinas y aminoácidos), o entrar en el ciclo central de nitrógeno, que incluye su transaminación a glutamato (Figura 6) (Godard et al., 2007). *S. cerevisiae* puede transformar cualquier molécula de nitrógeno en glutamato o glutamina de manera que estos aminoácidos son utilizados en la síntesis de todos los compuestos que contienen nitrógeno. Por otro lado, el amonio entra a través de tres permeasas (*MEP1, MEP2 y MEP3*) que sólo están activas cuando el amonio está presente en bajas concentraciones, y en cambio están reprimidas cuando en el medio hay buenas fuentes de nitrógeno.

Así pues, la captación de compuestos nitrogenados y su metabolismo está regulado en *S. cereviciae* por el mecanismo de represión catabólica por nitrógeno (NCR) (Magasanik and Kaiser, 2002; ter Schure et al., 2000). Este mecanismo regula la captación de los compuestos nitrogenados por parte de la levaduradurante la fermentación alcohólica, ya que cuando las fuentes de nitrógeno preferidas están presentes en el medio (como amonio o glutamina), se reprime la expresión de genes necesarios para la captación y catabolismo de fuentes de nitrógeno menos preferidas (Beltran et al., 2004).

Durante los primeros días de la fermentación, la concentración de las fuentes de nitrógeno preferidas va disminuyendo y, en consecuencia, el mecanismo NCR se desreprime, utilizando entonces el resto de compuestos nitrogenados menos preferidos (Beltran et al., 2004; 2005; Godard et al., 2007; Ljungdahl and Daignan-Fornier, 2012).

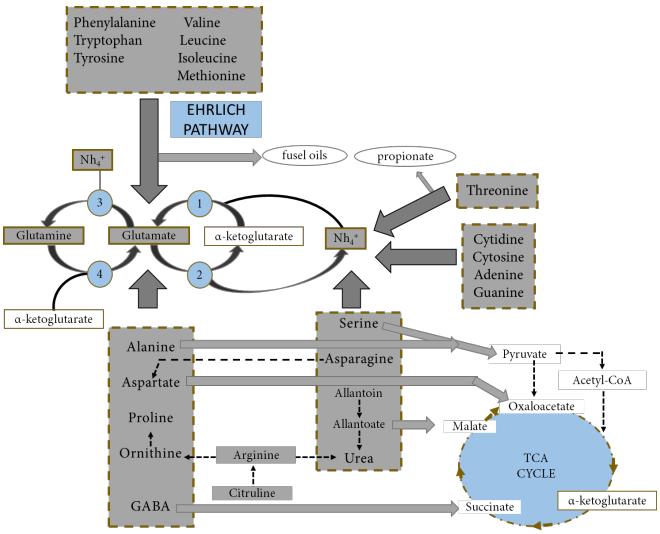

Figura 6. Principales reacciones relacionadas con la utilización de fuentes nitrogenadas en la levadura *S. cerevisiae* (Godard et al., 2007).

Aunque las levaduras pueden utilizar una gran variedad de compuestos nitrogenados diferentes, suelen mostrar preferencia por algunos de ellos. Así pues, se ha investigado profundamente a lo largo de los últimos años el consumo y las preferencias de nitrógeno que presenta la levadura fermentadora por excelencia, *S. cerevisiae*. Se conoce que algunas de sus fuentes de nitrógeno preferidas, como el amonio, la glutamina y la asparagina, promueven su crecimiento celular. En cambio, las menos deseables, como la prolina y la urea, dan como resultado un bajo crecimiento cuando son las únicas fuentes de nitrógeno (Bell et al., 1979;

Beltran et al., 2005; Gutiérrez et al., 2012; Jiménez-Martí et al., 2007; Jiranek et al., 1995; Mendes-Ferreira et al., 2004; Torija et al., 2003; Vilanova et al., 2007).

En los últimos años se ha incrementado el número de estudios sobre las preferencias nitrogenadas por parte de levaduras no-*Saccharomyces* debido a que han aumentado las evidencias científicas de sus contribuciones positivas al vino (Tabla 2).

Tabla 2. Resumen de las preferencias nitrogenadas de *Saccharomyces cerevisiae* (Sc), *Torulaspora delbrueckii* (Td), *Lachancea thermotolerans* (Lt), *Metschnikowia pulcherrima* (Mp), *Starmerella bacillaris* (Sb), *Pichia membranifaciens* (Pm), *Hanseniaspora uvarum* (Hu) y *Hanseniaspora vineae* (Hv) durante vinificaciones a 20°C, 25°C y 28°C. Referencias 1: Andorrà et al. (2010); 2: Kemsawasd et al. (2015a); 3: Gobert et al. (2017); 4: Lleixà et al. (2019); 5: Prior et al. (2019); 6: Su et al. (2020); 7: Gobert et al. (2019) (adaptada de Gobert et al. (2019)).

Nitrogen source	Wine yeasts	References
Alanine	Sc, Hu, Mp, Sb, Td	2, 3, 7
Ammonium	Sc, Mp, Td	2, 7
Arginine	Sc, Lt, Hu, Td	2, 7
Asparagine	Sc, Lt, Pm, Td	2, 3, 7
Aspartat	Sc	7
Aspartic acid	Sc, Hu, Lt, Td	2, 6, 7
Cysteine	Sc, Hu, Hv	1, 4, 7
GABA	Sc	7
Glutamate	Sc, Td	6, 7
Glutamic acid	Sc, Hu, Lt, Mp, Hv	1, 2, 4, 7
Glutamine	Sc, Hu, Lt Mp, Td	1, 2, 3, 6, 7
Glycine	Sb, Pm	1, 3
Histidine	Sc, Mp, Pm, Hv, Td	3, 4, 6, 7
Isoleucine	Sc, Lt, Mp, Td, Hv	2, 3, 4, 6, 7
Leucine	Sc, Hu, Lt, Mp, Hv, Td	1, 2, 3, 4, 5,6, 7
Lysine	Sc, Mp, Sb, Pm, Hv, Td	3, 4, 6, 7
Methionine	Sc, Hu, Mp, Td	1, 3, 6, 7
Phenylalanine	Hu, Lt, Hv, Td	2, 4, 5
Serine	Sc, Lt, Pm, Td	3, 2, 5, 6, 7
Threonine	Sc, Mp, Pm, Td, Lt	3, 5, 7
Tryptophan	Sb	1
Tyrosine	Lt	2
Valine	Hu, Td, Lt	2, 5, 6

Los resultados obtenidos han demostrado que las especies no-*Saccharomyces* tienen perfiles de consumo de aminoácidos específicos, que dependen en gran medida de la composición de nitrógeno del medio (Gobert et al., 2017; Englezos et al., 2018; Rollero et al., 2018a; Gobert et al., 2019; Rollero et al., 2019; Su et al., 2020) y de la cepa utilizada (Gobert et al., 2017; Rollero et al., 2018b; Su et al., 2020).

1.2.3. La producción de aromas

El aroma del vino está formado por los aromas primarios o varietales (originarios de las uvas), los aromas secundarios o fermentativos (producidos por levaduras y bacterias) y los aromas terciarios o post-fermentativos (que aparecen durante el envejecimiento) (Fleet and Heard, 1993). Los compuestos volátiles que contribuyen en mayor medida al *bouquet* de fermentación son los ácidos orgánicos, los alcoholes superiores y los ésteres, y en menor medida, los aldehídos (Figura 7). Los compuestos aromáticos más negativos son los compuestos reducidos de azufre (Lambrechts and Pretorius, 2000).

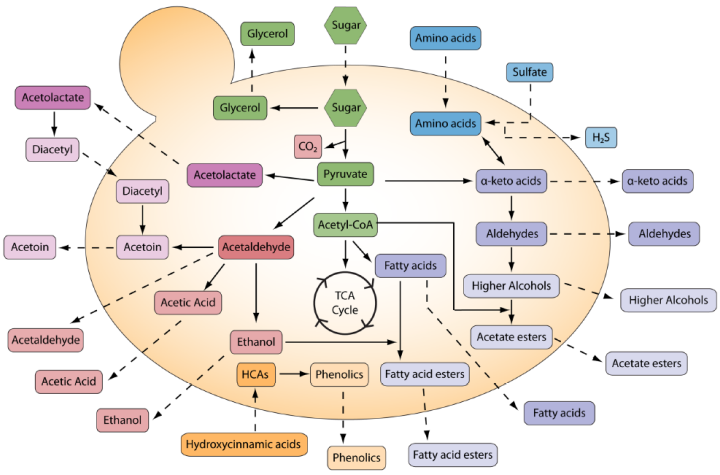

Figura 7. Producción de compuestos aromáticos en *S. cerevisiae* (Dzialo et al., 2017).

Los alcoholes superiores y los aldehídos se generan a través de la vía de Ehrlich. Esta vía consta de tres pasos, una primera etapa de transaminación, seguida de la descarboxilación de los α-ceto ácidos en aldehído, y finalmente la reducción de éste (Figura 8). Los aminoácidos que sirven como sustrato por la vía de Ehrlich (valina, leucina, isoleucina, metionina y fenilalanina) se absorben lentamente a lo largo de la fermentación (Zhang et al., 2016). La vía de Ehrlich funciona simultáneamente con la vía de la desmetilación como dos ramas competidoras que convierten los aminoácidos en alcoholes (Hazelwood et al., 2008).

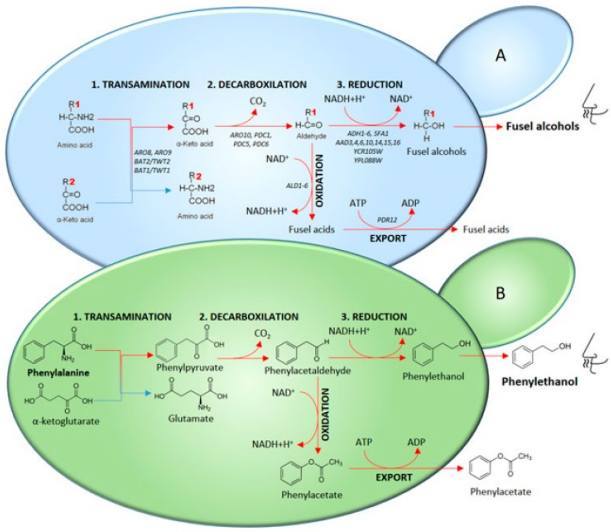

Figura 8. Vía de Ehrlich en *S. cerevisiae*. (A) Genes que codifican enzimas implicadas en el proceso. (B) Catabolismo de la fenilalanina por la vía de Erhlich (Belda et al., 2017b).

La capacidad de producir alcoholes superiores depende de la cepa de levadura (Belda et al., 2017a; du Plessis et al., 2019). Estos compuestos pueden reconocerse por un olor y un sabor fuerte y penetrante. Según la concentración en la que se encuentren influirán en el carácter del vino de manera positiva, contribuyendo en la complejidad del vino (por debajo de

300 mg/L) o de manera negativa (por encima de 400 mg/L) (Ribéreau-Gayon et al., 2006). De los producidos durante la fermentación alcohólica cabe destacar los alcoholes alifáticos (propanol, alcohol isobutílico, alcohol amílico activo (2-metil-1-butanol) y alcohol isoamílico (3-metil-1-butanol)) y los aromáticos (tirosol, triptofol y 2-fenil etanol) (Lambrechts and Pretorius, 2000). La producción de algunos de ellos ha sido descrita como una característica de especies de levaduras no-*Saccharomyces,* como el aumento en la producción de 2-feniletanol, considerado un aroma agradable, producido por *M. pulcherrima* (Clemente-Jimenez et al., 2004), *L. thermotolerans* (Beckner Whitener et al., 2015), y *S. bacillaris* (Andorrà et al., 2010).

Los ésteres se producen por la combinación de acetil-CoA (originará ésteres de acetato) o acil-CoA (dará lugar a ésteres etílicos de ácidos grasos) y un alcohol mediante la acción de las enzimas alcohol-O-acetil (o acil)-transferasas (Dizalo et al., 2017). Los más importantes son los ésteres de acetato de alcoholes superiores (derivados del ácido acético): acetato de etilo (olor afrutado), acetato de isoamilo (olor a banana), acetato de isobutilo (olor a manzana), acetato de hexilo (olor a hierba) y acetato de 2-feniletilo (olor a rosas, floral); y ésteres etílicos de ácidos grasos saturados de cadena lineal: butanoato de etilo (olor afrutado, floral), hexanoato de etilo (olor a manzana verde y violetas), octanoato de etilo (olor a piña y pera), decanoato de etilo (aroma floral) y dodecanoato de etilo (pera, afrutado, floral, hoja) (Lambrechts and Pretorius, 2000). Los niveles de ésteres producidos por las levaduras no-*Saccharomyces* son generalmente

mucho más bajos que los producidos por de *S. cerevisiae* (Rojas et al., 2001, 2003).

Los aldehídos se asocian a olores similares a los de la manzana y son importantes para el aroma y el *bouquet* del vino pese a sus bajos niveles de detección. De entre ellos, el acetaldehído constituye más del 90% del contenido total de aldehídos en el vino y su cantidad puede variar entre 10 mg/L hasta 300mg/L, aunque en grandes cantidades no suele ser positivo en el vino (Lambrechts y Pretorius, 2000). Las cepas de *S. cerevisiae* suelen producir niveles más altos (5-120 mg/L) que las no-*Saccharomyces* (hasta 40 mg/L) (Fleet y Heard, 1993; Romano et al., 2003).

La acidez volátil de los vinos corresponde al 10-15% de la acidez total. La conforman una amplia variedad de ácidos grasos de cadena corta, como el ácido propanoico, el ácido butanoico y el ácido acético, siendo este último el mayor constituyente (más del 90%) (Radler, 1993). Los ácidos propanoico y butanoico están asociados al metabolismo bacteriano (Ribéreau-Gayon, 2006). Aunque las levaduras no-*Saccharomyces* producen cantidades variables de ácido acético (Romano et al., 2003; (Benito et al., 2014), se ha observado una baja acidez volátil por parte de cepas de *T. delbrueckii* (Renault et al., 2009; Comitini et al., 2011), *L. thermotolerans* (Kapsopoulou et al., 2007) y *S. bacillaris* (Englezos et al., 2016) en comparación con *S. cerevisiae*.

Otros compuestos que contribuyen en la calidad sensorial del vino son los ácidos grasos volátiles, los carbonilos y los compuestos de azufre (Lambrechts and Pretorius, 2000; Moreira et al., 2005). Por lo general, se asocian a aromas tropicales, como maracuyá y pomelo (Swiegers and

Pretorius, 2007). Entre ellos, cabe destacar los "tioles volátiles tropicales" 4-mercapto- 4-metilpentano-2-ona (4-MMP), 3-mercapto-hexanol (3-MH) y 3-mercaptohexilacetato (3-MHA), que contribuyen al perfil aromático varietal de algunos vinos blancos (Darriet et al., 1995; Swiegers and Pretorius, 2005). Estos aromas se encuentran en la uva en su forma precursora (no volátil) conjugadas con cisteína (Tominaga et al., 1998, 1995) o glutatión (Fedrizzi et al., 2009). Las levaduras producen la forma volátil durante la fermentación alcohólica (Dubordieu et al., 2006) mediante enzimas carbono-azufre liasas que rompen el precursor (Dubordieu et al., 2006; Howell et al., 2004) (Figura 8).

En *S. cerevisiae* la enzima β-liasa está codificada por el gen *IRC7* (Roncoroni et al., 2011; Thibon et al., 2008) el cual tiene dos alelos (uno está completo y el otro tiene una deleción y es más corto, cosa que hace que la enzima sea menos funcional) (Roncoroni et al, 2011). Entre las cepas de *S. cerevisiae* el alelo mayoritario es el más corto (Belda, et al., 2016b), que genera enzimas con baja actividad. Además, la NCR afecta a los genes responsables de asimilar el precursor (Subileau et al., 2008) y de su posterior escisión (Thibon et al., 2008). Todo esto hace que se libere un bajo porcentaje de los precursores de tioles de la uva (Coetzee and du Toit, 2012).

La cepa de levadura *S. cerevisiae* utilizada para llevar a cabo la fermentación es uno de los factores más importantes que afectan a la liberación de tioles (Cordente et al., 2012; Maggu et al., 2007). También se han descrito levaduras no-*Saccharomyces* de las especies *S. bacillaris*, *P. kluyveri* (Anfang et al., 2009), *T. delbrueckii*, *M. pulcherrima* y *L. thermotolerans* (Renault et al., 2016; Zott et al., 2011) que son capaces de producir

cantidades significativas de tioles volátiles 3MH y 3MHA, aunque también depende de la cepa (Belda et al., 2016b; Zott et al., 2011).

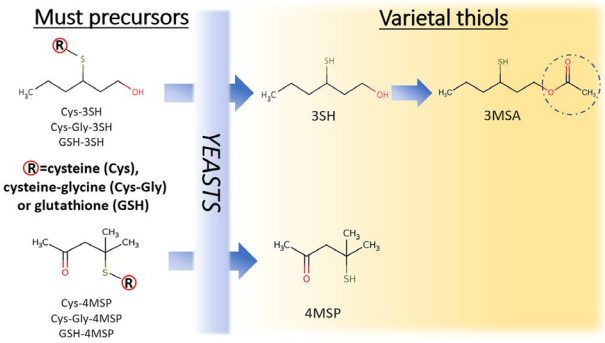

Figura 8. Biosíntesis de tioles en *S. cerevisiae* (Ruiz et al., 2019).

2. Factores que afectan las levaduras y la fermentación

2.1. Nutrientes

Las levaduras son organismos quimioorganótrofos y necesitan nutrientes orgánicos como fuentes de carbono y energía para crecer (Ribéreau-Gayon et al., 2006). El crecimiento de las levaduras, así como el correcto rendimiento de la fermentación, dependerá de la disponibilidad de nutrientes esenciales como azúcares y compuestos nitrogenados, entre otros.

2.1.1. Composición del mosto de uva

Los granos de uva constituyen la materia prima para la elaboración del vino. Antes de la cosecha, la maduración de este fruto es el resultado de varias transformaciones bioquímicas que aumentan la concentración de azúcar y disminuyen la acidez. La madurez consiste en la proporción óptima entre azúcares y ácidos, aunque el criterio para considerar la uva madura y lista

para cosechar variará según el objetivo que se persiga. Por ejemplo, para producir vinos blancos secos se necesitan uvas con alta concentración de compuestos aromáticos y suficiente acidez. En cambio, para elaborar un vino tinto de calidad, la vendimia se realizará cuando los compuestos fenólicos sean fácilmente extraíbles (Ribereáu-Gayon et al., 2006).

Los componentes principales del mosto son los azúcares, esencialmente la glucosa y la fructosa, pero también puede haber xilosa, arabinosa, ramnosa, maltosa, rafinosa y sacarosa, pero a muy baja concentración. La concentración de azúcares puede llegar a ser entre 150 y 240 g/L. Por otro lado, también están los ácidos, siendo mayoritarios el ácido tartárico, el ácido málico y el ácido cítrico. Existen muchas diferencias en las concentraciones de los ácidos según el clima, y la variedad y la madurez de la uva (Ribéreau-Gayon et al., 2006).

Además, el mosto contiene otros nutrientes, como los compuestos nitrogenados y los micronutrientes, explicados a continuación.

2.1.2. Compuestos nitrogenados

El nitrógeno en el mosto está presente de dos formas: inorgánica (amoniacal) y orgánica (aminoácidos). Así pues, las fuentes de nitrógeno que contiene el mosto son el amonio (3-10%), los aminoácidos (25-30%), los polipéptidos (25-40%) y las proteínas (5-10%) (Ribéreau-Gayon et al., 2006), aunque sólo el amonio, los aminoácidos y pequeños péptidos son fuentes de nitrógeno asimilables por la levadura (Yeast Assimilable Nitrogen o YAN). De entre los amoniácidos, la prolina requiere de oxígeno para su asimilación y metabolismo, por lo que en condiciones de

fermentación no se considera asimilable (Ingledew et al., 1987). Además, el consumo de algunos aminoácidos viene regulado por el sistema NCR, y su asimilación va a depender de la cantidad de amonio o fuentes preferentes de nitrógeno presentes en el medio (Beltran et al. 2004, 2005).

Para un buen desarrollo de la fermentación se considera óptima una concentración de aproximadamente 300 mgN/L de YAN, pero 140 mg/L de YAN es el mínimo necesario para completar la fermentación (Bell and Henschke, 2005; Marsit et al., 2015, Martínez-Moreno et al., 2012).

2.1.3. Micronutrientes

Los micronutrientes son aquellas sustancias esenciales para la levadura, necesarias en cantidades muy pequeñas, las cuales tienen otras funciones que no son la de aportar energía. En este grupo encontramos los minerales, los oligoelementos, las vitaminas y los factores de supervivencia o anaerobiosis.

2.1.3.1. Minerales y oligoelementos

Los oligoelementos son minerales que están presentes en pequeñas cantidades, pero son esenciales para el crecimiento de la levadura, y tanto su ausencia como exceso puede ser perjudical para el organismo. Estos compuestos están involucrados en reacciones bioquímicas en las cuales interactúan con protones e iones hidróxido. Por ejemplo, los iones de potasio estabilizan las cargas negativas dentro de las células y activan procesos metabólicos importantes como la traducción de proteínas; y el calcio realiza funciones enzimáticas, estructurales y de señalización dentro

de las células (Cyert and Philpott, 2013). De forma general, en el mosto hay cantidad suficiente de minerales para asegurar un correcto desarrollo de la levadura (Ribéreau-Gayon et al., 2006).

Solo se conoce la función exacta de una pequeña parte de los oligoelementos (Ribéreau-Gayon et al., 2006). Por ejemplo, el cobre, el hierro y el manganeso son determinantes en la estructura de proteínas y tienen la función de actuar como cofactores enzimáticos (Cyert and Philpott, 2013). El zinc interviene en el crecimiento y el metabolismo de las levaduras (Walker, 2004). Tiene un papel importante en proteínas y ácidos nucleicos. Por ejemplo, interviene en proteínas de unión al ADN con los conocidos como dedos de zinc; y también participa en la integridad de la membrana celular, promoviendo la floculación de la levadura estabilizando así la membrana (de Nicola et al., 2009). Además, es cofactor de un gran número de enzimas como la fosfatasa alcalina, la anhidrasa carbónica, la superóxido dismutasa, varias carboxipeptidasas y la alcohol deshidrogenasa, siendo esencial en el metabolismo fermentativo de las levaduras. Una deficiencia de zinc detiene el crecimiento celular y, en consecuencia, la fermentación puede volverse lenta o incompleta (Walker, 2004). La suplementación con zinc en la fermentación alcohólica mejora el estrés de la levadura y la tolerancia al etanol (Zhao and Bai, 2012).

2.1.3.2. Vitaminas

Las vitaminas son factores de crecimiento que afectan a la reproducción y a la actividad celular. Una deficiencia en ellas tiene un efecto negativo sobre el metabolismo de las levaduras debido a que son componentes esenciales

de varias coenzimas. El mosto natural contiene una amplia variedad de vitaminas (Tabla 3) (Ribéreau-Gayon et al., 2006).

Tabla 3. Concentraciones máximas y mínimas (µg/L) de vitaminas presentes en el mosto (Ribéreau-Gayon et al., 2006).

Vitamins	Grape musts	White Wines	Red Wines
Thiamine	160 – 450	2 – 58	103 – 245
Riboflavin	3 – 60	8 – 133	0.47 – 1.9
Pantothenic acid	0.5 – 1.4	0.55 – 1.2	0.13 – 0.68
Pyridoxine	0.16 – 0.5	0.12 – 0.67	0.13 – 0.68
Nicotinamide	0.68 – 2.6	0.44 – 1.3	0.79 – 1.7
Biotin	1.5 – 4.2	1 – 3.6	0.6 – 4.6
Mesoinositol (mg/L)	380 – 710	220 – 730	290 – 334
Cobalamine	0	0 – 0.16	0.04 – 0.10
Choline	19 - 39	19 - 27	20 - 43

La piridoxina es una vitamina con un papel importante en la síntesis de una gran cantidad de metabolitos como la acetil CoA (Bataillon et al., 1996). La nicotinamida y el ácido nicotínico son precursores del NAD^+ (Panozzo et al., 2002). El ácido pantoténico participa en la síntesis de la coenzima A y en la síntesis de lípidos. La biotina está implicada en la carboxilación del piruvato y en el metabolismo del nitrógeno y de los lípidos (Oura and Suomalainen, 1978; Ribéreau-Gayon et al., 2006).

La tiamina actúa como cofactor de varias enzimas implicadas en diferentes vías metabólicas, incluidas la glicólisis, el ciclo del ácido tricarboxílico y la vía de las pentosas fosfato. Es esencial para la biosíntesis de intermediarios de la síntesis de aminoácidos aromáticos, la biosíntesis de lípidos y la producción de ribosa-5-fosfato para la síntesis de ADN y ARN (Hohmann and Meacock, 1998). Además, tiene una función de protección frente al estrés (Kowalska et al., 2012; Wolack et al., 2014; Kartal et al., 2018; Li et al., 2019). La mayoría de las levaduras asimilan la tiamina exógena mediante

un sistema de transporte activo, pero también pueden sintetizarla de *novo*, aunque con un gran coste energético. Las levaduras pueden utilizar más tiamina (600-800 µg/L) de la que el mosto contiene. *S. cerevisiae* es capaz de consumir hasta 800 µg/L de tiamina durante las primeras seis horas posteriores a su inoculación (Bataillon et al., 1996). Una deficiencia de tiamina en condiciones enológicas (considerada inferior a 250 µg/L) provoca un crecimiento deficiente, muerte celular y paradas de fermentación (Bataillon et al., 1996; Bisson, 1999). Su adición, en forma de hidrocloruro de tiamina, mejora la cinética de fermentación (Guzzon et al., 2011) y es una práctica común en bodega (concentración máxima permitida de 0.6 g/L en la Unión Europea y Sudáfrica) (OIV, 2021).

2.1.3.3. Factores de anaerobiosis

La levadura requiere la presencia de compuestos lipídicos denominados factores de anaerobiosis o de supervivencia. Son esteroles y ácidos grasos de cadena larga que intervienen en el metabolismo de las levaduras y en la síntesis de la membrana celular. Es importante que estén disponibles en el medio durante la fermentación ya que *S. cerevisiae* necesita oxígeno para poder sintetizarlos, y las condiciones anaeróbicas de la fermentación hace difícil su síntesis. Éstos compuestos suelen estar presentes en mostos naturales ya que provienen de la uva, encontrándose en mayor cantidad en mostos de uvas tinta (ya que se vinifica con las pieles), y deben tenerse en cuenta a la hora de utilizar mosto sintético a escala de laboratorio, donde deberán añadirse. Uno de los esteroles más abundantes en *S. cerevisiae* es el ergosterol, que contribuye a la integridad de la membrana. El ácido oleico y el ácido palmitoleico son los ácidos grasos más comunes en *S. cerevisiae*.

(Klug and Daum, 2014). Estos compuestos contribuyen a acelerar la velocidad de la fermentación (Ribéreau-Gayon et al., 2006).

También se ha visto que en fermentaciones mixtas los factores de anaerobiosis pueden afectar al crecimiento de *S. cerevisiae* según la cantidad añadida. Mientras a dosis bajas inducen la imposición de *S. cerevisiae* sobre *T. delbrueckii*, a dosis altas esta imposición se ve alterada (Brou et al., 2018).

2.1.4. Suplementos nutricionales

El déficit de compuestos nitrogenados en el mosto es una de las causas de problemas como fermentaciones lentas o incompletas (Bisson, 1999; Varela et al., 2004; Medina et al., 2012; Lleixà et al., 2016; Rollero et al., 2018a). Una manera de evitar estos problemas es suplementar el mosto con sales de amonio (Gutiérrez et al., 2012; Martínez-Moreno et al., 2014; Medina et al., 2012). Otra manera rápida, cómoda y eficaz de suplementar y mejorar la fermentación y las características organolépticas del vino en bodega es utilizar los preparados comerciales de levadura seca inactiva (IDY, Inactive Dry Yeasts) que se obtienen de cultivos de *S. cerevisiae* inactivados térmicamente. Se pueden clasificar en cuatro tipos:

1. Levaduras inactivas (se obtienen por inactivación térmica).
2. Autolisados de levaduras (se obtienen por inactivación térmica y una incubación que permite que las enzimas se liberen de las vacuolas, degradando así parte del contenido intracelular).
3. Paredes de levaduras (el extracto insoluble de las levaduras formado por las paredes, sin el contenido citoplasmático)

4. Extractos de levadura (el extracto soluble de las levaduras después de la degradación total del contenido citoplasmático).

Todos estos productos derivados de la levadura son ofrecidos por diferentes empresas. Cada uno promete diferentes mejoras específicas en el vino. El uso de preparados de IDY se ha correlacionado con un consumo más rápido de azúcar y un mayor número de levaduras viables en las fermentaciones alcohólicas (Pozo-Bayón et al., 2009).

2.2. Otros factores

2.2.1. Oxígeno

Las levaduras llevan a cabo la fermentación alcohólica en condiciones donde el oxígeno está limitado. Su disponibilidad será decisiva en la supervivencia de las diferentes especies de levaduras a lo largo de la fermentación. *S. cerevisiae* es capaz de crecer en estas condiciones, siendo ésta una ventaja a la hora de imponerse respecto al resto de levaduras (Bisson, 1999). En cambio, especies de levaduras no-*Saccharomyces*, como *T. delbrueckii* y *L. thermotolerans,* son menos tolerantes a niveles bajos de oxígeno (Hansen et al., 2001; Nissen and Arneborg, 2003). Sin embargo, se ha visto que *T. delbrueckii* compensa la falta de oxígeno produciendo más glicerol cuando es cultivada en un medio con baja cantidad de nitrógeno (Brandam et al., 2013) y además es capaz de crecer y completar la fermentación en condiciones anaerobias (Taillandier et al., 2014).

2.2.2. Temperatura

La temperatura es uno de los factores que afectan a la fermentación debido a que tiene un efecto en el crecimiento de las levaduras. A temperaturas por encima de 15-20°C, junto a la presencia de etanol, se reduce el crecimiento de las levaduras debido a que se ve afectada la integridad y la permeabilidad de la membrana celular (Albergaria and Arneborg, 2016). En el caso de *S. cerevisiae,* el incremento de la temperatura es ventajoso ya que puede crecer bien, a diferencia de las levaduras no-*Saccharomyces* (Salvadó et al., 2011). Existen algunas levaduras no-*Saccharomyces*, como especies del género *Hanseniaspora* spp., que son capaces de sobrevivir y dominar la fermentación a temperaturas inferiores a 20°C (Alonso-del-Real et al., 2017; Ciani et al., 2006). Así pues, las bajas temperaturas en la fermentación podrían favorecer el crecimiento y la supervivencia de especies no-*Saccharomyces* durante un tiempo más largo.

Las fermentaciones se realizan a baja temperatura para obtener vinos con características específicas de aroma y sabor, especialmente en vinos blancos y rosados (Llauradó et al., 2002; Torija et al., 2003; Beltran et al., 2008; Deed et al., 2017). Entre los 10°C y 15°C se incrementa la producción, y también la retención, de algunos compuestos volátiles producidos a lo largo del proceso de fermentación (Gamero et al., 2013; Killian and Ough, 1979; Kopsahelis et al., 2012). Además, el crecimiento de algunas bacterias se ve afectado negativamente a bajas temperaturas, y, en consecuencia, producen menos metabolitos indeseables durante la fermentación alcohólica (Ribéreau-Gayon et al., 2006).

2.2.3. SO_2

La adición de dióxido de azufre (SO_2) es una práctica común para evitar contaminaciones microbianas. El SO_2 entra en la célula por difusión y, una vez en el citosol, se disocia y reacciona con enzimas, coenzimas y vitaminas. Como resultado, el crecimiento celular se detiene y finalmente la célula muere (Ribereau-Gayon et al., 2006).

Se ha demostrado que las cepas de *S. cerevisiae* son bastante tolerantes al SO_2 en general, en comparación con otras especies, aunque muestran una tolerancia al SO_2 muy diversa (Divol et al., 2006; Nardi et al., 2010; Pilkington and Rose, 1988; Warth, 1985). Entre las levaduras no-*Saccharomyces* también hay mucha variedad entre las especies que toleran o no el SO_2, destacando cepas de las especies *Zygosaccharomyces bailii* (Divol et al. 2006), *Schizosaccharomyces pombe* (Stratford et al., 1987) y de *L. thermotolerans* (Comitini et al., 2011).

2.3. Interacciones entre levaduras

Como se ha mencionada anteriormente, el interés por las levaduras no-*Saccharomyces* ha aumentado debido a su contribución positiva a la calidad del vino. La estrategia de utilizarlas en fermentaciones mixtas junto a *S. cerevisiae* implica la existencia de interacciones entre ellas. Es por ello que en los últimos años se ha intensificado la investigación sobre las interacciones entre las levaduras.

Existen diferentes tipos de interacciones que pueden darse entre las levaduras (Figura 9). Por un lado estan las interacciones que pueden tener

efectos positivos en la población, como el mutualismo y el comensalismo. Entre ellas se encuentran las interacciones por las que un metabolito o producto sintetizado por una levadura es utilizado por otra levadura que se beneficia. Por otro lado, estan las que tienen efectos negativos, como la competencia y el amensalismo. Entre estas estan la competencia por nutrientes y la producción de compuestos antimicrobianos (Zilelidou and Nisioutou, 2021).

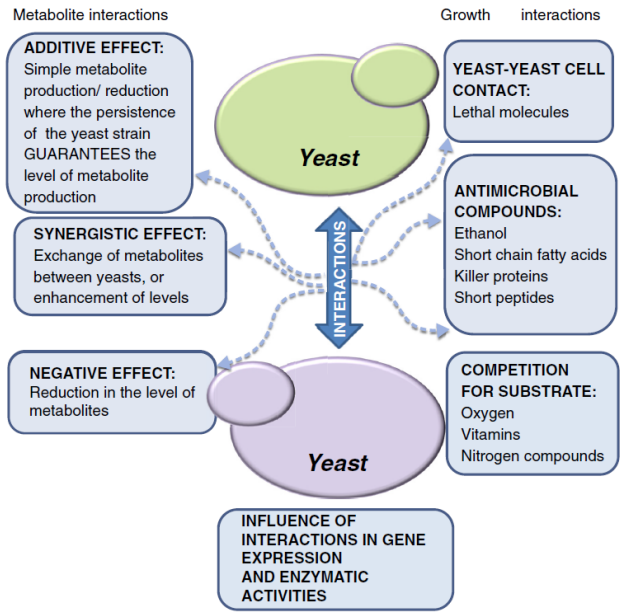

Figura 9. Tipos de interacciones entre levaduras(Ciani and Comitini, 2015).

También pueden clasificarse como interacciones directas, donde estarían las interacciones de contacto célula-célula que se deben al contacto físico entre las células, y indirectas, que englobarían aquellas interacciones que tienen un efecto derivado de otro, como por ejemplo la muerte por déficit de nutrientes debido a que otra especie lo ha consumido.

Cabe mencionar que también pueden diferenciarse las interacciones entre levaduras no-*Saccharomyces* (NS-NS) de las interacciones entre levaduras no-*Saccharomyces* y *S. cerevisisae* (NS-Sc). Estas se encuentran resumidas en la Tabla 4.

2.3.1. Competencia por nutrientes

Durante la fermentación, las levaduras compiten entre ellas para consumir nutrientes. La disponibilidad de nutrientes puede influir en la dinámica poblacional a lo largo de la fermentación, así como en el rendimiento de ésta (Bisson, 1999).

Aunque algunas levaduras no-*Saccharomyces* con actividad proteolítica pueden enriquecer el medio (Ciani et al., 2015), en general, su crecimiento resulta en un empobrecimiento del medio, ya que pueden consumir nutrientes esenciales para *S. cerevisiae*. En inoculaciones secuenciales, este efecto es mayor que en co-inoculación, ya que, en éstas últimas, al inocularse ambas especies simultáneamente, *S. cerevisiae* puede afectar negativamente al crecimiento de las cepas no-*Saccharomyces* desde el inicio de la fermentación (Medina et al., 2012; Taillandier et al., 2014).

Tabla 4. Interacciones entre diferentes especies de levaduras Referencias: 1: Peña et al. (2020); 2: Johnson et al. (2020); 3: Seguinot et al. (2020); 4: Melvydas et al. (2020); 5: Valera et al. (2019); 6: Bagheri et al. (2019); 7: Yan et al. (2020); 8: Branco et al. 2014; 9: Pietrafesa et al. (2020); 10: Sgouros et al. 2020; 11: Kemsawasd et al. 2015b; 12: Renault et al. 2016; 13: Brou et al. 2018; 14: Shekhawat et al. 2017; 15: Branco et al. 2014 (adaptada de Zilelidou and Nisiotou, (2021)).

Tipo de interacción	Levaduras * involucradas	Descripción de la interacción	Referencias
NS-NS	Ci-Db	Ci produce péptidos contra Db	1
	Ci-Pg	Ci produce péptidos contra Pg	1
	Lt-Hu	La presencia de Lt inhibe el crecimiento de Hu	2
NS-Sc	Mp-Sc	Mp modifica el balance NAD^+/NADH	3
		Mp consume el hierro del medio	4
	Sb-Sc	Cambios en la dinámica poblacional según las concentraciones de triptofol y melatonina	5
	Hv-Sc	Incremento de la supervivencia de Hv en presencia de Sc	6
		Hv consume el oxígeno del medio	7
	Hu-Sc	Sc produce péptidos contra Hu	8
		Hu pierde viabilidad en contacto con Sc	9
	Lt-Sc	Sc probablemente consume nutrientes más rápido que Lt	10
		Lt pierde viabilidad en contacto con Sc	11
	Td-Sc	Td libera Cys-$3SH^2$ que es captado por Sc	12
		Td estimula el crecimiento de Sc por contacto	13
		Cambios en la dinámica poblacional según las concentraciones de triptofol y melatonina	5
		Td consume oxígeno y produce biomasa	14
		Sc produce péptidos contra Td	15

*Sc, *Saccharomyces cerevisiae*; Hu, *Hanseniaspora uvarum*; Hv, *Hanseniaspora vineae*:; Mp, *Metschnikowia pulcherrima*; Td, *Torulaspora delbrueckii*; Sb, *Starmerella bacillaris*; Lt, *Lachancea thermotolerans*; Ci, *Candida intermedia*; Pg, *Pichia guilliermondii*; Db, *Dekkera bruxellensis*;. NS, no-*Saccharomyces*.

La competencia por compuestos nitrogenados entre levaduras no-*Saccharomyces* y *S. cerevisiae* (NS-Sc) fue de las primeras hipótesis a tenerse en cuenta, y a lo largo de los años, diferentes estudios la han confirmado (Medina et al., 2012; Taillandier et al., 2014; Lleixà et al., 2016; Renault et al., 2016; Wang et al., 2016; Gobbert et al., 2017; Rollero et al., 2018a; Petitgonnet et al., 2019; Binati et al., 2020). También se ha visto que la deficiencia de otros tipos de nutrientes como las vitaminas (especialmente la tiamina) (Medina et al., 2012; Maisonnave et al. 2013; Rollero et al. 2018a), minerales (como el zinc) (Walker, 2004; Maisonnave et al. 2013) y factores de anaerobiosis (Brou et al., 2018) se asocia a paradas de fermentación y fermentaciones lentas, por lo que se da una competencia entre las levaduras no-*Saccharomyces* y *S. cerevisiae* por consumir estos nutrientes.

2.3.2. Compuestos antimicrobianos

El crecimiento de las levaduras puede verse afectado negativamente por la liberación de compuestos tóxicos o antimicrobianos, como dióxido de azufre, etanol, ácidos grasos de cadena media y toxinas killer, por parte de otras levaduras (Bisson, 1999).

Por un lado, *S. cerevisiae* produce etanol, un metabolito con efecto tóxico para otras especies. Así pues, esta levadura es capaz de dominar la fermentación hacia la mitad y el final del proceso, es decir, a medida que aumenta el contenido de etanol (Ciani and Comitini, 2015). Por otro lado, los ácidos octanoico y decanoico inhiben el crecimiento de las levaduras ya

que disminuyen la tasa máxima de crecimiento y el rendimiento de biomasa durante la fermentación alcohólica (Viegas et al., 1989).

Las levaduras pueden secretar toxinas proteicas que pueden matar cepas sensibles a ellas. El fenotipo asesino o killer ha sido ampliamente descrito en *S. cerevisiae*. Existen levaduras que presentan el fenotipo neutro, es decir, son resistentes, pero no producen la toxina, y levaduras con fenotipo sensible, que son sensibles a la toxina que producen otras levaduras. Las cepas killer a su vez pueden también morir a causa de una toxina diferente a la que ellos producen y a la que no tienen resistencia. El fenotipo killer está codificado en un virus dsARN. Hay dos tipos de genomas víricos (VLP). Uno es el genoma M (1.3–1.9 kb) que codifica la toxina K y el factor de inmunidad que aporta resistencia. El otro es el genoma L (4.5 kb) que codifica una ARN polimerasa y la cápside proteica que encapsula los dos genomas. Ambas partículas virales (M y L) son necesarias para que la levadura exprese el fenotipo killer, ya que el genoma L es necesario para el mantenimiento del M. Se han estudiado los VLP correspondientes a los diferentes fenotipos que puede poseer *S. cerevisiae:* K1, K2, K28 y Klus. Cada uno de ellos codifica para la toxina killer de su mismo nombre y su respectiva autoinmunidad. En condiciones enológicas, solamente la toxina K2 es activa (porque está activa en un rango de pH (2.8-4.8) que incluye el pH del vino). Esta toxina es una glicoproteína de 16 kDa que ataca a las células sensibles al unirse a un receptor en la pared celular. Las células sensibles se ven afectadas por varias alteraciones funcionales como la interrupción del transporte acoplado de aminoácidos y protones, la acidificación del contenido celular y la fuga de potasio y ATP, lo que da

lugar a la formación de poros en la membrana plasmática y posterior muerte celular (Ribereau-Gayon et al., 2006; Rodríguez-Cousiño et al., 2011).

La secreción de toxinas killer no es una habilidad exclusiva de *S. cerevisiae*. Algunas cepas de las especies *T. delbrueckii* (Sangorrin et al., 2007; Sangorrin et al., 2008; Ramírez et al., 2015; Velázquez et al., 2015), *P. kluyveri* (Labbani et al., 2015; Middelbeek et al., 1980), *H. uvarum* (Mendoza et al., 2019) y *P. membranifaciens* (Santos et al., 2009) también son capaces de producirlas.

En la última década se ha descubierto que las levaduras pueden secretar otro tipo de proteínas con propiedades antimicrobianas. Por un lado, Pérez-Nevado et al. (2006) sugirieron que *S. cerevisiae* era capaz de producir uno o más compuestos tóxicos (que no fueron identificados) que provocaban la muerte temprana de *H. guilliermondii* en cultivos mixtos. Por otro lado, Albergaria et al. (2010) descubrieron que la cepa *S. cerevisiae* CCMI 885 era capaz de secretar una pequeña fracción proteica conocida como péptidos antimicrobianos (AMP) que inhibía el crecimiento de algunas levaduras no-*Saccharomyces*. Más tarde se vio que estos péptidos corresponden a fragmentos de la proteína gliceraldehído 3-fosfato deshidrogenasa (GAPDH) (Branco et al., 2014; Branco et al., 2017). Recientemente, se ha descrito la posible participación de vesículas extracelulares en las interacciones entre levaduras durante la vinificación. Concretamente se observó que unas fracciones extracelulares de *T. delbrueckii* tenían un

efecto negativo en el crecimiento de otras especies de levaduras como *S. cerevisiae* (Mencher et al., 2020).

2.3.3. Contacto célula-célula

La densidad celular tiene importantes funciones reguladoras en el crecimiento de las células en eucariotas superiores. Por medio del contacto entre células, conocido como "contacto célula-célula", las células en crecimiento saben qué hacer, es decir, si continuar o detener su crecimiento (Caveda et al., 1996; Fiore and Degrassi, 1999; Hirano et al., 2001). En las levaduras, el control de la densidad celular (así como los cambios morfológicos, la formación de biopelículas y la patogénesis), está relacionado con la secreción de ciertas moléculas que son reconocidas por otras levaduras de manera que modifican su comportamiento. Este tipo de comunicación entre las levaduras es lo que se conoce como quorum sensing (Fuqua et al., 1994; Wuster and Babu, 2008).

Se han observado interacciones por contacto célula-célula en las levaduras durante la fermentación. Nissen et al. (2003) observaron que una elevada población de células de *S. cerevisiae* afectaba negativamente el crecimiento de *L. thermotolerans* durante una fermentación coinoculada. Se descartaron la limitación de nutrientes y la acumulación de compuestos inhibidores del crecimiento como posibles responsables de esta detención temprana del crecimiento de *L. thermotolerans*. La causa propuesta fue el mecanismo de contacto célula-célula dependiente de altas concentraciones de células viables de *S. cerevisiae* (Nissen et al., 2003). Años después, se demostró que la muerte de *L. thermotolerans* en cultivos mixtos con *S.*

cerevisiae era causada por una combinación de contacto célula-célula y péptidos antimicrobianos (Kemsawasd et al., 2015b). Más tarde, Petitgonnet et al. (2019) observaron que el contacto célula-célula entre *L. thermotolerans* y *S. cerevisiae* modificaba el metabolismo de las levaduras haciendo que el perfil de compuestos volátiles fuera significativamente diferente que el obtenido en la fermentación secuencial sin contacto célula-célula. También se confirmó el efecto negativo del mecanismo por contacto célula-célula entre *S. cerevisiae* y *T. delbrueckii*, donde el contacto entre estas especies indujo la muerte temprana de *T. delbrueckii* (Renault et al., 2013). Actualmente se siguen realizando estudios para profundizar el conocimiento sobre este tipo de interacciones.

Resultados

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Elena Roca Mesa

Capítulo I

Estudio de los requerimientos nitrogenados de levaduras no-Saccharomyces y su impacto sobre el crecimiento, el desarrollo de la fermentación y el vino final.

Capítulo I. 1

"Nitrogen preferences during alcoholic fermentation of different non-*Saccharomyces* yeasts of oenological interest"

Capítulo I. 2

"The effect of must nitrogen composition on yeast growth and volatile thiol release in sequential fermentations "

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Capítulo I. 1

Nitrogen preferences during alcoholic fermentation of different non-*Saccharomyces* yeasts of oenological interest

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Abstract:

Non-*Saccharomyces* yeasts have long been considered spoilage microorganisms. Currently, oenological interest in those species is increasing, mostly due to their positive contribution to wine quality. In this work, the fermentative capacity and nitrogen consumption of several non-*Saccharomyces* wine yeast (*Torulaspora delbrueckii, Lachancea thermotolerans, Starmerella bacillaris, Hanseniaspora uvarum,* and *Metschnikowia pulcherrima*) were analyzed. For this purpose, synthetic must with three different nitrogen compositions was used: a mixture of amino acids and ammonium, only organic or inorganic nitrogen. The fermentation kinetics, nitrogen consumption, and yeast growth were measured over time. Our results showed that the good fermentative strains, *T. delbrueckii* and *L. thermotolerans*, had high similarities with *Saccharomyces cerevisiae* in terms of growth, fermentation profile, and nitrogen assimilation preferences, although *L. thermotolerans* presented an impaired behavior when only amino acids or ammonia were used, being strain-specific. *M. pulcherrima* was the non-*Saccharomyces* strain least affected by the nitrogen composition of the medium. The other two poor fermentative strains, *H. uvarum* and *S. bacillaris*, behaved similarly regarding amino acid uptake, which occurred earlier than that of the good fermentative species in the absence of ammonia. The results obtained in single non-*Saccharomyces* fermentations highlighted the importance of controlling nitrogen requirements of the wine yeasts, mainly in sequential fermentations, in order to manage a proper nitrogen supplementation, when needed.

Keywords: wine fermentation; *Torulaspora delbrueckii*; *Lachancea thermotolerans; Starmerella bacillaris*; *Hanseniaspora uvarum; Metschnikowia pulcherrima*; amino acids; ammonium

1. Introduction

Wine is a common product that has been consumed since antiquity and is the result of several biochemical reactions produced principally by yeasts. During alcoholic fermentation, yeasts transform sugars into ethanol and carbon dioxide. Additionally, several metabolites are involved in this process and are present in the final product, contributing to the final quality and complexity of the wine (Ribéreau-Gayon et al., 2006). Although the main yeast responsible for wine fermentation is *Saccharomyces cerevisiae*, there are also non-*Saccharomyces* yeast species involved in the process. Those non-*Saccharomyces* species can be found on the grape surface and, for many years, have been considered spoilage microorganisms. Currently, it has been demonstrated that some non-*Saccharomyces* yeasts can positively contribute to the organoleptic profile of wines, producing volatile compounds not produced by *Saccharomyces* strains, consequently providing different characteristics to the final product (Albertin et al., 2017; Andorrà et al., 2010; Lleixà et al., 2016; Renault et al., 2016). Therefore, the use of different non-*Saccharomyces* species in mixed fermentation with *S. cerevisiae* is a good alternative to improve certain wine characteristics. For example, it has been shown that *Torulaspora delbrueckii* enhances the complexity and fruity notes of wines (Lleixà et al., 2016), *Hanseniaspora vineae* enriches wines with fruity and flowery aromas (Renault et al., 2015), *Lachancea thermotolerans* increases the total acidity (Gobbi et al., 2013), and *Metschnikowia pulcherrima* reduces the ethanol levels and enhances varietal aromas (Contreras et al., 2014; Quirós et al., 2014). However, to optimize the use of non-*Saccharomyces* yeasts in sequential and

coinoculated fermentations with *Saccharomyces* spp., it is necessary to better understand their metabolism and nutrient requirements. During a sequential inoculation, the initial consumption of nutrients by non-*Saccharomyces* yeasts could affect the growth and survival of *Saccharomyces* yeasts, inoculated later (Lleixà et al., 2016; Medina et al., 2012; Rollero et al., 2018a).

Nitrogen compounds are the nutrients mostly assimilated by yeasts, after carbon compounds, during alcoholic fermentation. They are involved in the metabolism and growth of yeasts, affecting the correct evolution of the fermentation (Bisson, 1999; Ribéreau-Gayon et al., 2006), and the production of volatile compounds (Carrau et al., 2008; Fairbairn et al., 2017; González et al., 2018). In grape musts, nitrogen composition can be highly variable, both in concentration and in the types of nitrogen compounds present (Jiranek et al., 1995). Furthermore, only some of these nitrogen compounds, known as yeast assimilable nitrogen (YAN), are metabolized by yeast. In *S. cerevisiae,* YAN sources include amino acids and ammonia (Bell and Henschke, 2005). YAN can be classified into two categories: preferred and non-preferred nitrogen sources (Gobert et al., 2017). The preferred nitrogen sources, such as ammonia, glutamine, and asparagine, promote yeast growth. Instead, non-preferred sources, such as proline and urea, result in low growth when they are the only nitrogen sources. Nitrogen deficiencies are the common causes of sluggish or stuck fermentation (Bisson, 1999; Butzke and Bisson, 2000; Ingledew and Kunkee, 1985). For this reason, in some cases, natural musts are supplied with external nutrients, typically ammonium salts, to avoid fermentative

problems (Martínez-Moreno et al., 2014). However, Gutiérrez et al. (2012) showed that different commercial *S. cerevisiae* strains had different nitrogen needs and proposed the use of supplements containing organic nitrogen sources.

While nitrogen consumption and preferences are well researched in *S. cerevisiae* (Bell et al., 1979; Beltran et al., 2005; Gobert et al., 2019; Gutiérrez et al., 2012; Jiménez-Martí et al., 2007; Jiranek et al., 1995; Mendes-Ferreira et al., 2004; Monteiro and Bisson, 1991; Torija et al., 2003; Vilanova et al., 2007), nutrient uptake by non-*Saccharomyces* yeasts has not been extensively studied. Available studies have analyzed the preferential nitrogen sources of some non-*Saccharomyces* yeasts, focusing on their capacity to assimilate different nitrogen compounds, their rate of consumption, or their influence in aroma production (Englezos et al., 2018; Gobert et al., 2017; Kemsawasd et al., 2015a; Lleixà et al., 2016; Prior et al., 2019; Rollero et al., 2019; Rollero et al., 2018a; Seguinot et al., 2020; Su et al., 2020). The firsts studies reported that the nitrogen sources directly affect the fermentation performance and yeast growth in a species-specific manner (Andorrà et al., 2012; Kemsawasd et al., 2015a). Later, some studies have revealed that non-*Saccharomyces* yeasts have specific profiles for amino acid consumption, concluding that the different nitrogen composition of the media strongly influences the assimilation order of each compound, both in natural (Gobert et al., 2017) and synthetic must (Rollero et al., 2019; Rollero et al., 2018b; Su et al., 2020).

Different studies have analyzed the possible competition for nutrients between *Saccharomyces* and non-*Saccharomyces* yeasts (Alonso-del-Real

et al., 2019; Curiel et al., 2017; Medina et al., 2012; Rollero et al., 2018a; Taillandier et al., 2014). Rollero et al. (2018a) observed different consumption profiles of *S. cerevisiae* depending on the non-*Saccharomyces* yeasts used in the sequential culture, suggesting that this behavior could be explained by the competition for nutrients. Moreover, recent studies have demonstrated that under conditions of co-cultivation with some non-*Saccharomyces* species, *S. cerevisiae* partially relieves the nitrogen and glucose catabolite repression, in order to increase the flux of nutrients and reduce their availability for other yeast species (Rollero et al., 2018a; Taillandier et al., 2014). Indeed, the presence of other *Saccharomyces* species, such as *Saccharomyces kudriavzevii,* can also produce metabolic stimulation in *S. cerevisiae* (Alonso-del-Real et al., 2019).

Thus, the increasing popularity of non-*Saccharomyces* yeasts in winemaking makes it necessary to know more about them, especially about their nitrogen preferences. With this knowledge, winemakers can improve non-*Saccharomyces* implementation and avoid problems during alcoholic fermentation. Some of these non-*Saccharomyces* species, such as *Torulaspora delbrueckii*, *Lachancea thermotolerans,* and *Metschnikowia pulcherrima,* are already on the market as active dry yeast (Prior et al., 2019), but other species with great potential in the enological industry are still being evaluated.

The aim of this study was to analyze the nitrogen consumption and fermentative kinetics of commercial and non-commercial non-*Saccharomyces* yeasts. We performed fermentations on a laboratory scale using synthetic must with different nitrogen sources: only amino acids, only

ammonium, or a combination of amino acids and ammonium. The fermentation kinetics, yeast growth, and nitrogen consumption were monitored throughout the fermentation, comparing the results of these non-*Saccharomyces* with those of a commercial *S. cerevisiae* strain used as a control.

2. Materials and Methods

2.1. Yeast strains

Six yeast species were used in this study. The non-*Saccharomyces* strains were *Torulaspora delbrueckii* Viniferm NS-TD (Agrovin S.A., Spain) (Td), *Lachancea thermotolerans* Lt2 provided by Agrovin S.A. (Lt), *Hanseniaspora uvarum* CECT 1444 (from Spanish Type Culture Collection) (Hu), and *Metschnikowia pulcherrima* CECT 13131 (Mp) and *Starmerella bacillaris* CECT 13129 (Sb) isolated from Priorat DOQ (Qualified Designation of Origin) (URV collection). *Saccharomyces cerevisiae* Viniferm Revelación (Agrovin S.A.) (Sc) was used as a control. To confirm the nitrogen preferences of *L. thermotolerans,* other strains were analyzed: Laktia (Lallemand S.L.) (LtL), Lt1 provided by Agrovin S.A. (Lt1) and *L. thermotolerans* ICVV1131 (Lt78), ICVV1132 (Lt79) and ICVV1133 (Lt80), from the ICVV collection (Logroño, Spain).

All strains were preserved in YPD liquid medium (2% (w/v) glucose, 2% (w/v) bacto peptone, and 1% (w/v) yeast extract; Cultimed, Barcelona, Spain) with 40% (v/v) glycerol, at −80 °C. Before their use, they were streaked on YPD agar plates (YPD liquid with 2% (w/v) agar). Isolated colonies from these pure cultures were grown in YPD at 28 °C and 120 rpm in an orbital shaker for 24 h and used as a preculture for inoculating the

fermentations. The microscopic counting of the cells by a Neubauer chamber was used to calculate the cell concentration in the precultures.

2.2. Fermentation conditions and sampling

Single inoculum fermentations were performed in synthetic must, as described in Beltran et al. (2004) (Supplementary Table S1). The initial nitrogen content was 300 mg N/L in all cases (Supplementary Table S2), but different nitrogen compositions were used: 1) the control condition with 40% ammonium and 60% amino acids (SM-Mix), 2) amino acids as a sole nitrogen source (SM-AA), and 3) only ammonium (SM-NH_4^+). Synthetic must was inoculated to a final concentration of 2×10^6 cells/mL, for each yeast species: *S. cerevisiae* (Sc), *T. delbrueckii* (Td), *L. thermotolerans* (Lt), *H. uvarum* (Hu), *M. pulcherrima* (Mp), and *S. bacillaris* (Sb). Fermentations were performed in triplicate at 22 °C and 120 rpm in 250 mL borosilicate glass bottles containing 220 mL of medium and capped with closures that enabled carbon dioxide to escape and samples to be removed. Fermentations were conducted under semi-anaerobic conditions since some aeration was necessary for harvesting samples for subsequent analysis.

Fermentation kinetics were monitored by measuring the daily must density with an electronic densimeter (Densito 30PX Portable Density Meter (Mettler Toledo, España)), total yeast population by optical density at 600 nm (OD_{600}), and viable yeast population by plating serial dilutions of samples on YPD and Wallerstein laboratory nutrient agar (WLN) medium (DifcoTM, Sparks, Nevada, USA). We selected the endpoint of fermentation when sugars were below 2 g/l, or in the case of stuck or

sluggish fermentations, samples at 240 h were collected as end-point. For the analysis of the nitrogen compounds or organic metabolites, 1.5 mL of the supernatant was collected at 12, 24, 48, and 72 h and at the endpoint of fermentation (216 or 240 h) and stored at −20 °C, until analysis. Fermentations with different Lt strains were followed by lost weight in 40 mL of each medium.

2.3. Nitrogen analysis

The nitrogen content was analyzed by HPLC (high-performance liquid chromatography), according to the method of Gómez-Alonso et al. (2007). The HPLC (Agilent 1100, Agilent Technologies, Germany) was equipped with a DAD ultraviolet detector and a fluorescence detector (Agilent Technologies, Germany), and separation was performed on a Hypersil ODS C18 column (Agilent Technologies, Germany) with a particle size of 5 μm (250 mm × 4.6 mm) and thermostated at 20 °C. The mobile phase (A) consisted of 2.05 g/L of sodium acetate anhydrous and 0.2 g/L of sodium azide with MilliQ water (Millipore Q-PODTM Advantage A10) adjusted to pH 5.8 with glacial acetic acid, and mobile phase (B) consisted of 80% (v/v) acetonitrile and 20% (v/v) methanol. Chromatograms were analyzed using Agilent ChemStation Plus software (Agilent Technologies, Germany). Amino acid and ammonium concentrations were transformed into yeast assimilable nitrogen (YAN, expressed as mg N/L) according to the nitrogen atoms of each amino acid. The ammonia concentration of SM-$NH4^+$ was also analyzed with a multi analyzer Miura One (TDI, Barcelona, España), using an ammonia nitrogen enzymatic kit (TDI, Barcelona, España).

2.4. Metabolite analysis

The concentration of organic metabolites (glucose, fructose, glycerol, ethanol, and acetic acid) was determined at 48 and 240 h (216 h in Sc), following the protocol described by Quirós et al. (2010), using an Agilent 1100 HPLC (Agilent Technologies, Germany) equipped with a Hi-Plex H, 300 mm x 7.7 mm column inside a 1260 MCT (Infinity II Multicolumn Thermostat) connected to both an MWC and an RID (G1365B multi-wavelength detector and 1260 Infinity II refractive index detector) (Agilent Technologies, Germany). The column was maintained at 60 °C, and 5 mM H_2SO_4 was used as the mobile phase at a flow rate of 0.6 mL/min. Previously, samples were filtered through 0.22 µm pore size filters (Dominique Dutscher, Brumath, France).

2.5. Statistical analysis

Data are expressed as the mean and standard deviation of triplicates as data points. ANOVA and Tukey's test analyses using XLSTAT 2019 software (Addinsoft, New York, New York, USA) were performed to determine significant differences between different nitrogen sources, media, and strains. The results were considered statistically significant at a *p-value* of less than 0.05. The GraphPad Prism 7 program (GraphPad Software, San Diego, California, USA) was used for graphical data modeling.

3. Results

3.1. Fermentation kinetics

Single fermentations with the five non-*Saccharomyces* species (*T. delbrueckii* (Td), *L. thermotolerans* (Lt), *H. uvarum* (Hu), *M. pulcherrima* (Mp), and *S. bacillaris* (Sb)), as well as a *S. cerevisiae* (Sc) strain as a control,

were performed in synthetic must with different nitrogen sources: inorganic nitrogen only (SM-NH_4^+), organic nitrogen only (SM-AA), and a mixture of organic and inorganic nitrogen (SM-Mix) (Figure 1).

Among the non-*Saccharomyces* strains, only Td was able to finish the fermentation under all tested nitrogen conditions (10 days for all of them), although the best growth was obtained in SM-Mix. Lt growth and fermentation were impaired in SM-NH_4^+ and SM-AA. In these cases, the fermentations were sluggish and stuck at approximately 1040 g/L density, while the fermentation was complete in 10 days in the SM-Mix condition. For Hu and Mp, although cell growth was favored in SM-NH_4^+, the fermentations were stuck in all conditions at a similar density (between 1020 and 1040 g/L). Sb was not able to finish the fermentation under any of the nitrogen conditions, and the least amount of sugars were consumed in the SM-Mix fermentation. As expected, the fermentations with Sc were faster than those with the non-*Saccharomyces* strains finishing in 7 days for SM-Mix and in 9 days for SM-AA and SM-NH_4^+ (Figure 1).

We observed significant differences in growth between media in Lt, Td, and Hu (Table 1), being SM-Mix the best medium for Td and Lt growth, and SM-NH_4^+ the best for Hu. Moreover, Td was the non-*Saccharomyces* strain that grew best in all media, and Hu and Sb the ones with the lowest growth.

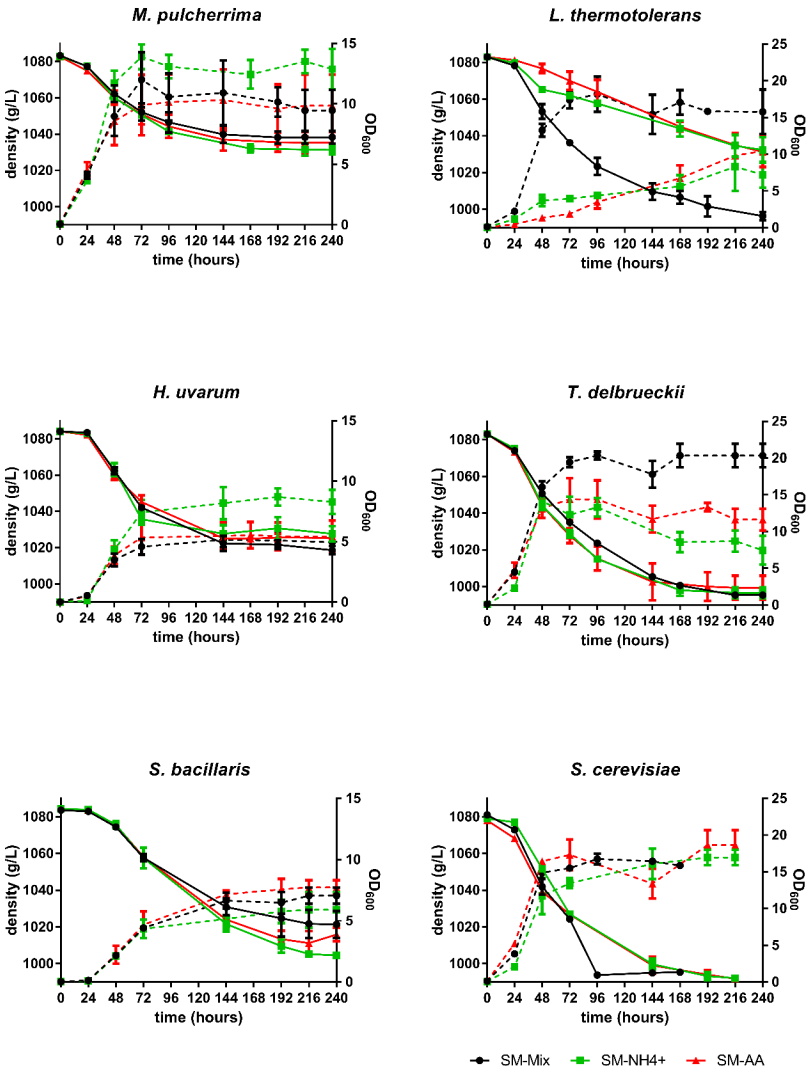

Figure 1. Fermentation kinetics and yeast growth of each species in synthetic must with different nitrogen sources: inorganic nitrogen only (SM-NH_4^+), organic nitrogen only (SM-AA), and a mixture of organic and inorganic nitrogen (SM-Mix). Solid lines refer to density and dotted lines refer to OD_{600}. Error bars represent standard deviation.

Table 1. Maximum growth (expressed as OD_{600}) during single fermentations under three nitrogen conditions (SM-Mix, SM-NH_4^+, and SM-AA), for each yeast species: *M. pulcherrima* (Mp), *L. thermotolerans* (Lt), *T. delbrueckii* (Td), *H. uvarum* (Hu), *S. bacillaris* (Sb), and *S. cerevisiae* (Sc).

	SM-Mix	SM-NH_4^+	SM-AA
Mp	12.00 ± 2.27 ^{A}	13.91 ± 1.03 ^{A}	10.33 ± 2.54 ^{A}
Lt	18.17 ± 1.96 ^{A}	8.29 ± 3.27 ^{B}	10.45 ± 2.15 ^{B}
Td	20.67 ± 0.56 ^{A}	14.13 ± 0.60 ^{B}	14.40 ± 2.87 ^{B}
Hu	5.16 ± 0.51 ^{B}	8.71 ± 0.71 ^{A}	5.47 ± 1.12 ^{B}
Sb	7.06 ± 0.67 ^{A}	5.91 ± 1.45 ^{A}	7.73 ± 0.59 ^{A}
Sc	16.76 ± 0.70 ^{A}	16.95 ± 1.05 ^{A}	18.65 ± 2.03 ^{A}

Capital letters indicate significant differences in each species between the three media. SM-Mix: a mixture of organic and inorganic nitrogen; SM-NH_4^+: inorganic nitrogen only; AM-AA: organic nitrogen only.

The species most affected by the nitrogen composition in the medium was Lt, as it presented good growth in the SM-Mix but poor growth when only ammonia or only amino acids were present, which was related to the sluggish fermentation observed under these conditions (Figure 1, Table 2). To verify this profile, additional strains of *L. thermotolerans* were analyzed (Figure 2). Our results showed that nitrogen preferences were strain-dependent, as some of the strains exhibited better growth in SM-Mix (Lt2 and Lt79) and others in the medium with only amino acids (SM-AA, Lt78, and LtL). In general, ammonium was the nitrogen source that resulted in a low growth, except for the strain Lt80, which also fermented faster in this medium (Supplementary Figure S1).

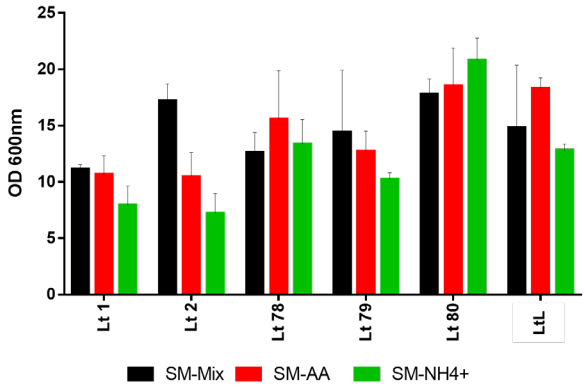

Figure 2. Maximal growth (expressed as OD_{600}) of different *L. thermotolerans* strains at the end of fermentation in synthetic must with different nitrogen sources. Error bars represent standard deviation. * Indicates statistically significant differences between media ($p < 0.05$).

3.2. Nitrogen consumption and preferences

Nitrogen consumption was measured throughout the different experimental fermentations (Figure 3). Sc, Td, and Sb showed similar consumption profiles, regardless of the nitrogen composition of the medium. Sc and Td depleted the nitrogen in 48 h, while Sb consumed most of the YAN during the first 72 h.

The other three species exhibited different consumption profiles of the YAN present in the media, depending on its composition. Lt depleted all YAN but showed different rates, with the fastest in SM-Mix and the slowest in SM-AA. On the other hand, none of the fermentations performed with Mp were able to consume all of the nitrogen, with residual nitrogen levels in the medium between 60 mg/L YAN (SM-NH_4^+) and 120 mg/L YAN (SM-Mix and SM-AA). Surprisingly, Hu consumed much less nitrogen in SM-Mix than in the other two media, although this difference was not reflected in the fermentation or growth kinetics (Figure 1).

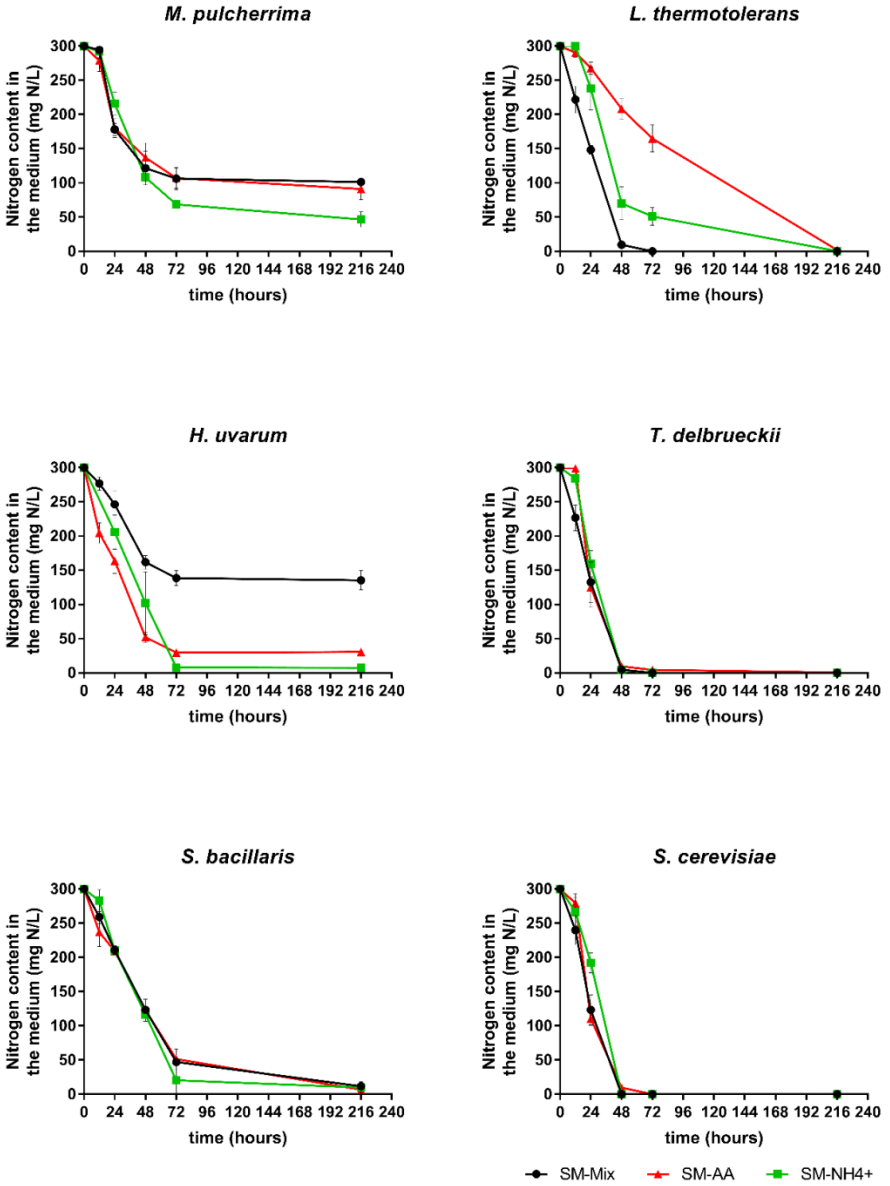

Figure 3. Nitrogen consumption (mg N/L) profiles of single fermentations in three different media: SM-Mix, SM-AA, and SM-NH_4^+. Error bars represent standard deviation.

The preference between organic and inorganic nitrogen in SM-Mix differed among the species (Figure 4). Inorganic and organic nitrogen were similarly

consumed by Sc, Td, and Lt, depleting all the nitrogen in less than 48 h. In contrast, the other three species (Mp, Hu, and Sb) consumed less than 200 mg N/L in the same period, with Hu being the strain with the lowest YAN uptake, and Sb the strain with the lowest amino acid consumption in 48 h.

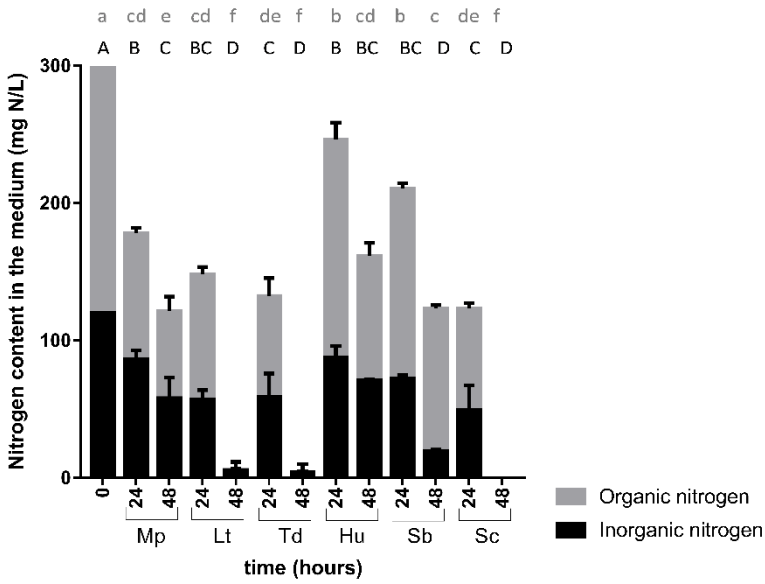

Figure 4. Organic and inorganic nitrogen (mg N/L) present in the medium at 0, 24, and 48 h during single fermentations in SM-Mix. Capital letters indicate significant differences in inorganic nitrogen levels. Lowercase letters indicate significant differences in organic nitrogen levels.

To better understand the amino acid preferences of each species and how the presence of ammonium could modulate their uptake, we compared the amino acid consumption patterns of different species in the presence or absence of ammonium (Figure 5, Supplementary Table S3 and S4).

As expected, the amino acid consumption profile in SM-Mix differed from that in SM-AA. In SM-Mix, Td and Lt behaved similarly to Sc and were the fastest non-*Saccharomyces* species to consume all amino acids. Td and Lt

first consumed tyrosine, methionine, cysteine, and isoleucine (in less than 12 h). Additionally, the consumption of aspartic acid, histidine, glycine, and alanine occurred mostly when ammonium was exhausted from the media (at 48 h, Figure 6). Surprisingly, in SM-AA, no amino acid uptake was observed during the first 12 h for Td and Lt (except for histidine). In this medium, Lt exhibited a strong delay in amino acid consumption, requiring more than 72 h for its depletion, except for glycine, which was not completely consumed under this condition. Indeed, the uptake of this amino acid was also delayed in Td, with its consumption beginning at 72 h. As mentioned above, Sb was able to use all nitrogen in all media, while Hu fermentation had higher residual nitrogen levels in SM-Mix (135.36 ± 11.46 mg N/L) than in the other fermentations (Figure 3). Nevertheless, both species presented a similar amino acid consumption profile in both media, with depletion of isoleucine in less than 12 h, and a relatively fast amino acid uptake in the absence of ammonia (SM-AA). In fact, when ammonium was present, practically half of the organic nitrogen (mainly formed by alanine, glutamine, and arginine) remained in the medium in the fermentations with Hu, which even excreted some amino acids, such as alanine and histidine (Supplementary Table S3). In the fermentations with Sb, we also observed a delay in the uptake of the amino acids, probably linked to the presence of ammonia. The least consumed amino acids by this species were glycine, tyrosine, aspartic acid, and valine.

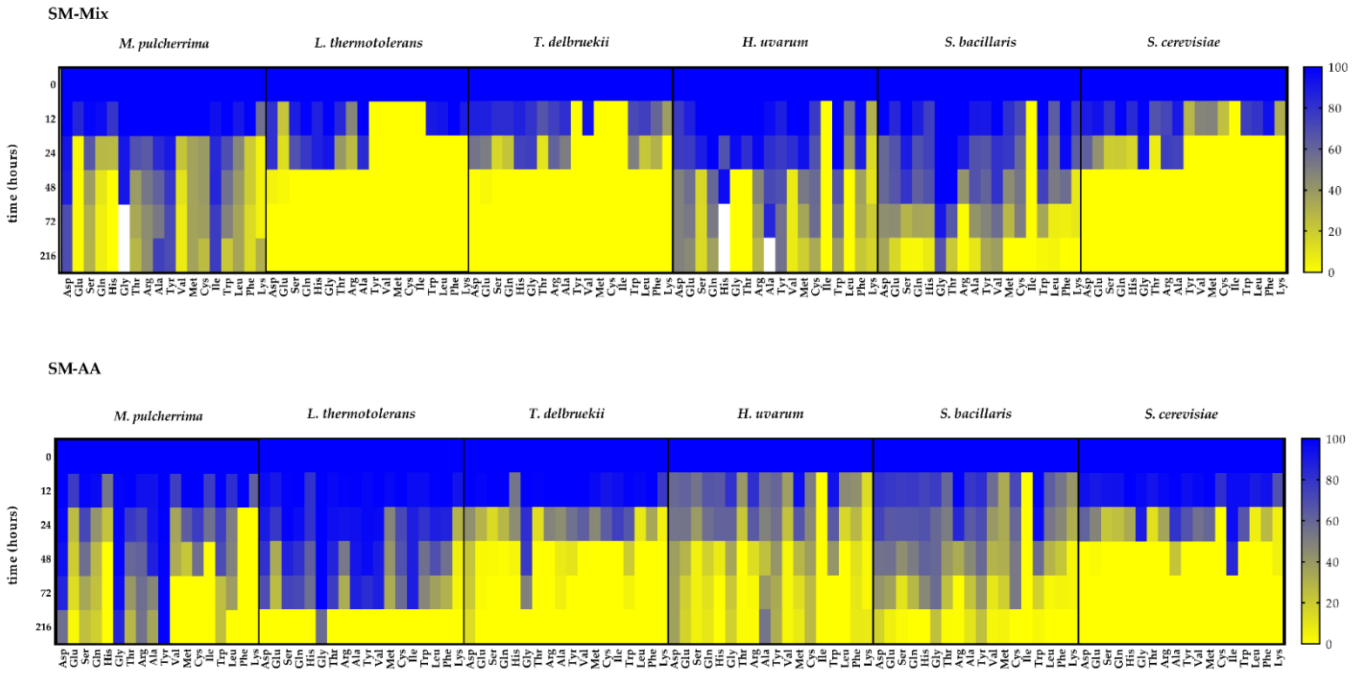

Figure 5. Amino acid concentration (%) present in the medium at different time points of the alcoholic fermentation in SM-Mix and SM-AA. The initial concentration of each amino acid is expressed as 100%. White color represents more than 100%.

Finally, the presence of ammonia in the media did not greatly affect the amino acid uptake in Mp. As observed in Sb, Mp was also not able to consume some amino acids, such as aspartic acid, tyrosine in any media, glycine in SM-AA, and alanine or isoleucine in SM-Mix.

Ammonium consumption was analyzed at the same time points as that of amino acids (Figure 6, Supplementary Table S5). Sc and Td depleted ammonium in less than 48 h in both media. Lt and Sb fermentations consumed ammonium slowly in SM-NH_4^+. In contrast, Hu and Mp were not able to consume all ammonium when amino acids were present. Indeed, Mp was the only strain with residual ammonium present in SM-NH_4^+.

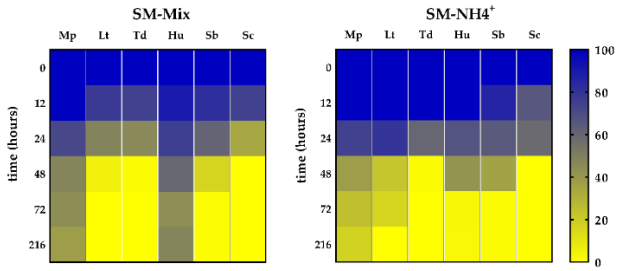

Figure 6. Ammonium concentration (%) present in the medium at different time points of the alcoholic fermentation in SM-Mix and SM-NH_4^+. The initial concentration of ammonium is expressed as 100%.

For further analysis of these data, principal components analysis (PCA) was applied to correlate the different variables at 48 h and highlight if there were grouping patterns within the different species (Figure 7). The PCs explained 78.62% of the variance, and the variables that were positively and negatively correlated in each component are listed in Supplementary Table 6. The six analyzed species clustered into five groups since Sc and Td grouped

together and apart from the others due to their higher growth and ethanol contents and their lower glucose levels in all media, compared with those of other species (Component 1). On the other hand, Component 2 differentiated the other four species. The high levels of fructose in SM-AA and SM-NH_4^+, together with the low consumption of some amino acids in SM-AA, clearly separated Lt from the other species. However, when PCA was performed for each medium separately (Supplementary Figure S2), Lt grouped with Sc and Td in the SM-Mix medium. Additionally, Hu and Sb were clustered on the negative axis of Component 2 due to their lower consumption of amino acids in SM-Mix than in the other media.

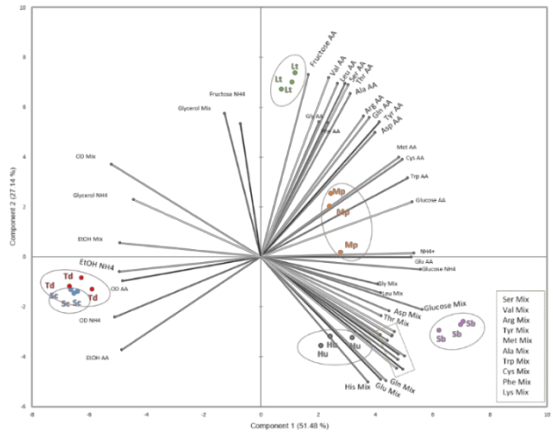

Figure 7. Biplot of principal component analysis (PCA) using nitrogen concentration, OD_{600}, and the glucose, fructose, glycerol, and ethanol contents at 48 h in all media as variables. The explicative variables were distributed along the PCA axes as follows: Component 1 (+): glucose, aspartic acid (Asp), glutamic acid (Glu), serine (Ser), glutamine (Gln), histidine (His), glycine (Gly), threonine (Thr), arginine (Arg), alanine (Ala), tyrosine (Tyr), valine (Val), methionine (Met), cysteine (Cys), tryptophan (Trp), leucine (Leu), phenylalanine (Phe), lysine (Lys) (SM-Mix); glucose, Asp, Glu, Tyr, Met, Cys, Trp (SM-AA); glucose, ammonium (SM-NH_4^+). (−): OD_{600}, ethanol (SM-Mix); OD_{600}, ethanol (SM-AA), OD_{600}, glycerol, ethanol (SM-NH_4^+). Component 2 (+): glycerol (SM-Mix); fructose, Ser, Gln, Gly, Thr, Arg, Ala, Val, Leu, Phe, (SM-AA); fructose (SM-NH_4^+).

Finally, to compare the fermentative behavior of the different species in the three media used, some compounds of oenological interest were analyzed in the final wine (Table 2). In general, glucose was preferably consumed to fructose, although as expected, Sb showed a clear fructophilic behavior, and Hu seemed to consume fructose slightly faster than it consumed glucose. Sc and Td obtained the highest ethanol contents in all the media, although the ethanol yield was very similar in most species, except for Mp and Lt, which exhibited slightly lower yields in some media. In fact, Mp was the strain with the highest glycerol production in all media after 10 days of fermentation and was also the highest at 48 h (Supplementary Table S7). SM-AA was the medium exhibiting the lowest glycerol production. On the other hand, the acetic acid content varied between 0.18 and 1.90 g/L, and Mp was also the strain that produced high acetic acid levels in all conditions, even though its lower sugar consumption, and Lt the one with the lowest production yield, in SM-Mix.

Table 2. Concentration of compounds of oenological interest at the end of each single fermentation under three nitrogen conditions (SM-Mix, SM-NH_4^+, and SM-AA) for each yeast species: *M. pulcherrima* (Mp), *L. thermotolerans* (Lt), *T. delbrueckii* (Td), *H. uvarum* (Hu), *S. bacillaris* (Sb), and *S. cerevisiae* (Sc).

		Glucose	Fructose	Glycerol	Glycerol Yield	Acetic Acid	Acetic Acid Yield	Ethanol	Ethanol Yield
		g/L	g/L	g/L	g/g	g/L	mg/g	% (v/v)	g/g
	SM-Mix	31.81 ± 2.68	49.85 ± 2.88	10.30 ± 0.50	0.06 ± 0.04	1.90 ± 0.10	15.91 ± 0.99	5.36 ± 0.11	0.36 ± 0.01
	SM-AA	30.77 ± 3.24	51.76 ± 2.80	6.87 ± 0.76	0.05 ± 0.01	0.98 ± 0.16	7.61 ± 0.20	5.59 ± 0.70	0.32 ± 0.05
Mp	SM-NH_4^+	25.35 ± 2.00	43.46 ± 2.69	8.32 ± 0.25	0.06 ± 0.00	0.86 ± 0.44	8.04 ± 2.66	6.03 ± 0.32	0.36 ± 0.01
	SM-Mix	3.15 ± 0.12	2.12 ± 2.99	10.10 ± 0.40	0.03 ± 0.02	0.23 ± 0.20	1.20 ± 0.84	9.55 ± 1.34	0.39 ± 0.05
	SM-AA	22.14 ± 2.15	54.78 ± 5.22	4.25 ± 1.38	0.03 ± 0.01	1.01 ± 0.29	8.74 ± 0.21	6.3 ± 0.94	0.34 ± 0.06
Lt	SM-NH_4^+	22.65 ± 0.16	50.08 ± 0.34	6.034 ± 1.12	0.04 ± 0	0.27 ± 0.08	1.58 ± 0.13	5.84 ± 0.48	0.32 ± 0.08
	SM-Mix	3.03 ± 0.11	0.52 ± 0.17	7.72 ± 0.59	0.04 ± 0	1.16 ± 0.14	5.88 ± 0.59	9.92 ± 0.76	0.40 ± 0.02
	SM-AA	3.10 ± 0.40	1.60 ± 2.20	4.81 ± 0.02	0.02 ± 0	1.32 ± 0.32	7.65 ± 0.31	10.44 ± 0.75	0.42 ± 0.03
Td	SM-NH_4^+	2.60 ± 0.28	3.10 ± 1.60	7.80 ± 1.11	0.04 ± 0	0.52 ± 0.21	3.31 ± 0.20	10.54 ± 0.29	0.43 ± 0.01
	SM-Mix	38.78 ± 3.98	24.38 ± 2.29	5.70 ± 0.63	0.04 ± 0.01	0.18 ± 0.06	1.33 ± 0.40	6.79 ± 0.18	0.39 ± 0.02
	SM-AA	36.80 ± 1.00	28.92 ± 0.95	3.95 ± 0.60	0.03 ± 0	0.18 ± 0.03	1.29 ± 0.17	6.80 ± 0.46	0.37 ± 0.06
Hu	SM-NH_4^+	32.98 ± 3.91	22.26 ± 1.99	6.31 ± 0.29	0.04 ± 0	0.30 ± 0.14	2.00 ± 0.99	7.47 ± 0.54	0.38 ± 0.03
	SM-Mix	46.77 ± 6.72	1.24 ± 0.35	6.32 ± 1.21	0.04 ± 0.01	0.51 ± 0.26	3.60 ± 1.43	8.61 ± 1.32	0.41 ± 0.01
	SM-AA	17.60 ± 18.86	0.00 ± 0.00	6.08 ± 1.83	0.03 ± 0.01	0.39 ± 0.06	2.12 ± 0.16	9.09 ± 0.76	0.39 ± 0.02
Sb	SM-NH_4^+	61.72 ± 12.05	0.00 ± 0.00	8.60 ± 0.97	0.06 ± 0.02	0.24 ± 0.20	2.65 ± 0.56	7.51 ± 0.83	0.43 ± 0.03
	SM-Mix	3.17 ± 0.50	1.97 ± 1.90	7.00 ± 0.37	0.04 ± 0	0.66 ± 0.22	3.37 ± 0.95	9.73 ± 0.19	0.39 ± 0.01
	SM-AA	3.05 ± 0.09	2.10 ± 1.40	5.38 ± 0.45	0.03 ± 0	0.71 ± 0.28	3.64 ± 1.18	10.28 ± 0.19	0.42 ± 0.01
Sc	SM-NH_4^+	3.61 ± 0.41	2.36 ± 1.74	7.98 ± 0.97	0.04 ± 0	0.82 ± 0.11	4.19 ± 0.44	9.85 ± 0.43	0.40 ± 0.01

4. Discussion

Nitrogen utilization and metabolism have been extensively researched in *S. cerevisiae*. However, in the last decade, the use of non-*Saccharomyces* (NS) species has been widely spread in winemaking and has been used in mixed and/or sequential starter cultures due to their desirable properties, which could positively contribute to the quality of wine (Ciani et al., 2010; Jolly et al., 2014). For this reason, knowledge of the utilization of nitrogen by NS species is critical for proper fermentation development in mixed or sequential cultures. Nevertheless, only a few and recent studies have evaluated the differential utilization of nitrogen by some NS yeasts (Gobert et al., 2017; Kemsawasd et al., 2015a; Lleixà et al., 2016; Prior et al., 2019; Rollero et al., 2018b), suggesting that nitrogen consumption and nitrogen preferences are dependent on the strain and fermentation conditions. Thus, in this study, we analyzed the nitrogen preferences of five NS strains belonging to yeast species of oenological interest (*M. pulcherrima, T. delbrueckii, L. thermotolerans, H. uvarum,* and *S. bacillaris*) in different nitrogen compositions of the media. We used strains that have not been previously analyzed, some of which are natural isolates from DOQ Priorat, to broaden the understanding of nitrogen metabolism of these species and improve the management of their implementation in wine fermentation.

S. cerevisiae was the fastest yeast to ferment all tested media, due to its well-known good fermentative capacity (Bauer and Pretorius, 2000; Beltran et al., 2002; Bisson, 1999; Torija et al., 2001). In SM-Mix, *T. delbrueckii* and *L. thermotolerans*, which also had good fermentation performance, exhibited a similar nitrogen consumption pattern to that of *S. cerevisiae*. These results

were consistent with those of previous studies (de Koker, 2015; Kemsawasd et al., 2015a; Prior et al., 2019; Rollero et al., 2018b), where strong fermenter species, such as *T. delbrueckii, L. thermotolerans, K. marxianus,* or *S. paradoxus*, assimilated nitrogen in a similar manner as *S. cerevisiae*, while poorly fermenting species, such as *M. pulcherrima, P. kluyveri, P. burtonii,* or *Zygoascus meyerae*, displayed a low nitrogen uptake.

In *T. delbrueckii* and *L. thermotolerans*, the assimilation of some amino acids, known to be repressed by the nitrogen catabolism repression (NCR) system in *S. cerevisiae* (aspartic acid, alanine, arginine, and histidine), was also delayed and linked to ammonium depletion, suggesting a nitrogen regulation system similar to the one that is well described in *S. cerevisiae* (Beltran et al., 2002; Jiranek et al., 1995; Lleixà et al., 2019; Rollero et al., 2018b). For *S. cerevisiae*, the assimilation of preferred nitrogen sources can be explained mainly by the regulation of nitrogen transport, including the Ssy1p-Ptr3p-Ssy5 (SPS) system (Lleixà et al., 2019) and the nitrogen catabolism repression (NCR) system (Beltran et al., 2004; Marini et al., 1997). These mechanisms have been scarcely explored in NS wine yeasts, as only recent studies had been done in the *Hanseniaspora* genus (Lleixà et al., 2019; Seixas et al., 2019). In fact, Lleixa et al. (2019) demonstrated that the NCR mechanism could also be present in some NS species, specifically in *H. vineae*. However, Seixas et al. (2019) evidenced that almost all *S. cerevisiae* amino acid-specific permeases were absent in *Hanseniaspora guilliermondii*, *Hanseniaspora opuntiae,* or *H. uvarum*, suggesting that *Hanseniaspora* species might favor the utilization of general nitrogen permeases instead of specific ones. Another recent study performed in

Kluyveromyces marxianus (Rollero et al., 2019) evidenced that the nitrogen regulation in this species was dissimilar to the one in *S. cerevisiae*, lacking some key ammonium permeases, such as Mep1 and Mep2. Thus, although the presence of an NCR system should not be ignored in those species, more studies would need to be performed. On the other hand, the absence of ammonium in the medium resulted in a delay in the amino acids' consumption for *T. delbrueckii, L. thermotolerans,* and *S. cerevisiae*, with higher differences among them, especially in *L. thermotolerans*, which could not finish the fermentation under this condition. These results do not agree with Prior et al. (2019), who reported no difference in amino acids' uptake in *L. thermotolerans* or *T. delbrueckii,* with and without ammonium. Under this condition, the latest amino acid consumed by *T. delbrueckii* and *L. thermotolerans* was glycine, which has been previously described as a poor nitrogen source for several yeast species (Cooper, 1982; Kemsawasd et al., 2015a; Ljungdahl and Daignan-Fornier, 2012; Rollero et al., 2018b; Su et al., 2020). Indeed, glycine was also one of the less preferred amino acids for *M. pulcherrima* and *S. bacillaris*, being even excreted/produced by *M. pulcherrima* in SM-Mix. In contrast, *H. uvarum* showed a preference for this amino acid, especially in SM-Mix. Kemsawasd et al. (2015a) also observed that glycine favored the consumption of glucose and the production of ethanol in *H. uvarum*, indicating that the preference for this amino acid is specific for this yeast species.

Ammonium is shown to be a good nitrogen source to support fermentation in *T. delbrueckii*, being consumed early in SM-Mix or SM-NH_4^+ (Kemsawasd et al., 2015a; Su et al., 2020), although yeast growth was

impaired when fermenting with ammonia as a single nitrogen source. On the other hand, although *L. thermotolerans* also exhibited early assimilation of ammonia in mixed media, it was severely delayed in SM-NH_4^+. These results correlated with low yeast growth and fermentation kinetics in SM-NH_4^+, suggesting that this *Lachancea* strain might be favored by the presence of complete media containing organic and inorganic nitrogen. However, our fermentations performed with other *Lachancea* strains showed different growth and fermentation kinetics, indicating that the nitrogen preferences in *Lachancea* spp. seem to be strain-specific more than species-specific (de Koker, 2015; Gobert et al., 2017; Kemsawasd et al., 2015a). Some studies performed with other *Lachancea* strains confirmed this variability in this species, i.e., the *L. thermotolerans* strain BBMCZ7-FA20 completed the fermentation process slowly, in 21 days, in white must (de Koker, 2015), and the *L. thermotolerans* Viniflora·ConcertoTM strain fermented well in musts with different nitrogen sources (Rollero et al., 2018b).

H. uvarum, *M. pulcherrima,* and *S. bacillaris* resulted in stuck fermentations under all conditions, which agrees with previous works performed with other strains of these species in single cultures (Andorrà et al., 2012; Englezos et al., 2018; Gobert et al., 2017). Despite this, *S. bacillaris* was able to consume all nitrogen under all conditions. The nitrogen consumption profile of *S. bacillaris*, as well as that of *H. uvarum*, revealed a high delay in the assimilation of most amino acids in the presence of ammonium, which was not observed in *S. cerevisiae*, *T. delbrueckii,* or *L. thermotolerans*. Englezos et al. (2018) described a similar behavior of two

strains of *S. bacillaris*, suggesting differences in the regulation of nitrogen uptake between *S. bacillaris* and *S. cerevisiae*. Different explanations are proposed by these authors, such as less-efficient SPS-control methods of amino acid permeases, an inhibitory mechanism mediated by ammonium, or the use of an additional efficient system for ammonium uptake in *S. bacillaris* (Englezos et al., 2018). Indeed, the three species seemed to prefer a single inorganic nitrogen source, as they consumed more YAN in SM-NH_4^+ than in the other fermentation groups. Surprisingly, higher nitrogen consumption did not always correlate with higher growth or better fermentation kinetics. In fact, in our study, nitrogen uptake seemed to be decoupled from growth or fermentation under some conditions for *T. delbrueckii, H. uvarum,* or *S. bacillaris*. In particular, the growth of *S. bacillaris* was better sustained by amino acids than by ammonium, although similar consumption of YAN was observed. Those results indicated a less efficient conversion of inorganic nitrogen to growth by *S. bacillaris*. Gutiérrez et al. (2016) previously described a similar decoupled behavior between nitrogen uptake and growth in *S. cerevisiae* strains. These authors justified that part of the assimilated nitrogen was used by yeast to replenish intracellular pools rather than being utilized for reproduction. Therefore, our results demonstrated that, as observed in *S. cerevisiae*, nitrogen assimilation, yeast growth, and fermentation kinetics were not always correlated in non-*Saccharomyces* species, and it is important to study the effect of a nitrogen source on all these parameters.

The amino acid preferences of *M. pulcherrima* correlated with those outlined in previous studies performed with other strains (Gobert et al.,

2017; Kemsawasd et al., 2015a; Su et al., 2020), showing lysine, glutamine, valine, and glutamic acid as good nitrogen sources, and glycine, tyrosine, alanine, and aspartic acid as poor ones (Su et al., 2020). On the other hand, the consumption of isoleucine, which we and other authors observed as a good nitrogen source for most NS yeasts (Gobert et al., 2017; Kemsawasd et al., 2015a; Su et al., 2020), seemed to be impaired in our *M. pulcherrima* strain in SM-Mix. This could be due to modifications of the matrix or fermentation conditions, as observed by Gobert et al. (2019) with different temperatures, or to some genetic variations occurring among strains of the same species, as previously described for *S. cerevisiae* (Cubillos et al., 2009; Gutiérrez et al., 2013; Marullo et al., 2007; Salinas et al., 2012; Zimmer et al., 2014).

Interestingly, we also observed an increase of some amino acids after 72 h for *H. uvarum* (histidine, alanine, and lysine) and *M. pulcherrima* (glycine, alanine, and lysine), mainly in the presence of ammonia. However, we discarded this release of amino acids as a consequence of autolysis because it mainly occurred during the early stationary phase without an evident loss of viability. Previous studies have also shown a release of some amino acids in different NS species, such as *M. pulcherrima, T. delbrueckii,* and *Pichia kluyveri* (Gobert et al., 2017), or *S. bacillaris* (Englezos et al., 2018), although in some cases, this release was before uptake began, possibly due to a response to the stress of inoculation (Hagman et al., 2014).

Overall, our results confirmed the high similarities between *S. cerevisiae* and *T. delbrueckii* under all conditions in terms of growth, fermentation profile, and nitrogen assimilation preferences. These similarities between *T.*

delbrueckii and *S. cerevisiae* were not unexpected since these species are believed to have evolutionarily diverged approximately 100–150 million years ago (Hagman et al., 2014), being very close genetically (Masneuf-Pomarede et al., 2016). In fact, *T. delbrueckii* was previously included in *Saccharomyces* spp. as *Saccharomyces rosei* (Bely et al., 2008). All these similarities could suggest that both species could share a similar nitrogen regulation system. Indeed, both species presented fast nitrogen assimilation, which could provide them with a competitive edge relative to other yeasts present in fermenting musts (Crépin et al., 2012; García-Ríos et al., 2014).

On the other hand, the *L. thermotolerans* strain was also very similar to *S. cerevisiae* and *T. delbrueckii* but only in the complete medium. When only organic or inorganic nitrogen was used, its behavior changed drastically, resulting even in stuck fermentation, highlighting the importance of fermentation conditions when characterizing NS strains for their potential use in winemaking.

M. pulcherrima was the NS strain least affected by the nitrogen composition of the medium, presenting a similar nitrogen assimilation profile, as well as fermentation kinetics in all tested media. Although this species could not finish the fermentations, it had the highest production of glycerol and acetic acid and the lowest ethanol yield. In fact, this species has been described as a Crabtree-negative yeast, with a preference for respiratory metabolism under aerobic conditions (Contreras et al., 2015; Quirós et al., 2014; Venturin et al., 1995), and it is a good candidate for lowering the ethanol content in wines. This respiro-fermentative metabolism is linked to an

increase in glycerol and acetic acid contents, which correlates with our results.

The other two poor fermentative strains, *H. uvarum* and *S. bacillaris*, shared a preference for fructose as carbon source, especially *S. bacillaris*, which has been previously described as a highly fructophilic yeast (Englezos et al., 2018, 2016; Mestre Furlani et al., 2017). Moreover, both strains behaved similarly regarding amino acid uptake, which occurred earlier than that of the good fermentative species in the absence of ammonia. These results could imply a different nitrogen regulation system for these species, as already suggested by Englezos et al. (2018), although more studies are needed to confirm this hypothesis.

These species would be used in winemaking as mixed or sequential inocula, as their fermentative capacity is, in general, limited. In sequential fermentations, *S. cerevisiae* is usually added after 24 or 48 h of NS inoculation. Thus, the knowledge of the nitrogen consumption by the NS strains during this period is crucial to avoid the fermentative problem. In this work, we differentiated two behaviors regarding nitrogen consumption. One group included the good fermentative strains, *T. delbrueckii*, *L. thermotolerans*, which consumed practically all nitrogen in 48 h (as *S. cerevisiae*). This high consumption of nitrogen by those species might compromise the success of a sequential fermentation with *S. cerevisiae*, as suggested by Rollero et al. (2018a), making the nitrogen supplementation an essential practice for the correct development of the fermentations. Indeed, when using good fermentative NS strains, the recommended inoculation time of *S. cerevisiae* would be around 24 h. The

other group included the weaker fermentative strains, *M. pulcherrima*, *S. bacillaris,* and *H. uvarum*, which only consumed part of the nitrogen present in the must at 48 h, leaving more than 100 mg N/L in the media. This residual nitrogen could be enough for the proper growth of *S. cerevisiae*, making the nitrogen supplementation less necessary. In order to allow those weaker fermenters to have an impact on the final wines, the inoculation of *S. cerevisiae* should preferably be done after 48 h.

In conclusion, nitrogen consumption and preferences are species-dependent, and the nitrogen composition of the must has a direct effect on the fermentation profile and yeast growth. This work highlighted the importance of controlling nitrogen availability in sequential fermentations, as some nitrogen compounds might be depleted by non-*Saccharomyces* yeasts before *Saccharomyces* inoculation, impairing its growth and the performance of the fermentation. Thus, it is important to control the nitrogen availability in mixed fermentations before inoculating *Saccharomyces* to avoid problems, such as stuck or sluggish fermentations.

Author Contributions

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

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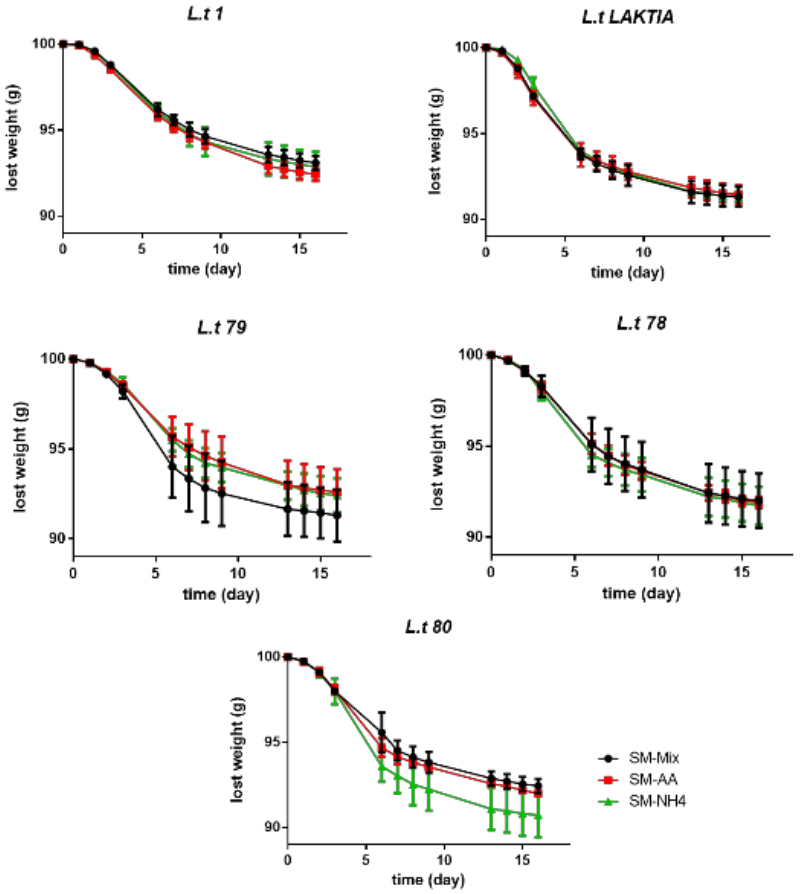
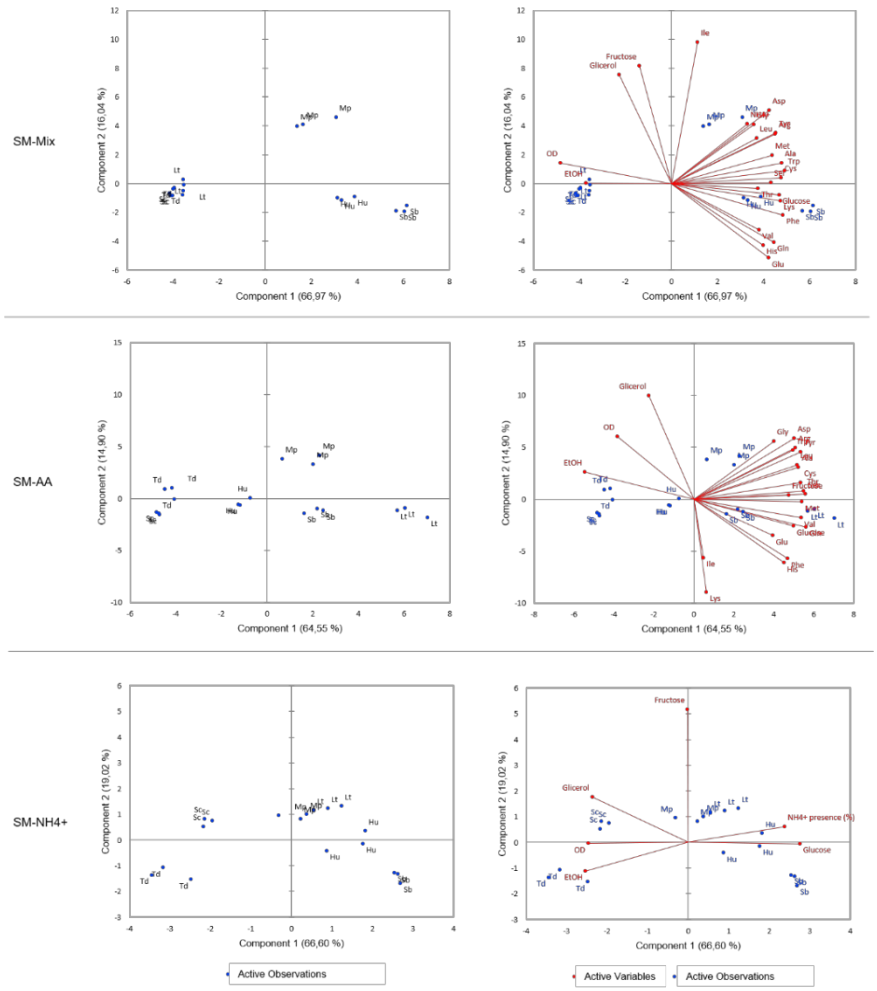
Conflicts of Interest

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

Supplementary Material



Supplementary Figure S1: Fermentation kinetics of different *Lachancea* strains in the three media.



Supplementary Figure S2: Biplot of principal components analysis using nitrogen concentration, OD_{600}, glucose, fructose, glycerol, and ethanol at 48 h in each medium as variables.

All the supplementary Tables except for Supplementary Table S1 and Supplementary Table S2 are not included in the present thesis due to format incompatibility, and are available online at: https://www.mdpi.com/2076-2607/8/2/157 **Supplementary TableS3**: Nitrogen presence (mgN/L) in SM-Mix medium during single fermentations. **Supplementary Table S4**: Nitrogen presence (mgN/L) in SM-AA medium during single fermentations. **Supplementary Table S5**: Ammonium presence (%) in SM-NH_4^+ and SM-Mix media during single fermentations. **Supplementary Table S6**: Correlated variables of PCA, and **Supplementary Table S7**: Compounds of oenological interest present at 48 h in SM-Mix, SM-AA, and SM-NH_4^+ media.

Supplementary Table S1: Synthetic must composition.

		Concentration (g/L)
Sugars	Glucose	100
	Fructose	100
Acids	Citric acid	0.5
	Malic acid	5
	Tartaric acid	3
Salts	KH_2PO_4	0.750
	K_2SO_4	0.500
	$MgSO_2$ 7 H_2O	0.250
	$CaCl_2$ 2 H_2O	0.155
	NaCl	0.200
Oligo-elements	$MnSO_4$ H_2O	0.004
	$ZnSO_4$ 7 H_2O	0.004
	$CuSO_4$. 5 H_2O	0.001
	KI	0.001
	$CoCl_2$ 6 H_2O	0.0004
	H_3BO_3	0.001
	$(NH_4)_6Mo_7O_{24}$	0.001
Vitamins	Myo-inositol	0.02
	Pantothenate calcium	0.0015
	Thiamine hydrochloride	0.00025
	Nicotinic acid	0.002
	Pyridoxine	0.00025
	Biotine	0.000003
Anaerobic factors	Ergosterol	0.015
	Oleic acid	0.0045
	Tween 80	0.535
	Ethanol (absolute)	0.395
Nitrogen (300 mg N/L)		
SM-Mix		
	NH_4Cl (120 mg N/L)	0.46
	Amino acid solution (180 mg N/L)	6.25 mL *
SM-AA		
	NH_4Cl (0 mg N/L)	0.00
	Amino acid solution (300 mg N/L)	10.42 mL *
SM-$NH4^+$		
	NH_4Cl (300 mg N/L)	0.214 g
	Amino acid solution (0 mg N/L)	0.00 mL *

* Volum added from Amino acids stock solution (Supplementary Table S2).

Supplementary Table S2: Ammonium content and amino acid stock solution content expressed as g/L and the corresponding nitrogen concentration in synthetic must in mg N/L.

Amino acid	g/L	mg N/L
Asp	4.42	2,91
Glu	11.96	7,12
Ser	7.80	6,50
Gln	49.92	59,84
His	3.38	1,91
Gly	1.82	2,12
Thr	7.54	5,54
Arg	36.79	55,50
Ala	14.56	14,31
Tyr	1.95	0.94
Cis	2.08	1.50
Val	4.42	3.31
Met	3.12	1.83
Trp	17.42	7.47
Phe	3.77	2.00
Ile	3.25	2.17
Leu	4.81	3.21
Lys	1.69	2.03
Pro	45.60	0,00
Total aas		180.22
Ammonia (NH_4Cl)	0.46	120.00
Total N		300.82

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Elena Roca Mesa

Capítulo I. 2

The effect of must nitrogen composition on yeast growth and volatile thiol release in sequential fermentations

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Manuscrito en preparación

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Abstract:

The use of non-*Saccharomyces* yeasts in mixed fermentations has increased in recent decades. In this work, we analyzed the nitrogen preferences and requirements of different non-*Saccharomyces* yeasts, determining the effect of different nitrogen sources, such as a mixture of amino acids and ammonium, ammonium only, or ammonium with the addition of Inactive Dry Yeasts (IDY), on non-*Saccharomyces* yeast growth. Non-*Saccharomyces* species presented different growth values depending on the nitrogen source and concentration. Supplementation with IDY improved yeast growth, mainly in musts with low nitrogen content. Then, sequential fermentations were performed in Verdejo grape must, using *T. delbrueckii* and *S. cerevisiae* strains, with and without the addition of IDY. Our results showed that *S. cerevisiae* was not the predominant strain by the end of sequential fermentations with *T. delbrueckii*. On the other hand, the inoculation strategy and the addition of commercial IDY-based nutrients did not show a clear effect on the release of volatile thiols, probably due to the high nitrogen content of the grape must used in this study.

Keywords: winemaking; non-*Saccharomyces*; *Torulaspora delbrueckii;* varietal thiols; inactive dry yeasts (IDY)-based supplements

1. Introduction

Although non-*Saccharomyces* yeasts have long been considered undesirable or spoilage yeasts, there is currently a growing interest in the use of some species of these yeasts in winemaking due to the particular characteristics that they provide to the final product. They can contribute to enhancing primary and secondary aromas, reducing the ethanol content in wine, and releasing mannoproteins or wine colour stabilization compounds (Ciani et al., 2010; Jolly et al., 2014; Morata et al., 2020; Padilla et al., 2016; Ruiz et al., 2020). In fact, some of these yeasts are already being used commercially in the wine industry and are available as active dry yeasts (*Torulaspora delbrueckii*, *Metschnikowia pulcherrima*, *Lachancea thermotolerans*, etc.) (Vejarano and Gil-Calderón, 2021).

Interest in the use of non-*Saccharomyces* yeasts as starter cultures promoted several studies to investigate the optimal conditions for their development throughout fermentation, their nutritional requirements, as well as the relationship between the composition of the medium, the capacity of imposition and survival, and the synthesis of metabolites of oenological interest, in both fermentations with pure and mixed cultures. *T. delbrueckii* is a non-*Saccharomyces* yeast that has interesting applications in winemaking (Arslan et al., 2018; Belda et al., 2015; Canonico et al., 2019; Escribano-Viana et al., 2019; Muñoz-Redondo et al., 2021; Velázquez et al., 2019), such as reducing the production of acetic acid and ethanol, increasing the amount of mannoproteins, or modulating the overall aromatic profile of wines by enhancing the fresh fruit odour descriptors and decreasing the perception of vegetal flavours (Belda et al., 2017b; Benito, 2018a; Tufariello et al., 2021).

One of the main limiting nutrients in alcoholic fermentation is nitrogen. The adequate supply of this nutrient allows yeasts to undergo adequate growth and fermentation. Nitrogen deficiency in the must might result in stuck or sluggish fermentations, but an excess of nitrogen can cause microbial instability and an accumulation of ethyl carbamate (Bell and Henschke, 2005). Preferences for certain nitrogen compounds depend on the strain and the type of starter culture (Crépin et al., 2012; Gutiérrez et al., 2012; Mendes-Ferreira et al., 2004). In recent years, several studies have focused on knowledge about nitrogen preferences and metabolism in non-*Saccharomyces* species. It has been shown that the nitrogen needs of non-*Saccharomyces* yeasts differ from those of *S. cerevisiae* (Andorrà et al., 2010; Prior et al., 2019; Roca-mesa et al., 2020; Su et al., 2020). These different nutrient requirements have to be accounted for in mixed inoculum fermentations, mainly in sequential inoculations, in which the growth of the non-*Saccharomyces* species before the inoculation of *S. cerevisiae* can compromise the subsequent imposition of *S. cerevisiae* (Andorrà et al., 2012; Lleixà et al., 2016; Medina et al., 2012; Rollero et al., 2018a).

The uptake and utilization of nitrogen compounds is regulated in *S. cerevisiae* by a mechanism called nitrogen catabolic repression (NCR) (Magasanik and Kaiser, 2002; ter Schure et al., 2000). In the presence of preferred nitrogen sources, such as ammonium or glutamine, the transcription of the genes necessary for the transport and use of less preferred nitrogen sources is suppressed. Once the preferred nitrogen sources become limited in the medium, genes responsible for the utilisation of nonpreferred nitrogen sources are gradually derepressed and NCR is

lifted (Cooper, 2002; ter Schure et al., 2000). Thus, the uptake of amino acids such as arginine, alanine, asparagine, glycine, tyrosine and glutamate occurs only when ammonium and other preferred nitrogen sources have been depleted from the medium, which usually is at the end of the growth phase or during the stationary phase, as long as no excess nitrogen content has been added (Beltran et al., 2004). However, the classification of nitrogen source preferences is not absolute, and can vary between yeast strains (Magasanik and Kaiser, 2002). NCR is mainly mediated by four transcription factors (GATA factors), as well as the regulatory protein Ure2p (Cooper, 2002). Deletion of *URE2* causes an increase in nitrogen consumption during alcoholic fermentation and biomass production (Dufour et al., 2013) and has also been related to an increase in the synthesis of volatile thiols, such as 4-mercapto-4-methylpentane-2-one (4-MMP), 3-mercaptohexan-1-ol (3-MH) and 3-mercaptohexyl acetate (3-MHA), since Ure2p regulates the transcription of the *IRC7* gene, which encodes the enzyme cysteine-β-lyase, responsible for the release of thiol aromas from their non aromatic cysteine precursors (Dubourdieu et al., 2006; Roncoroni et al., 2011; Thibon et al., 2008). Dufour et al. (2013) showed that the use of a natural *URE2* mutation can be used to design a yeast starter with a higher capacity for the production of volatile thiols. Moreover, Ruiz et al. (2021) reported new genetic and phenotypic signatures to explain the distribution of the *IRC7*^{S} allele (which encodes a shorter enzyme with lower activity) in the great majority of *S. cerevisiae* wine strains. Therefore, the NCR system appears to be the main regulatory mechanism for thiol synthesis in *S. cerevisiae* (Subileau et al., 2008; Thibon et al., 2008).

The effect of NCR on *S. cerevisiae* under alcoholic fermentation conditions has been extensively studied (Beltran et al., 2005, 2004; Carrasco et al., 2003; Godard et al., 2007; Gutiérrez et al., 2013; Tesnière et al., 2015). However, there is little knowledge about NCR in non-*Saccharomyces* yeasts. *Hanseniaspora vineae* showed a similar amino acid consumption and gene expression profile as *S. cerevisiae*, suggesting similar nitrogen regulation in both species and therefore the presence of the NCR mechanism in *H. vineae* (Lleixà et al., 2019). In a previous work, the nitrogen assimilation profile shown by *T. delbrueckii,* in which the consumption of some NCR-regulated amino acids was also delayed and associated with ammonium depletion (Roca-Mesa et al., 2020), suggested a nitrogen regulation system similar to that of *S. cerevisiae* (Beltran et al., 2002; Jiranek et al., 1995; Lleixà et al., 2019; Rollero et al., 2018b). Moreover, in mixed fermentations, the nitrogen dose showed an impact on aromatic compound concentrations due to the presence of *T. delbrueckii*, which affected the *S. cerevisiae* transcriptional response (Ruiz et al., 2021). However, little is known about the relationship between nitrogen metabolism and the release of volatile thiols by non-*Saccharomyces* yeast in sequential fermentations. Indeed, the *IRC7* gene has been recently identified in *T. delbrueckii*, and mixed fermentations with *T. delbrueckii* showed improvements in thiols release (Belda et al., 2017a).

A better understanding of nitrogen utilization among yeasts of different species is important to increase the efficiency, predictability, and quality of wine production, as well as the release of varietal thiols. Thus, the objective of this study is the analysis of nitrogen metabolism in different yeasts of oenological interest, determining the nitrogen needs and preferences of

different non-*Saccharomyces* species, as well as the effect of supplementation with inactive yeasts on fermentation and the release of varietal thiols.

2. Materials and Methods

2.1. Yeast strains, inactive yeasts and media

The strains used in this study were two strains of *Torulaspora delbrueckii* (Viniferm NS-TD (Td5) and TdP), *Saccharomyces cerevisiae* (Viniferm Diana (ScD) and Viniferm Revelación (ScR)) and *Lachancea thermotolerans* (Lt1 and Lt2). All strains were provided by Agrovin S.A. (Spain).

In some fermentations, nutrient supplementation was performed by adding inactive dry yeasts (IDY) at 0.2 or 0.4 g/L. IDY1-IDY5 were composed of *S. cerevisiae* yeasts thermally inactivated and completely autolyzed by a thermal gradient, with IDY1, the commercial Actimax Natura (Agrovin S.A., Spain). Instead, IDY6 consisted of yeasts that were only thermally inactivated.

All strains were rehydrated following the manufacturer's instructions and stored in YPD liquid medium (2% (w/v) glucose, 2% (w/v) bactopeptone, and 1% (w/v) yeast extract; Cultimed, Barcelona, Spain) with 40% (v/v) glycerol, at –80 °C. They were streaked on YPD agar plates (YPD liquid with 2% (w/v) agar) before use. From these pure cultures, isolated colonies were grown in YPD at 28 °C and 120 rpm for 24 h, and the cell concentration was calculated by microscopic counting using a Neubauer chamber. These precultures were used to inoculate the fermentations.

2.2. Microplate growth monitoring

Fermentations were performed in 96-well microplates at 28 °C to assess the effect of nitrogen sources and nitrogen concentration on *S. cerevisiae*, *T. delbrueckii* and *L. thermotolerans* growth. In all cases, the tested nitrogen concentrations were obtained by diluting a stock solution of 10 g N/L of each nitrogen source: amino acids and ammonium (SM-Mix), only ammonium (SM-NH4) and ammonium with IDY at 0.2 g/L (SM-IDY). Additionally, to study the effect of IDY dose on yeast growth, this compound was also added at 0.4 g/L in some of the conditions. Each strain was inoculated at a concentration of 1 x 10^5 cells/mL in synthetic must. Yeast growth was measured after preshaking the microplate for 30 seconds at 600 rpm using a SPECTROstar Nano microplate reader (BMG LABTECH, Germany) every 30 minutes until the stationary phase. For each growth curve, the generation time, growth efficiency and duration of the lag phase were calculated.

2.3. Fermentation conditions and sampling

Single and sequential fermentations were performed in Verdejo must. The initial nitrogen content was 514.67 mg of yeast assimilable nitrogen (YAN) per litre (Supplementary Table S1), the pH was 3.17 and the density was 1089.5 g/L.

In some fermentations, nutrient supplementation was conducted by the addition of IDY1 (Actimax Natura) at 0.2 g/L.

Fermentations were performed in triplicate at 16 °C in 1000 mL borosilicate glass bottles containing 800 mL of medium and capped with closures that enabled carbon dioxide to escape and samples to be removed. Single

fermentations were inoculated at 2 x 10^6 cells/mL, while in sequential fermentations, the first yeast species (*T. delbrueckii*) was inoculated at 2 x 10^6 cells/mL, and the second species (*S. cerevisiae*) was also inoculated at 2 x 10^6 cells/mL, when the density decreased by approximately 15 units.

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

2.4. Nitrogen analysis

The nitrogen content was analyzed by HPLC (high-performance liquid chromatography), according to the method of Gómez-Alonso et al. (2007). The HPLC (Agilent 1100, Agilent Technologies, Germany) was equipped with a DAD ultraviolet detector and a fluorescence detector (Agilent Technologies, Germany). Separation was performed on a Hypersil ODS C18 column (Agilent Technologies, Germany) with a particle size of 5 μm (250 mm × 4.6 mm) and temperature of 20 °C. The mobile phase (A) consisted of 2.05 g/L anhydrous sodium acetate and 0.2 g/L sodium azide

with Milli-Q water (Millipore Q-PODTM Advantage A10) adjusted to pH 5.8 with glacial acetic acid. The mobile phase (B) consisted of 80% (v/v) acetonitrile and 20% (v/v) methanol. Agilent ChemStation Plus software (Agilent Technologies, Germany) was used to analyze the chromatograms. Yeast assimilable nitrogen (YAN, expressed as mg N/L) was calculated according to the nitrogen atoms of each amino acid.

2.5. Metabolite analysis

The concentrations of some organic metabolites (glucose, fructose, glycerol, ethanol, and acetic acid) were determined by HPLC at the end of fermentation, following the protocol described by Quirós et al. (2010). We used an Agilent 1100 HPLC (Agilent Technologies, Germany) equipped with a Hi-Plex H, 300 mm x 7.7 mm column inside a 1260 MCT (Infinity II Multicolumn Thermostat) connected to both an MWC and an RID (G1365B multiwavelength detector and 1260 Infinity II refractive index detector) (Agilent Technologies, Germany).

The concentration of varietal thiols was analyzed by the Nyséos Laboratory (Montpellier, France) using gas chromatography/ion trap mass spectrometry (GC–ITMS/MS) following the protocol described by Roland et al. (2010).

2.6. Statistical analysis

Data were expressed as the mean and standard deviation (SD) of triplicates as data points. ANOVA and Tukey's test analyses using XLSTAT 2019 software (Addinsoft, New York, New York, USA) were performed to determine significant differences between fermentation conditions. The results were considered statistically significant with a *p-value* less than 0.05.

Graphical data modelling was performed using the GraphPad Prism 7 program (GraphPad Software, San Diego, California, USA).

3. Results

3.1. Nitrogen composition of different inactive dry yeasts

The nitrogen composition of the commercial nutrient Actimax Natura (IDY1) and 5 samples of different inactive yeasts supplied by Agrovin S. A. were analyzed (IDY2-IDY6) (Table 1). Samples IDY1-5 showed similar nitrogen contents, higher than 100 mg N/g. Conversely, sample IDY6, which was an inactive yeast product without the autolytic process, showed a very different profile from the rest, with a much lower nitrogen concentration (approximately 20 mg N/g of product) than that of the samples in which the autolytic process was induced during their production. For all IDY samples analyzed, proline and alanine were the amino acids found in the highest proportion, representing between 40 and 50% of the total nitrogen present in the product, although proline is hardly assimilated by yeast in fermentative conditions, and usually considered as non assimilable nitrogen. These were followed by glutamine, arginine, cysteine, isoleucine and tryptophan, which accounted for another 25% of the total nitrogen. The inorganic nitrogen concentration (NH_4Cl) differed between the different samples (between 0 and 4% of the total nitrogen).

Table 1. Nitrogen content (expressed in mg N/g of product) of Actimax Natura (IDY1) and other experimental batches of inactive yeasts (IDY2-IDY6) supplied by Agrovin S. A. Mean of triplicates and standard deviation (SD).

	IDY1	**IDY2**	**IDY3**	**IDY4**	**IDY5**	**IDY6**
Asp	2.61 ± 0.18	2.29 ± 0.18	2.39 ± 0.94	2.45 ±0.02	2.54 ± 0.05	0.87 ± 0.08
Glu	0.16 ± 0.01	0.17 ± 0.01	0.18 ± 0.05	0.16 ± 0.01	0.16 ± 0.00	0.04 ± 0.00
Ser	2.58 ± 0.19	2.33 ± 0.19	3.17 ± 0.99	2.58 ± 0.08	2.86 ± 0.06	0.48 ±0.04
Gln	5.49 ± 0.36	5.01 ± 0.44	6.82 ± 2.03	5.80 ± 0.20	6.13 ± 0.21	0.86 ± 0.07
His	0.67 ± 0.05	0.61 ± 0.05	0.93 ± 0.50	0.71 ± 0.01	0.74 ± 0.03	0.40 ± 0.03
Gly	2.93 ± 0.20	2.61 ± 0.33	4.06 ± 1.87	3.07 ± 0.10	3.16 ± 0.08	0.28 ± 0.04
Thr	2.46 ± 0.12	2.41 ± 0.30	3.69 ± 1.87	2.69 ± 0.18	2.79 ± 0.18	0.91 ± 0.31
Arg	4.96 ± 0.32	4.84 ± 0.63	7.13 ± 2.99	5.68 ± 0.91	5.90 ± 0.71	1.23 ± 0.34
Ala	17.65 ± 1.67	23.22 ± 6.86	24.92 ± 6.95	21.77 ± 5.25	22.77 ± 6.24	3.35 ± 1.20
Pro	26.14 ± 3.85	36.16 ± 9.65	37.61 ± 10.55	33.79 ± 8.02	31.12 ± 6.19	8.51 ± 2.33
Tyr	1.66 ± 0.27	1.70 ± 0.18	2.16 ± 0.26	1.74 ± 0.17	1.90 ± 0.02	0.64 ± 0.27
NH_4Cl	1.94 ± 1.78	4.81 ± 4.19	5.58 ± 4.13	nd*	4.52 ± 2.27	1.00 ± 0.12
Val	2.80 ± 0.40	2.99 ± 0.08	3.22 ± 0.72	3.32 ± 0.16	2.58 ± 0.28	1.19 ± 0.91
Met	2.96 ± 0.67	3.44 ± 0.86	3.52 ± 0.61	3.52 ± 1.11	2.88 ± 0.73	0.92 ± 0.16
Cys	9.28 ± 2.88	4.40 ± 0.89	7.28 ± 3.10	6.80 ± 4.52	4.53 ± 4.93	3.05 ± 1.60
Ile	5.44 ± 0.46	5.76 ± 0.62	6.66 ± 1.57	7.18 ± 2.76	6.65 ± 0.98	0.72 ± 0.29
Trp	5.16 ± 0.32	5.51 ± 0.81	6.40 ± 1.41	5.30 ± 0.64	6.65 ± 1.30	1.37 ± 0.90
Leu	4.03 ± 0.10	4.44 ± 0.60	5.34 ± 1.18	4.30 ± 0.55	4.80 ± 0.15	0.51 ± 0.12
Phe	1.42 ± 0.26	1.76 ± 0.29	2.77 ± 0.78	1.95 ± 0.30	1.27 ± 0.19	0.69 ± 0.22
Lys	3.99 ± 0.09	3.78 ± 0.29	4.85 ± 1.07	3.77 ± 0.26	4.44 ± 0.13	0.87 ± 0.19
Total Nitrogen	**104.34 ± 5.65**	**118.23 ± 23.19**	**138.69 ± 38.07**	**116.57 ± 16.71**	**118.40 ± 11.42**	**27.91 ± 6.06**
Total YAN **	78.20 ± 1.80	82.07 ± 13.53	101.08 ± 27.52	82.78 ± 9.11	87.28 ± 5.29	19.40 ± 3.90

* nd (not detected)

** Total YAN (Yeast Assimilable Nitrogen) calculated as total nitrogen minus Proline

3.2. Effect of the nitrogen composition and IDY supplementation on yeast growth

The growth of different yeast species was analyzed using synthetic must with different nitrogen compositions (SM-Mix, SM-NH4, SM-IDY) and concentrations (40-200 mg N/L). Among the three media, we observed that *T. delbrueckii* exhibited the highest growth (ODmax), followed by *S. cerevisiae*, while the *L. thermotolerans* Lt1 strain presented the lowest growth (Figure 1a, Supplementary Table S2). Comparing nitrogen sources, medium with ammonium (SM-$NH4^+$) produced lower growth in all tested yeasts (Figure 1b), and the mixed nitrogen combination (SM-Mix) resulted in a higher biomass production in concentrations above 140 mg/L of nitrogen, similar to that obtained with SM-IDY.

Regarding the nitrogen required to reach the maximal growth in each condition and strain (limiting nitrogen concentration), all *S. cerevisiae* and *T. delbrueckii* indeed increased their maximal growth with the increase of nitrogen, at all tested concentrations (from 40 to 200 mg/L), although this increase was generally less pronounced after 140 mg/L (Supplementary Figure S1). Conversely, the growth of *L. thermotolerans* increased slightly above 80-100 mg N/L, indicating that the lower populations reached by these strains were not due to nitrogen limitation.

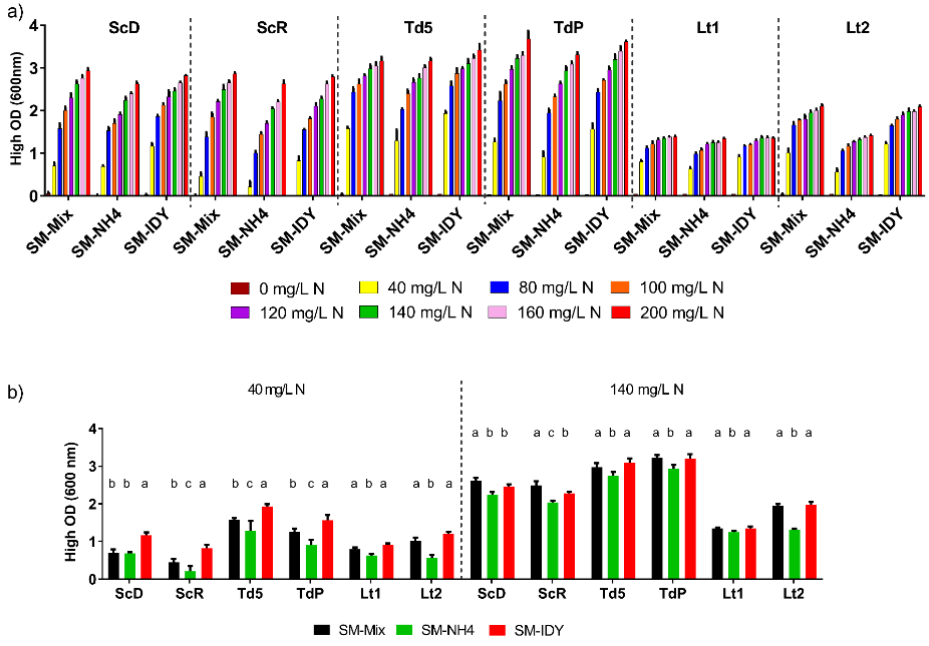

Figure 1. a) Maximal population (high OD_{600nm}) obtained with different yeast species and strains (*S. cerevisiae* (Sc), *T. delbrueckii* (Td) and *L. thermotolerans* (Lt)), in synthetic must containing different nitrogen sources (mixture of ammonium and amino acids (SM-Mix), ammonium (SM-NH4), and ammonium and IDY (SM-IDY)) and at different nitrogen concentrations (from 40 to 200 mg/L). IDY was added at 0.2 g/L in medium with increasing concentrations of ammonium. b) Maximal growth obtained with the different nitrogen sources for each yeast strain, at 40 and 140 mg N/L. Letters indicate significant differences between the maximal growth obtained with the different nitrogen sources in each yeast strain at 40 and 140 mg N/L.

However, supplementation with IDY improved yeast growth, mainly at low nitrogen concentrations, reaching growth levels similar to or higher than those obtained with mixed nitrogen composition (Figure 1b). The better yeast growth observed with the addition of IDY might be due in part to the increase in the assimilable nitrogen content of the must, but also to the contribution of other nutrients present in this product based on autolyzed inactive yeast.

Thereafter, we studied the effect of increasing IDY supplementation from 0.2 g/L to 0.4 g/L in a *S. cerevisiae* strain (Figure 2). Maximal growth increased with an increasing IDY dose in the medium, especially at low nitrogen concentrations (Figure 2a). As this improvement in yeast growth might be due to the increase in nitrogen in the medium, mostly in the form of organic nitrogen (Table 1), the total nitrogen was recalculated taking into account the amount of nitrogen provided by the added IDY (20 mg N/L in 0.2 g IDY/L, 40 mg N/L in 0.4 g IDY/L) (Figure 2b). In that case, similar growth was observed regardless of the IDY supplementation, akin to the values obtained with SM-Mix, but higher than the growth reached with only inorganic nitrogen (SM-NH4) at concentrations above 80 mg N/L. This result indicates that either the presence of organic nitrogen or of other components present in the inactive yeasts might help improve yeast growth in wine fermentations.

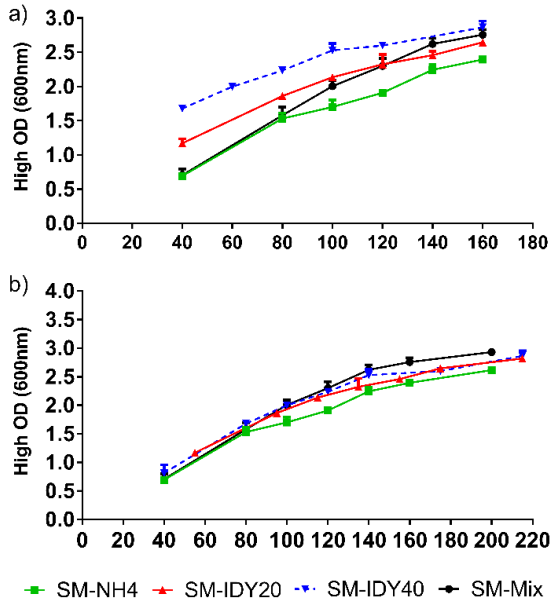

Figure 2. a) Maximal population (OD_{600nm}) obtained with *S. cerevisiae* (ScD), with different nitrogen sources in the medium, and at different nitrogen concentrations, from 40 to 200 mg N/L: ammonium (SM-NH4), ammonium and IDY at 0.2 or 0.4 g/L (SM-IDY20 and SM-IDY40), and a mixture of ammonium and amino acids (SM-Mix). (b) Maximal population (OD_{600nm}) obtained, considering the nitrogen supplemented by IDY in the total nitrogen concentration of each condition (20 mg N/L with 0.2 g/L and 40 mg N/L with 0.4 g/L).

3.3. Sequential fermentations in natural must

Subsequently, sequential fermentations with *T. delbrueckii* (Td5 and TdP) and *S. cerevisiae* (ScR) were performed, with and without IDY supplementation in a Verdejo must, to determine the effect of this nitrogen supplement on the fermentation and release of varietal thiols, which are known to be regulated by yeast nitrogen metabolism.

Single ScR fermentations finished in 19 days, with no effect of IDY supplementation (Figure 3a). All sequential fermentations finished in 34 days; however, the TdP strain showed a longer lag phase and slower initial fermentation kinetics than Td5 (Figure 3a). IDY supplementation did not show any effect on fermentation kinetics, except for a slight increase in the

fermentation rate of the *TdP , ScR* condition. The poor effect of this nutrient on the fermentative kinetics could be due to the high content of assimilable nitrogen present in the natural must (514.67 mg N/L). Population dynamics were also monitored during the sequential fermentations. Surprisingly, the presence of the Td5 strain did not allow *S. cerevisiae* to predominate in the fermentation, either with or without IDY addition (Figure 3b and c). ScR was only found in a higher proportion at the end points of TdP fermentation (Figure 3d and 3e). Indeed, the predominance of ScR over TdP was higher when the IDY addition was applied (Figure 3e).

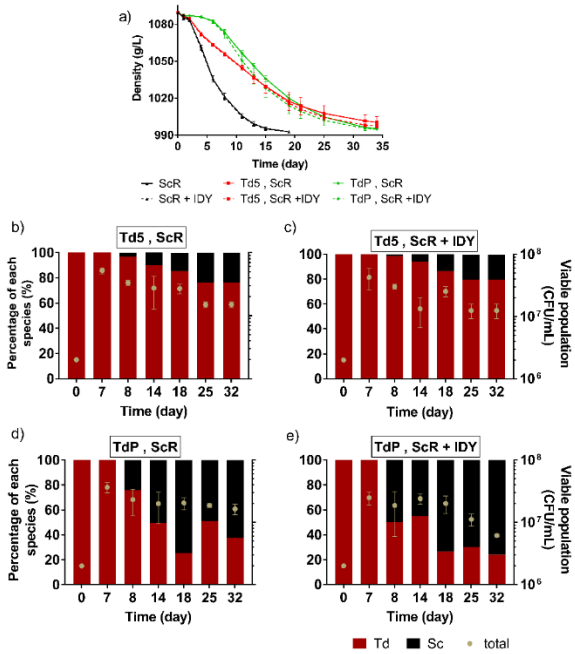

Figure 3. a) Must density during fermentations with natural Verdejo must, using *S. cerevisiae* (ScR) in single and sequential inoculation with *T. delbrueckii* (Td5, TdP), without (solid lines) and with (dropped lines) nutrient supplementation (IDY). Viable population and percentage of each species during sequential fermentations without (b and d) and with (c and e) nutrient supplementation (IDY).

Nitrogen consumption was followed during the first eight days of fermentation (Figure 4, Supplementary Table S3). In general, depletion of nitrogen in the medium was slower in all fermentations with IDY supplementation, probably due to the increased nitrogen content provided by such nutrients.

Nevertheless, fermentations performed with the same strain (with and without IDY) exhibited a similar nitrogen consumption pattern, although total nitrogen uptake was higher in IDY-supplemented conditions. Single *S. cerevisiae* fermentations revealed a faster nitrogen consumption profile than sequential fermentations, with threonine and alanine being the least preferred and slowest consumed amino acids. Moreover, there were some differences between *T. delbrueckii* strains used in sequential fermentations, since TdP consumed more nitrogen, especially organic nitrogen, before *S. cerevisiae* inoculation (Day 2) than Td5, although at the end of the process, the total nitrogen consumed by both strain combinations was similar. In contrast, larger differences in nitrogen consumption were detected between both sequential fermentations when IDY was added, since the *Td5 , ScR* combination consumed approximately 150 mg N/l more than *TdP , ScR.* Additionally, the lower ammonium consumption in the sequential fermentations (approximately half of that consumed in single *S. cerevisiae* fermentations) suggested that *T. delbrueckii* had a preference for organic nitrogen sources (Supplementary Table S3).

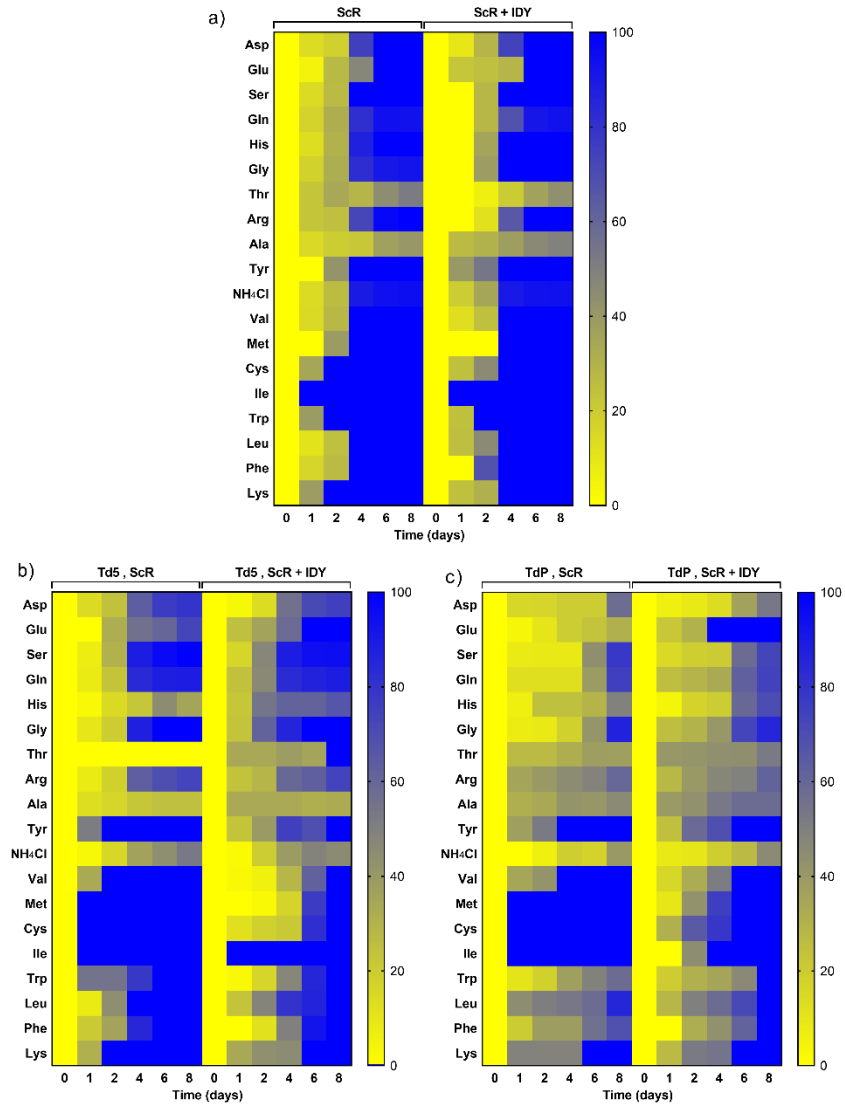

Figure 4. Nitrogen consumption during the fermentation of Verdejo must, using *S. cerevisiae* (ScR) in single (a) and sequential inoculation with *T. delbrueckii* Td5 (b) or with *T. delbrueckii* TdP (c), and with and without IDY supplementation.

Some organic compounds with oenological interest were analyzed at the end of the fermentations (Supplementary Table S4). Acetic acid concentrations were significantly higher in single ScR fermentations (values

above 0.4 g/L) than in mixed fermentations with *T. delbrueckii* (values between 0.12 and 0.2 g/L). Conversely, mixed fermentations with Td5 (with or without IDY) showed an increase in glycerol concentration and a slight decrease in ethanol content.

3.5. Metabolite contributions to aroma

Volatile varietal thiols of wines obtained by single and sequential fermentations, with and without IDY supplementation, were analyzed. The detected compounds were 3-mercaptohexan-1-ol (3-MH) and 3-mercaptohexyl acetate (3-MHA) (Figure 5).

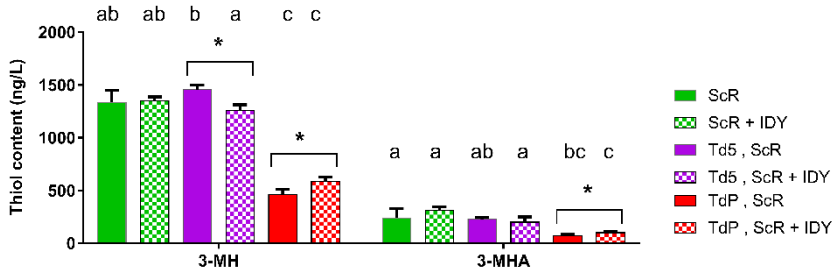

Figure 5. Concentration of thiols at the end of the fermentations of Verdejo must, using *S. cerevisiae* (ScR) in single and sequential inoculation with *T. delbrueckii* (Td5, TdP) with and without IDY supplementation.
Lowercase letters indicate significant differences between inoculation conditions. * Indicates significant differences between the condition with and without IDY supplementation in each compound. 3MH: 3-mercaptohexan-1-ol, 3-MHA: 3-mercaptohexyl acetate.

The results showed very high concentrations of 3MH, which were higher than 1200 ng/L in most cases. Surprisingly, the wines produced with the combination of *TdP , ScR* strains presented a much lower synthesis of this compound (between 400 and 600 ng/L). There was a significant effect of IDY addition on 3MH content in sequential fermentations, although it was dependent on the *T. delbrueckii* strain, as the presence of IDY significantly increased 3MH content in wines fermented with *TdP , ScR* but decreased it

in those fermented with *Td5 , ScR*. The 3-MHA concentration was also lower in mixed fermentations with the TdP strain, although there was a slight but significant increase when IDY was applied. Once again, the limited effect of this nutrient on the release of varietal thiols, as described above for fermentative kinetics, could be due to the high assimilable nitrogen content present in the Verdejo must.

4. Discussion

The use of non-*Saccharomyces* species in winemaking has become widely established in recent years due to the desirable characteristics that they can bring to wines. Their use in simultaneous and sequential fermentations could positively contribute to the quality of wines (Ciani et al., 2010; Jolly et al., 2014). Relatedly, volatile thiols, a group of highly appreciated aromatic compounds associated with tropical and fruity aromas, are present in some grape varieties in nonvolatile cysteine-bound forms, which are cleaved by a yeast β-lyase activity to release the corresponding volatile thiols during fermentation (Ruiz et al., 2019). The expression and activity of this enzyme in *S. cerevisiae* seems to be regulated by the NCR mechanism (Subileau et al., 2008; Thibon et al., 2008). Although *T. delbrueckii* species have been described as contributing to the release of varietal thiols (Belda et al., 2017a), little is known about the relationship between nitrogen metabolism and the release of these aromatic compounds by non-*Saccharomyces* yeasts. Thus, in this study, we first analyzed the nutrient requirements of several non-*Saccharomyces* species, under different nitrogen conditions, and then we evaluated the impact of nutrient supplementation (in the form of IDY) on fermentation kinetics and varietal

thiol release in sequential fermentations with *T. delbrueckii* and *S. cerevisiae*.

First, we analyzed yeast growth under different nitrogen concentrations and compositions. Regarding the nitrogen concentration required to achieve the maximum growth, in general, *S. cerevisiae* and *T. delbrueckii* strains needed approximately 140 mg N/L. These results agree with those of Martínez-Moreno et al. (2012), who established a nitrogen threshold concentration for *S. cerevisiae* of approximately 140 mg N/L in a must with 200 g/L of sugars, although this limiting nitrogen value increased with the increase in sugars in the must. Indeed, Su et al. (2020) also observed that *T. delbrueckii* and *S. cerevisiae* strains shared the same nitrogen source preferences to sustain growth and fermentation. Conversely, *L. thermotolerans* showed the lowest growth, and the maximum values were obtained at approximately 80 mg N/L, although, as previously described by Roca-Mesa et al. (2020), nitrogen preferences in *Lachancea* spp. could be highly strain dependent.

Regarding the effect of the nitrogen composition on yeast growth, in each yeast species the maximum biomass obtained depended on the nitrogen source, with the mixed nitrogen source generally producing the highest and ammonium the lowest biomass. These results agree with previous reports, which showed that in *S. cerevisiae* the combination of organic and inorganic nitrogen improves growth and fermentation performance (Arias-Gil et al., 2007; Martínez-Moreno et al., 2012). These results are also consistent with our previous study, where *T. delbrueckii* and *L. thermotolerans* reached the highest biomass in the medium with both types

of nitrogen sources (organic and inorganic), and grew less in the medium containing only ammonium (Roca-Mesa et al., 2020). However, IDY supplementation improved yeast growth, mainly at low nitrogen concentrations, which was not only due to the increase in assimilable nitrogen. The use of inactive yeast preparations has been correlated with faster sugar consumption and a higher number of viable yeasts in alcoholic fermentations (Pozo-Bayón et al., 2009). This enhancement is due to the increase in the assimilable nitrogen content of the must, but probably also to the presence of other kinds of metabolites released from yeast autolysis, such as amino acids, peptides, polysaccharides, nucleotides, fatty acids, yeast walls, vitamins and minerals (Pozo-Bayón et al., 2009).

Next, we performed sequential fermentations with *T. delbrueckii* and *S. cerevisiae* strains, using Verdejo grape must, which is known to be rich in volatile thiol precursors (Ruiz et al., 2019), to study the effect of IDY supplementation on the fermentation and release of varietal thiols. Sequential fermentations showed slower fermentation kinetics than individual fermentations of *S. cerevisiae*, probably due to the longer lag phase and lower fermentation rate of these non-*Saccharomyces* strains. Moreover, *T. delbrueckii* was able to reach populations of 2 – 4×10^7 cells/mL before inoculation of *S. cerevisiae*, which allowed these non-*Saccharomyces* yeasts to be present until the end of fermentation, compromising the dominance of *S. cerevisiae*. Previous studies have revealed that in mixed inoculations, *S. cerevisiae* usually dominates the fermentation process at the middle and final stages, both in simultaneous and sequential inoculations with non-*Saccharomyces* yeasts (Azzolini et al.,

2012; Beltran et al., 2002; Lleixà et al., 2016; Renault et al., 2016, 2015). Nevertheless, other studies have also shown that *Saccharomyces* is not always the dominant species at the end of mixed fermentations (Zhu et al., 2021), which could be due to nutrient competition (Medina et al., 2012; Rollero et al., 2018a), the presence of toxic compounds (Albergaria et al., 2010; Branco et al., 2014) or cell-to-cell contact inhibitions (Nissen et al., 2003; Petitgonnet et al., 2019).

Nitrogen consumption in mixed fermentations was lower and slower than in single *S. cerevisiae* fermentations. Moreover, inorganic nitrogen consumption was significantly lower in mixed fermentations, suggesting a *T. delbrueckii* preference for organic nitrogen sources as previously reported (Kemsawasd et al., 2015a; Su et al., 2020). Regardless, the grape must used in this study was very rich in nitrogen, and no nitrogen limitation occurred throughout the fermentation, indicating that the lack of dominance of *S. cerevisiae* strains in the mixed fermentations was not due to the lack of nitrogen, but rather to the limitation of other essential nutrients, or other microbial interactions with *T. delbrueckii*.

We also observed that the wines obtained by sequential inoculation presented lower concentrations of acetic acid and ethanol than those fermented only with *S. cerevisiae*. These results agree with previous studies that observed a decrease in volatile acidity (Bely et al., 2008), and ethanol content (Contreras et al., 2015; Belda et al., 2015; Zhu et al. 2020) in wines in which *T. delbrueckii* was involved.

In most non-*Saccharomyces* species, the β-lyase activity, the main activity related to thiol release, seemed to be scarce (Belda et al., 2016b; Zott et al.,

2011), however, some strains of *T. delbrueckii* stood out for their high contribution to the thiol content in wine fermentations, increasing the levels of some thiols, such as 3-sulfanylhexano-1-ol (3-SH), but with no detectable effect on the production of 4-mercapto-4-methylpentane-2-one (4-MSP) (Renault et al., 2016; Zott et al., 2011). Instead, Belda et al. (2016b) showed that the *T. delbrueckii* Viniferm NS-TD strain (Td5) presented high β-lyase activity, which increased not only the concentration of 3-SH but also 4-MSP. Therefore, we analyzed the production of volatile thiols in sequential fermentations with two *T. delbrueckii* strains, one of which was the Td5 strain. In our study, the mixed fermentation with the Td5 strain produced significantly higher concentrations of varietal thiol aromas (3-MH and 3-MHA) than the TdP condition but similar concentrations to the single *S. cerevisiae* fermentation. In Belda et al. (2017a), using similar fermentation conditions (the same combinations of strains (Td5 and ScR) and a Verdejo must), greater differences between single and mixed fermentations were observed, although they evaluated other volatile thiols, 3-SH and 4-MSP. Conversely, IDY addition presented a different impact on thiol release depending on the *T. delbrueckii* strain, since a slight increase in thiols was detected when IDY was added to the *TdP , ScR* fermentation; however, a decrease in 3-MH was detected in *Td5 , ScR*. It must be taken into account that the Verdejo must used in this study had a high content of assimilable nitrogen, which most likely diminished the effect of IDY supplementation on fermentation, as no limitation of nitrogen occurred during fermentation. Moreover, the release of thiols by the action of β-lyase can be inhibited by the presence of ammonium or rich nitrogen

sources (NCR system, catabolic repression by nitrogen) (Dubourdieu et al., 2006; Roncoroni et al., 2011; Thibon et al., 2008), which indicates that this enzyme might have been inhibited in our fermentative conditions. Therefore, a similar study using grape must with limited nitrogen concentration would help to further analyze and understand the effect of sequential inoculation and IDY supplementation on the release of varietal thiols.

In conclusion, we have evidenced that *T. delbrueckii* and *S. cerevisiae* present similar nitrogen requirements, while *L. thermotolerans* shows more differences at the strain level. Moreover, IDY supplementation enhanced yeast growth in all tested strains, mainly under nitrogen limiting conditions, which was not only due to the increase in total nitrogen. In sequential inoculations with *T. delbrueckii* and *S. cerevisiae*, the imposition of the *S. cerevisiae* strain could be compromised by the presence of the other species, either due to competition for nutrients, or other microbial interactions. Conversely, the release of volatile thiols was not greatly affected by either the inoculation strategy used or the supplementation with IDY, probably due to the high initial nitrogen content of the grape must used in this study. Therefore, further studies are necessary to analyze specific interactions between *T. delbrueckii* and *S. cerevisiae* strains when used in sequential fermentations.

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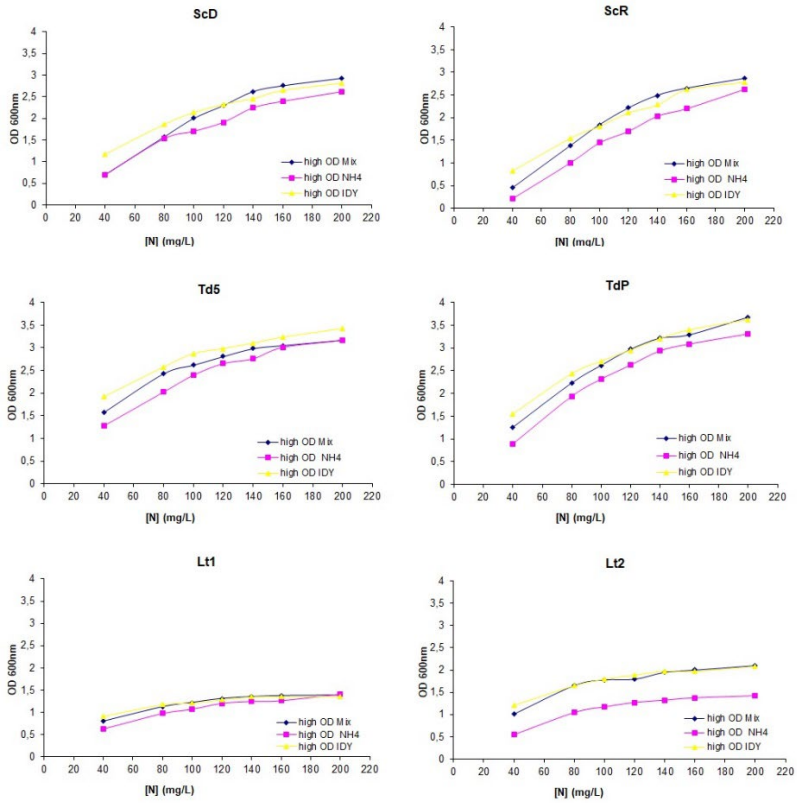
Supplementary Material

All supplementary material is attached below, except for Supplementary Table S2 that is not included in the present thesis due to format incompatibility, and is available online at: https://drive.google.com/drive/folders/1dd0-2fdjqn8ulcawWxGc5uchNeEmp0ed?usp=sharing.

Supplementary Table S2: Microplates data of yeast growth under different nitrogen media.



Supplementary Figure S1. Maximum growth (OD_{600}) obtained with different yeast species (*S. cerevisiae* (Sc), *T. delbrueckii* (Td) and *L. thermotolerans* (Lt)), in synthetic must containing different nitrogen sources (mixture of ammonium and amino acids (Mix), ammonium (NH4), and ammonium and IDY (IDY)) and at different nitrogen concentrations (from 40 to 200 mg/L). IDY was added at 0.2 g/L in a medium with increasing concentrations of ammonium.

Supplementary Table S1. Initial nitrogen content (mg N/L) of Verdejo must.

Nitrogen source	Concentration
Aspartic	7.03 ± 1.47
Glutamic	0.41 ±0.16
Serine	19.34 ±3.29
Glutamine	35.68 ±6.14
Histidine	4.73 ± 1.23
Glycine	18.69 ±2.01
Threonine	104.86 ±16.16
Arginine	62.74 ±12.23
Alanine	44.67 ±9.72
Tyrosine	3.83 ±0.67
Ammonia	190.94 ±2.45
Valine	3.35 ±0.34
Methionine	5.61 ±3.83
Cysteine	2.61 ±1.48
Isoleucine	1.62 ±0.89
Tryptophane	7.89 ±2.41
Leucine	4.82 ±0.72
Phenylalanine	5.45 ±1.20
Lysine	2.53 ±0.47
Total Nitrogen	514.67 ± 61.82
Organic Nitrogen	323.52 ± 49.37
Inorganic Nitrogen	190.94 ± 2.45

Supplementary Table S3. Nitrogen consumed (mg/L) along the first days of single and sequential fermentations of a Verdejo must, with and without IDY supplementation.

	Time (day)	Inorganic YAN	Organic YAN	Total YAN
ScR	1	22.53 ±4.28	103.01 ± 12.92	125.54 ±16.22
	2	44.81 3.67±	132.64 ± 12.06	162.51 ±20.05
	4	145.20 ± 1.00	250.10 ± 11.42	395.30 ±10.96
	6	150.96 ± 0.16	295.33 ± 8.53	446.29 ±8.55
	8	152.75 ±1.65	306.68 ± 13.16	459.43 ±12.92
ScR + IDY	1	33.93 ±1.11	51.16 ± 6.71	85.08 ±6.09
	2	59.63 ±2.52	139.72 ± 14.34	199.35 ±13.78
	4	150.49 ±1.56	332.74 ± 13.25	483.23 ±14.80
	6	153.51 ±0.98	415.92 ± 5.02	569.42 ±5.82
	8	154.39 ±0.78	427.66 ± 5.27	582.05 ±6.01
Td5 , ScR	1	3.17 ±1.63	67.55 ± 8.09	70.72 ±6.51
	2	27.92 ±2.78	111.73 ± 15.74	139.65 ±15.59
	4	56.86 ±6.34	220.99 ± 7.57	277.85 ±13.72
	6	69.97 ±5.24	241.87 ± 9.07	311.84 ±14.25
	8	85.12 ±0.16	247.07 ± 8.37	332.19 ±8.51
Td5 , ScR+IDY	1	3.49 ±0.60	145.97 ± 14.34	149.45 ±14.03
	2	33.07 ±0.41	186.24 ± 10.68	219.31 ±11.08
	4	62.68 ±1.93	248.07 ± 20.23	310.75 ±22.16
	6	76.60 ±6.94	323.23 ± 8.16	399.83 ±14.35
	8	80.82 ±5.67	472.31 ± 3.52	553.13 ±8.72
TdP , ScR	1	12.71 ±4.71	157.74 ± 10.20	170.45 ±6.28
	2	13.16 ±3.17	169.71 ± 10.60	182.86 ±13.69
	4	27.48 ±5.92	210.58 ± 11.96	238.06 ±17.56
	6	30.61 ±3.09	235.14 ± 14.99	265.75 ±17.81
	8	64.14 ±3.17	281.54 ± 16.26	345.68 ±15.27
TdP , ScR+IDY	1	12.66 ±3.96	167.67 ± 5.14	180.32 ±6.70
	2	19.33 ±7.72	223.03 ± 12.80	242.37 ±11.70
	4	29.83 ±7.80	254.22 ± 10.33	284.06 ±17.71
	6	44.50 ±4.51	325.84 ± 15.53	370.34 ±18.29
	8	72.30 ±6.54	326.73 ± 12.19	399.04 ±18.71

Supplementary Table S4. Oenological interesting compounds obtained at the end of single and sequential fermentations of a Verdejo must, with and without IDY supplementation.

	Sugars (g/L)	Glycerol (g/L)	Acetic acid (g/L)	Ethanol (%, v/v)
ScR	1.54 ± 0.84	4.61 ± 0.36^{b}	0.39 ± 0.01^{a}	12.80 ± 0.00^{a}
ScR + IDY	1.29 ± 0.14	5.42 ±0.20^{ab}	0.40 ± 0.02^{a}	12.92 ± 0.03^{a}
Td5 , ScR	0.89 ± 0.88	5.85 ±0.24^{a}	0.19 ± 0.06^{b}	12.22 ± 0.06^{b}
Td5 , ScR + IDY	0.48 ± 0.16	5.80 ±0.61^{a}	0.13 ± 0.01^{b}	12.42 ± 0.18^{b}
TdP , ScR	1.78 ± 1.51	4.59 ±0.24^{b}	0.17 ± 0.02^{b}	12.68 ± 0.10^{a}
TdP , ScR + IDY	0.72 ± 0.08	4.82 ±0.21^{b}	0.20 ± 0.03^{b}	12.68 ± 0.10^{a}

Lowercase letters indicate significant differences between fermentations in each compound.

Capítulo II

Estudio de las interacciones entre levaduras vínicas en fermentaciones mixtas.

Capítulo II.1

"Micronutrients and organic nitrogen importance in fermentations with *Torulaspora delbrueckii* and *Saccharomyces cerevisiae*."

Capítulo II.2

"The impact of nutrient supplementation and inoculum size in sequential fermentations with *Lachancea thermotolerans* and *Saccharomyces cerevisiae*."

Capítulo II.3

"Effect of the combined use of *Torulaspora delbrueckii* and *Lachancea thermotolerans* on fermentation performance and wine aroma composition."

UNIVERSITAT ROVIRA I VIRGILI
OPTIMIZACIÓN DEL USO DE LEVADURAS NO SACCHAROMYCES EN FERMENTACIONES MIXTAS: REQUERIMIENTOS NUTRICIONALES E INTERACCIONES MICROBIANAS
Elena Roca Mesa

Capítulo II. 1

Micronutrients and organic nitrogen importance in fermentations with *Torulaspora delbrueckii* and *Saccharomyces cerevisiae*

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Elena Roca Mesa

Abstract:

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

Keywords: winemaking; nutrients; vitamins; oligoelements; cell-to-cell contact.

1. Introduction

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

Winemakers must adapt wine production to consumer demands. For many years, one of the objectives of winemakers has been to obtain wines with predictable and reproducible quality and to place wines with similar characteristics on the market year after year. For this purpose, *S. cerevisiae* commercial yeasts have been widely used as single inocula in alcoholic fermentations, thus avoiding the presence and participation of non-*Saccharomyces* species during this process (Fleet and Heard, 1993). Currently, consumers and winemakers look for wines with special traits and characteristics, which can be obtained when several yeast species (also non-*Saccharomyces*) are involved in fermentation, e.g., in spontaneous or mixed fermentations. Although non-*Saccharomyces* yeasts have been considered spoilage microorganisms for many years, some of them are currently used in mixed fermentations with *S. cerevisiae* to improve certain wine characteristics (Jolly et al., 2014). For example, *Lachancea thermotolerans* produces relevant amounts of lactic acid, *Metschnikowia* spp. and *Starmerella bacillaris* enhance varietal aromas, *Torulaspora delbrueckii* and *Wickerhamomyces anomalus* enhance fruity notes, *Starmerella bacillaris* increases glycerol levels and reduces acetaldehyde and total SO_2, and

Kazachstania aerobia increases marzipan and floral aromas (Tufariello et al., 2021). Moreover, because of climate change, the oenological community is currently focused on finding strategies to reduce ethanol content in wines, being one of them the use of some non-*Saccharomyces* yeasts, such as *Metschnikowia pulcherrima, Zygosaccharomyces bailii* or *T. delbrueckii,* in combination with *S. cerevisiae* (Canonico et al., 2019; Contreras et al., 2015; Morales et al., 2015; Puškaš et al., 2020; Quirós et al., 2014; Zhu et al., 2020).

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

delbrueckii could produce fruity esters and higher alcohols, and *S. cerevisiae* could ensure the total consumption of sugars.

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

Other microbial interactions do not involve physical contact and are considered indirect interactions, such as competition for nutrients and the

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

The aim of this work was to investigate the possible causes that can compromise *S. cerevisiae* domination in mixed alcoholic fermentation with *T. delbrueckii*. For this purpose, fermentations with two commercial strains of *T. delbrueckii* and *S. cerevisiae* were carried out in synthetic must with and without cell-to-cell contact. Fermentation kinetics, population

dynamics and the synthesis of fermentative byproducts of pure cultures of each species were compared with sequential inoculations. The influence of different variables, such as species strain, inoculum size, nitrogen requirements and nutrient supplementations, were analyzed.

2. Materials and Methods

2.1. Yeast strains and media

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

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

2.2. Fermentation conditions and sampling

Single and sequential fermentations were performed in synthetic must as described in Beltran et al. (2004). The initial nitrogen content was 300 mg N/L in all cases, except for the high nitrogen condition (600 mg N/L) (Roca-Mesa et al., 2020). In some sequential fermentations, nutrient supplementation was performed before the inoculation of the second yeast

strain. The concentrations of the supplemented compounds (vitamins, amino acids, oligoelements, anaerobic growth factors, ammonium, thiamine, or zinc) were the same as in the initial synthetic must. Actimax Natura (Agrovin S.A., Spain) was used as an inactivated dried yeast (IDY) supplement, added at 20 g/HL. This concentration corresponds to 44 mg of yeast-assimilable nitrogen (YAN) per litre analized by HPLC.

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

Inoculations were performed as described in Table 1. Coinoculated fermentations were performed with three different ratios of ScR and Td5 strains (1:1, 1:9 and 9:1). In sequential fermentations, the second species (*S. cerevisiae*) was inoculated when the density decreased by approximately 15 units (g/L). In the sequential fermentation with cell contact, both species coexisted in the medium, while in the sequential fermentations without cell-to-cell contact, the medium was centrifuged and filtered to discard the cells of the first species before the inoculation of the second species.

Table 1. Inoculation strategies.

Inoculum Procedures	Name	Inoculum ratios (Td:Sc)	*T. delbrueckii* (Td)	*S. cerevisiae* (Sc)
Single inoculation	Td5 TdB	(1:0)	2×10^6	-
Single inoculation	ScR ScQA	(0:1)	-	2×10^6
Coinoculation	ScR:Td5	1:1	1×10^6	1×10^6
		1:9	1.8×10^6	2×10^5
		9:1	2×10^5	1.8×10^6
Sequential inoculation	Td_x , Sc_x	1:1	2×10^6	2×10^6
Sequential without contact*	Td_x / Sc_x	1:1	2×10^6	2×10^6

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

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

2.3. Microplate growth monitoring

To evaluate the effect of some nutrient supplementation on S*. cerevisiae* growth, different fermentation conditions were tested in 96-well

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

2.4. Nitrogen analysis

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

Yeast-assimilable nitrogen (YAN, expressed as mg N/L) was calculated according to the nitrogen atoms of each amino acid and ammonium.

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

2.5 Potential inhibitory compounds

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

To determine the presence of peptides or proteins produced by *T. delbrueckii* with potential inhibitory activity against *S. cerevisiae*, the protein profile of the medium after 48 h of fermentation was studied by high-resolution size-exclusion chromatography (HRSEC) (Canals et al., 1998). Briefly, 15 mL of the must was lyophilized, resuspended in 0.6 µL of 300 mmol/L ammonium acetate and centrifuged (12 000 × g for 10 min). The supernatant was filtered through a 0.22 µm pore size filter (Dominique

Dutscher, Brumath, France), and 100 µL was injected into the HPLC Agilent 1200 Series system (Agilent Technologies, Barcelona, Spain) with a diode array detector (DAD) to monitor the output at 230, 280 and 320 nm. Separation was carried out at 20 °C using an S 165 Shodex gel permeation HPLC column (OHpak 166 SB-803 HQ, 300 mm× 8 mm i.d.; Showa Denko). The mobile phase consisted of an aqueous solution of 300 mmol/L ammonium acetate applied at a constant flow of 0.6 mL/min for 70 min. The proteins were quantified according to the peak area for each fraction using bovine serum albumin as an external standard (Sigma–Aldrich) in the range between 0 and 10 mg/mL (r2 >0.99).

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

2.6. Metabolite analysis

The concentrations of some organic metabolites were determined by HPLC at the end of fermentation, following the protocol described by Quirós et al. (2010). These analyses were performed using an Agilent 1100 HPLC (Agilent Technologies, Germany) equipped with a Hi-Plex H, 300 mm x 7.7 mm column inside a 1260 Multicolumn Thermostat (Infinity II MCT) and connected to two detectors, a multiwavelength detector (G1365B MWC) and a 1260 Infinity II refractive index detector (Agilent Technologies, Germany). Chromatograms were analyzed using Agilent ChemStation Plus

software (Agilent Technologies, Germany). In some cases, residual sugars were also analyzed with a Y-15 multi analyzer using an enzymatic kit (BioSystems, Barcelona, Spain). Thiamine concentration was measured by a VitaFast® Vitamin B1 Microbiological Kit (R-Biopharm, AG, Germany).

2.7. Statistical analysis

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

3. Results

3.1. Single, coinoculated and sequential fermentations of T. delbrueckii and S. cerevisiae

The different fermentations performed (single, coinoculated and sequential fermentations) between Td5 and ScR strains are shown in Figure 1a. As expected, the *S. cerevisiae* single fermentation was faster than the *T. delbrueckii* single fermentation. Td5 finished the fermentation in fourteen days, while ScR only required ten days. The sequential fermentation finished all the sugars in fourteen days, similar to Td5 single fermentation, but it showed a higher fermentation rate, mainly after *S. cerevisiae* inoculation. In the case of coinoculated fermentations, three different inoculation ratios of ScR and Td5 (1:1, 9:1 and 1:9) were used. Surprisingly, the coinoculations with a higher population of *S. cerevisiae* cells (9:1 or 1:1)

finished faster than the ScR single fermentation, while the fermentation with a higher proportion of *T. delbrueckii* cells (1:9) required two more days, finishing at the same time as the ScR single fermentation (Figure 1a). Population dynamics were monitored in the different mixed inoculations (Figure 1b-e). We observed that in the sequential fermentation, even if ScR significantly increased its population after its inoculation at 48 h, reaching a maximal population of 2 x 10^7 CFU/mL, this species was not able to become the dominant yeast species, as Td5 was present at higher population levels until the end of the fermentation (8.9 x 10^7 CFU/mL) (Figure 1b). In the coinoculated fermentations, *S. cerevisiae* dominated the fermentation except when *T. delbrueckii* was inoculated at a higher proportion (9:1) (Figure 1c-e). When the two species were inoculated with the same ratio (1:1), Td5 was still present at the end of fermentation and comprised 40% of the total population.

To confirm the results obtained in the sequential inoculations and determine if the dominance of *T. delbrueckii* over *S. cerevisiae* was strain dependent, we performed sequential fermentations using two strains of each species. We observed similar results in all tested combinations, with *T. delbrueckii* being the dominant species throughout all the fermentations (Supplementary Figure S1). Indeed, ScR growth was impaired when TdB started the fermentation process, with no viable ScR cells detected on the plates after the 3rd day of fermentation, probably because the dilution factor needed to count TdB prevented the detection of ScR. Regardless, all sequential fermentations were able to deplete sugars (Supplementary Figure

S2), even though *T. delbrueckii* was the dominant species throughout the fermentation process.

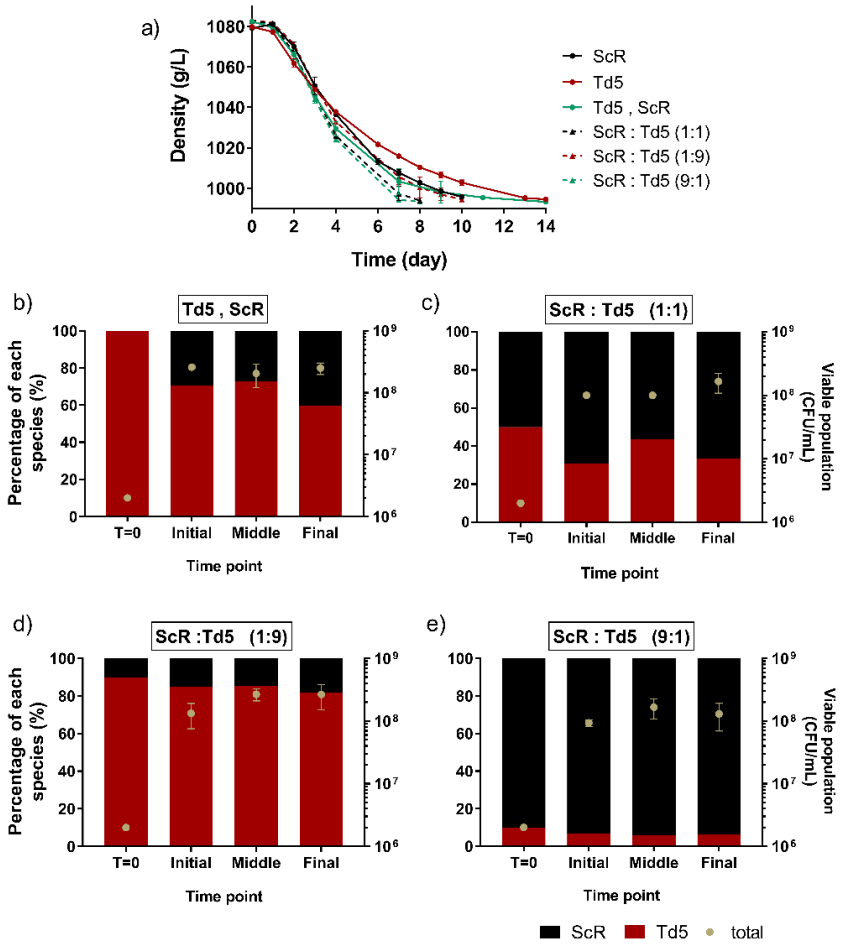

Figure 1. a) Must density of single inoculations with Td5 or ScR, sequential inoculation (Td5, ScR) and coinoculation at different ratios (ScR:Td5, 1:1, 1:9, 9:1) in synthetic must fermented at 16 °C. Total yeast viability (CFU/mL) and percentage of each species through the sequential (b) and coinoculated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

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

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

Surprisingly, when *T. delbrueckii* cells were removed by centrifugation and filtration prior to *S. cerevisiae* inoculation, all fermentations were stuck in all strain combinations (Figure 2a). Indeed, even if *S. cerevisiae* was able to grow after its inoculation in the centrifuged must, reaching up to 2.5 x 10^7 CFU/mL (Figure 2b), this population might not have been sufficient to complete the fermentation.

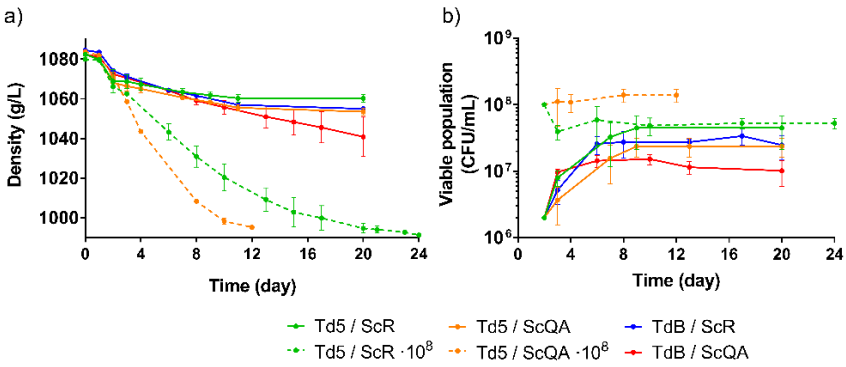

Figure 2. a) Must density and b) *S. cerevisiae* cell growth (CFU/mL) through the sequential fermentations performed with *T. delbrueckii* (Td5 and TdB) and *S. cerevisiae* (ScR and ScQA) strains, without contact between the two yeast species (*T. delbrueckii* cells were removed 48 h after fermentation,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

For this reason, sequential fermentations without cell-to-cell contact but with a higher *S. cerevisiae* inoculum size were performed using two different *S. cerevisiae* strains. Therefore, after removing *T. delbrueckii* cells at 48 h, we inoculated *S. cerevisiae* cells at 10^8 cells/mL instead of 2 x 10^6 cells/mL. Our results showed that with this inoculum size, fermentations finished, although ScQA needed 12 days to consume all the sugars, while ScR required 24 days (Figure 2a). These results suggested that the growth impairment of *S. cerevisiae* after the presence of *T. delbrueckii* in the media might be one of the reasons for the stuck fermentations.

3.3 Analysis of potential inhibitory compounds

A possible explanation for the growth impairment of *S. cerevisiae* could be the presence of killer toxins or other inhibitory compounds released by the *T. delbrueckii* strains. Therefore, we first tested the killer phenotype of the *T. delbrueckii* strains used in this study. The results obtained by the MB plates were confirmed by analyzing the presence of virus dsRNA molecules, indicating the absence of the killer viruses on those strains (Supplementary Figure S3) and thus discarding killer toxins as a possible cause for *T. delbrueckii* dominance. Then, the secretion of peptides or proteins with potential inhibitory activity against *S. cerevisiae* by *T. delbrueckii* was studied. For this purpose, the protein content in the medium after 48 h of fermentation with each strain was compared. A species-dependent profile was observed, with the media fermented with *S. cerevisiae* strains presenting the highest protein concentration compared to those fermented with *T. delbrueckii* strains. In both species, the most enriched protein fractions were those that contained the largest (the fraction of proteins with

molecular weights of more than 75 kDa) and the smallest proteins (proteins of less than 25 kDa) (Figure 3a).

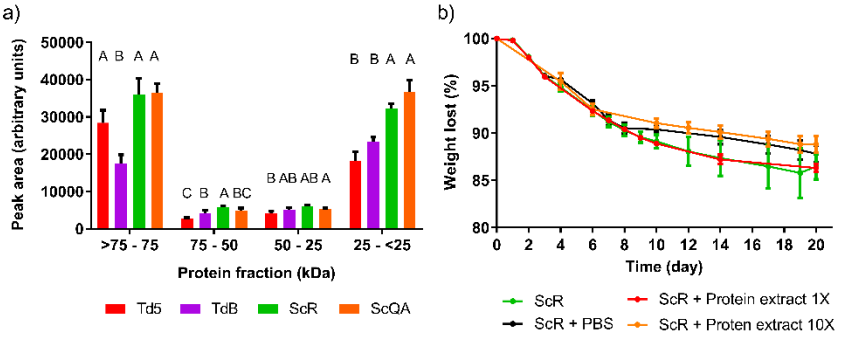

Figure 3. a) Protein fractions, expressed as peak areas (arbitrary units), obtained from the medium after 48 h of fermentation with different strains of *T. delbrueckii* (Td5 and TdB) and *S. cerevisiae* (ScR and ScQA). b) Fermentation kinetics of ScR (measured as weight loss normalized to the initial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

Finally, the protein extract, obtained by centrifugation and lyophilisation of the medium after 48 h of Td5 fermentation, was added to fresh synthetic must to determine if any substances present in this extract affected the ScR fermentation performance or even inhibited its growth (Figure 3b). The addition of Td5 protein extracts did not have an effect on the fermentation kinetics of ScR at any concentration (1x and 10x), thus indicating that the proteins secreted by Td5 during fermentation might not be a cause of the fermentation problems of *S. cerevisiae* strains.

3.4. Effect of nitrogen supplementation

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

nitrogen supplementation, to determine whether nitrogen limitation could explain the growth impairment of *S. cerevisiae*.

The analysis of the nitrogen in the medium revealed that *T. delbrueckii* strains consumed a large fraction of the nitrogen present in the must prior to *S. cerevisiae* inoculation at 48 h, with the remaining nitrogen being rather limited for *S. cerevisiae* growth (44.94 mgN/L in Td5 fermented medium and 89.44 mgN/L in TdB fermented medium) (Supplementary Table S1). Indeed, the patterns of nitrogen consumption were quite different between the two *T. delbrueckii* strains. While Td5 consumed amino acids and ammonia faster than *S. cerevisiae* strains, TdB consumption was similar to that of *S. cerevisiae* strains. As expected, when high-nitrogen must (600N) was used, more nitrogen was left in the medium at 48 h (Figure 4a, Supplementary Table S1), with 176.55 mg N/L still remaining when *S. cerevisiae* was inoculated.

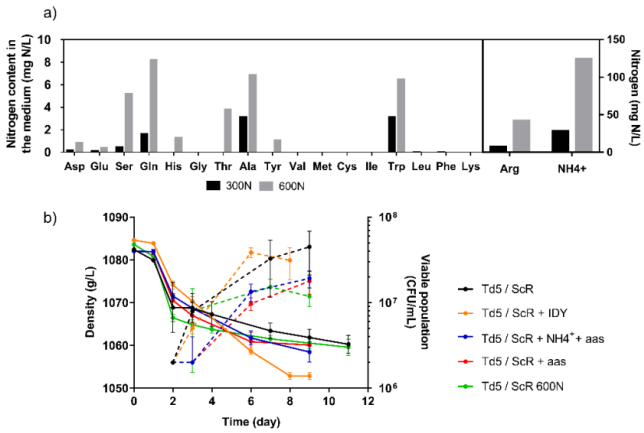

Figure 4. a) Nitrogen present in the medium at 48 h of Td5 fermentation in synthetic must with 300 and 600 mg N/L. b) Must density (solid lines) and ScR viable population (dotted lines) throughout sequential fermentations without cell contact, with the addition of different nitrogen sources when ScR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

Then, to reveal whether the nitrogen limitation for *S. cerevisiae* growth after *T. delbrueckii* presence was due to the lack of some specific amino acid, we tested the effect of adding each amino acid individually to the medium (previously fermented by *T. delbrueckii* for 48 h) on *S. cerevisiae* growth (Supplementary Table S2). No large differences were observed among the different amino acid supplementations, and the best growth was obtained with the supplementation of a mixture of all amino acids.

After that, we analyzed whether supplementation of the medium with more complex nitrogen sources at the time of *S. cerevisiae* inoculation, such as a commercial product based on inactive and autolysed yeast rich in nitrogen (IDY), a mixture of amino acids and ammonium, and a mixture of only amino acids was enough to prevent the growth impairment of these *S. cerevisiae* strains. Additionally, a sequential fermentation with high nitrogen content in the must was also performed (*Td5/ScR* 600N) (Figure 4b). Surprisingly, this surplus of nitrogen did not result in higher *S. cerevisiae* populations or higher fermentation capacity, with this fermentation also stuck after *S. cerevisiae* inoculation and showing similar fermentation and growth profiles as the conditions with lower nitrogen. Indeed, *S. cerevisiae* reached similar cell populations with all nitrogen supplementations (Figure 4b), and all of them resulted in stuck fermentations, indicating that nitrogen limitation was not the reason for the *S. cerevisiae* growth and fermentation impairments. In fact, the addition of IDY, which includes nutritional compounds other than nitrogen, seemed to be the most efficient supplement, both for growing and fermenting, although it was not sufficient to consume all sugars. These results suggest

that competition for other kinds of nutrients could be the reason for the stuck fermentations.

3.5. Effect of supplementation with other nutrients

To explore the impact of other nutrients on *S. cerevisiae* growth and sequential fermentations, the effects of vitamin, oligoelement and anaerobic growth factor supplementation were studied. In all cases, nutrients were added after removing *T. delbrueckii* from the medium at 48 h and at the same time as *S. cerevisiae* inoculation. The synergistic effect of adding vitamins, oligoelements, anaerobic growth factors and amino acids together was also tested (Figure 5a).

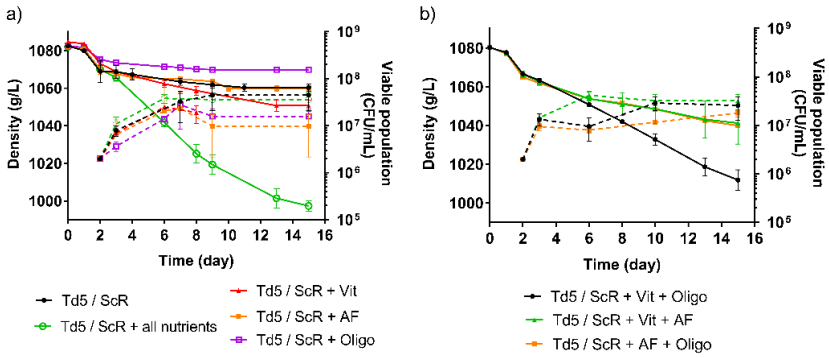

Figure 5. Must density (solid lines) and ScR viable population (dotted lines) throughout sequential fermentations without yeast contact, with the addition of different nutrients when ScR was inoculated: (a) individual addition of different nutrients compared to adding a mixture of all these nutrients;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

The addition of only one of the tested nutrients at a time also resulted in stuck fermentations. In contrast, when all nutrients were added simultaneously (*Td5/ScR* + all nutrients), the fermentation process finished correctly in fifteen days, suggesting that *S. cerevisiae* needs a complex

medium to finish the fermentation in sequential inoculations. After these results, we performed fermentation in synthetic must with high nitrogen content (to ensure that nitrogen was not limiting) with the addition of two of the tested nutrients at a time. The results revealed that the joint addition of vitamins and oligoelements was the combination with the best results in the fermentation kinetics (Figure 5b). Therefore, to decipher which vitamin and oligoelement were required for correct *S. cerevisiae* performance, screenings combining each vitamin and oligoelement at a time were performed in microfermentations. Our results showed that thiamine had the largest impact on ScR growth (Supplementary Table S3Figure). Thus, thiamine consumption by the different strains of T*. delbrueckii* and *S. cerevisiae* was analyzed at 24 and 48 h. The initial thiamine concentration in the must was 0.29 mg/L, and after 24 h of fermentation, the remaining thiamine in the medium in all strains and conditions was less than 0.08 mg/L, the detection limit of the technique (Supplementary Table S4), indicating that thiamine was depleted and could be limiting in all conditions.

Subsequently, we tested the effect of thiamine on the growth of *S. cerevisiae* in sequential fermentation by a single thiamine addition, combined with all the oligoelements or combined with only zinc, as zinc was the oligoelement that resulted in better growth in the previous experiment (Supplementary Table S3). Our results showed that thiamine alone had the lowest impact (Figure 6a).

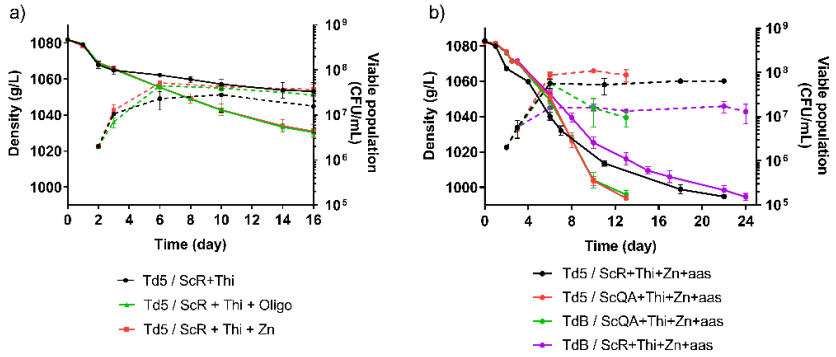

Figure 6. a) Must density (solid lines) and ScR viable population (dotted lines) in sequential fermentations without yeast contact, supplemented with thiamine (Thi) alone or combined with other nutrients: oligoelements (oligo) or zinc (Zn). b) Must density (solid lines) and *S. cerevisiae* viable populations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

However, the addition of thiamine with the mixture of oligoelements or with zinc had a positive and similar effect on the fermentation process and yeast growth, although fermentations were still incomplete.

Finally, we tested the addition of thiamine, zinc and amino acids in synthetic must using different *T. delbrueckii* and *S. cerevisiae* strains without contact between species. All fermentations were finished, demonstrating the positive effect of this supplementation (Figure 6b). ScQA was the fastest strain to ferment musts partly fermented by either TdB or Td5, depleting sugars in 13 days. In contrast, ScR needed between 22 and 24 days to consume all sugars in musts coming from Td5 or TdB fermentations, respectively. *S. cerevisiae* growth was similar in all fermentations performed without yeast species contact (Figure 6b). This supplementation was also tested in sequential fermentations between Td5 and ScR but without removing Td5 to determine if the imposition of *S. cerevisiae* increased after this addition of nutrients (Figure 7). Moreover,

this addition of thiamine, zinc and amino acids was compared to IDY supplementation. In all cases, *S. cerevisiae* strains grew more than in the conditions without nutrient supplementation (Figure 7, Figure 2b), although still without becoming the dominant yeast species after (Figure 7b-c), and the supplementation that most increased the *S. cerevisiae* imposition in both strains was IDY, which was close to 50% (Figure 7d-e).

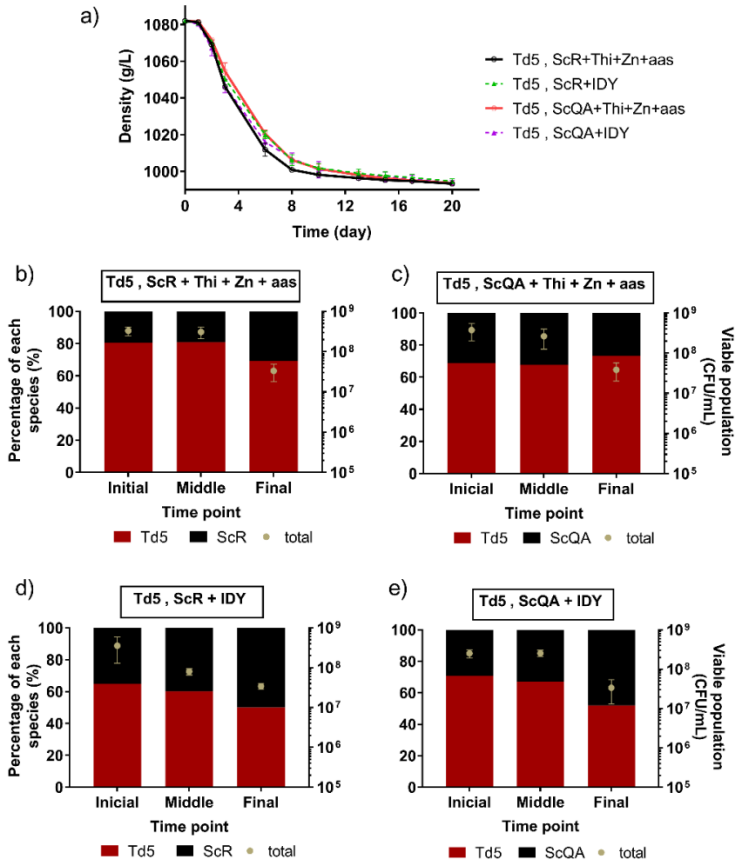

Figure 7. a) Must density of sequential fermentations performed with *T. delbrueckii* (Td5) and *S. cerevisiae* (ScR and ScQA) strains, supplemented with Thi, Zn and aas or with IDY when *S. cerevisiae* was inoculated (48 h). Population dynamics during sequential fermentations: (b) *Td5 , ScR*+Thi+Zn+aas,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

Compounds of oenological interest were analysed at the end of fermentations (Supplementary Table 5). Single fermentations using *S. cerevisiae* strains presented higher glycerol and acetic acid contents and a lower lactic acid content than wines fermented with single *T. delbrueckii* strains. When the coinoculation strategy was applied, the acetic, lactic, citric and tartaric acid contents decreased, while the malic and succinic acid contents increased in comparison with *S. cerevisiae* single fermentations. Furthermore, coinoculated wines presented lower ethanol and glycerol contents. Under the sequential inoculation strategy, in general, wines presented a higher content of malic acid and a lower content of lactic acid. No significant effect on ethanol content was observed due to the inoculation strategy.

4. Discussion

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

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

Although sequential fermentations proceeded normally and finished in the expected time, surprisingly, *S. cerevisiae* did not prevail over *T. delbrueckii* strains at the end of the fermentation. This fact was unusual because *S. cerevisiae* usually dominates the fermentation process in the middle and final stages of fermentation (Azzolini et al., 2012; Beltran et al., 2002; Lleixà et al., 2016; Renault et al., 2015, 2016), usually attributed to the different ethanol and sulfur dioxide (SO_2) sensitivities of the non-*Saccharomyces* yeasts (Ribéreau-Gayon et al., 2006). Similar results were obtained by Zhu et al. (2021), since in their study, *S. cerevisiae* failed to dominate sequential fermentations with a mixture of non-*Saccharomyces* species (*T. delbrueckii, Zygosaccharomyces bailii* and *M. pulcherrima*), with the *T. delbrueckii* strain being dominant at the end of the process, despite SO_2 treatment of the natural must. However, the same authors also tested sequential fermentations with single non-*Saccharomyces* species, observing the imposition or not of *S. cerevisiae* depending on the non-*Saccharomyces*

species used. The *S. cerevisiae* strains were able to clearly impose over the *M. pulcherrima* strain but not always over the *Lachancea thermotolerans* strain (Zhu et al., 2021). Yet, in the coinoculated fermentations, our results showed that *S. cerevisiae* was able to dominate the fermentation in all cases except when *T. delbrueckii* was inoculated at a higher ratio. In contrast, Zhu et al. (2021) showed that *S. cerevisiae* dominated at the end of the coinoculated fermentations regardless of the proportions of the non-*Saccharomyces* and *S. cerevisiae* used, and Taillander et al. also observed *S. cerevisiae* dominance, even when inoculated in mixed fermentations with a population 20 times lower than that of *T. delbrueckii* (Taillandier et al., 2014).

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

presence and persistence of particular species (Kemsawasd et al., 2015; Petitgonnet et al., 2019; Wang et al., 2016). A few years ago, a strain of *T. delbrueckii* with a killer phenotype (Kbarr-1) was described to be able to dominate fermentations in the presence of *S. cerevisiae* (Ramírez et al., 2015; Velázquez et al., 2015). However, in our study, we disproved that the cause of the impaired growth and fermentation of *S. cerevisiae* strains was the secretion of killer toxins since the tested strains of *T. delbrueckii* did not have the genetic requirements. Moreover, the presence of other potential toxic compounds, such as some antimicrobial peptides, was also ruled out since supplementation with protein extracts from *T. delbrueckii*-fermented medium did not seem to affect S. cerevisiae growth in this case.

Finally, to study whether this lack of imposition was due to some cell-to-cell contact mechanism, sequential fermentations without contact between the *T. delbrueckii* and *S. cerevisiae* cells were performed, and surprisingly, the strains of *S. cerevisiae* were unable to complete these fermentations. However, when these noncontact sequential fermentations were carried out with a larger population of the *S. cerevisiae* strain (10^8 cells/mL instead of 10^6 cells/mL, the normal inoculum size), they were able to finish the fermentation. Therefore, these results suggested that these stuck fermentations were caused by the impaired growth of *S. cerevisiae*, probably due to a lack of some essential nutrients, since when a larger inoculum size was applied and consequently, *S. cerevisiae* hardly needed to double to reach its maximum population, it was able to deplete sugars. Indeed, some authors have described that the fast and efficient intake of nutrients could negatively affect the fitness of a particular yeast species (Rollero et al., 2018;

Su et al., 2020). These results also correlated well with a greater effect in sequential fermentations since the previous growth of non-*Saccharomyces* strains reduced the nutrients available in the medium for *S. cerevisiae* strains, limiting their growth capacity. Instead, in coinoculations, as both species were inoculated simultaneously, this effect is reduced, and *Saccharomyces* can negatively affect the growth of non-*Saccharomyces* strains (Medina et al., 2012; Taillandier et al., 2014). Therefore, these results suggested a nutrient limitation or competition between *T. delbrueckii* and *S. cerevisiae* strains as a cause of *S. cerevisiae* growth impairment. Several studies supported this hypothesis, especially in relation to nitrogen limitation (Binati et al., 2020; Gobert et al., 2017; Lleixà et al., 2016; Medina et al., 2012; Renault et al., 2016; Rollero et al., 2018; Taillandier et al., 2014; Wang et al., 2016). This limitation could be especially relevant in the case of mixed fermentations performed with species considered stronger fermenters, such as *T. delbrueckii and L. thermotolerans*, because these species appear to compete more intensely with *S. cerevisiae* for nitrogen sources (de Koker, 2015; Medina et al., 2012). Indeed, Taillandier et al. (2014) observed growth arrest of *S. cerevisiae* due to nitrogen depletion by *T. delbrueckii* growth in a medium with low initial assimilable nitrogen but no effect on *S. cerevisiae* growth when the assimilable nitrogen content was higher. Similar results were obtained using other non-*Saccharomyces* species, such as *Hanseniaspora vineae* (Medina et al., 2012).

In our study, different nitrogen sources and concentrations were tested. However, supplementation with nitrogen alone was not enough to avoid stuck fermentations in sequential inoculations without cell contact. This

was in agreement with other studies, such as Lage et al. (2014), where *H. guillermondii* negatively interfered with the growth and fermentative performance of *S. cerevisiae*, regardless of the initial nitrogen concentrations, or Zhu et al. (2021), where a lack of dominance of *S. cerevisiae* in sequential fermentations was observed even after supplementing the medium with nitrogen before *S. cerevisiae* inoculation. Thus, the possibility of other limiting nutrients as the reason for *S. cerevisiae* growth impairment was analyzed. Several studies have associated vitamin limitation with sluggish fermentations (Bataillon et al., 1996; Guzzon et al., 2011; Wang et al., 2003). In fact, Medina et al. (2012) observed that the addition of diammonium phosphate with a vitamin mix increased the growth and fermentation rate of *S. cerevisiae* in sequential fermentations. Rollero et al. (2018) also suggested that there was competition for other kinds of nutrients, probably vitamins, between *Zygoascus meyerae* and *S. cerevisiae*, and it provoked stuck fermentations. Moreover, other nutrients, such as anaerobic growth factors, have been reported to also affect *S. cerevisiae* growth, although the results depended on the added dose. Low doses of anaerobic growth factors induced the dominance of *S. cerevisiae* over *T. delbrueckii*, though increasing concentrations upset this imposition (Brou et al., 2018). In our study, the joint addition of thiamine, zinc and amino acids showed a positive effect in the process, being the only combination of nutrients that allowed *S. cerevisiae* strains to complete the fermentation in sequential inoculation without contact. Thiamine, also referred to as vitamin B1, is involved in different metabolic processes for yeast growth, such as carbon assimilation

and the production of lipids, amino acids, antioxidants, DNA and RNA (Labuschagne and Divol, 2021). In particular, thiamine pyrophosphate (TPP) is a cofactor of several essential enzymes involved in central metabolism and wine fermentation, such as pyruvate dehydrogenase (Pdh) and pyruvate decarboxylase (Pdc) (Hohmann and Meacock, 1998). It has been described that the overexpression of THI4, involved in thiamine biosynthesis, improves glucose consumption, and therefore, fermentation efficiency (Shi et al., 2018; Ruiz et al., 2020). Additionally, thiamine has been reported to be involved in protection against thermal, osmotic and oxidative stresses (Kartal et al., 2018; Kowalska et al., 2012; Li et al., 2019; Wolak et al., 2014). Although yeasts are able to synthesize thiamine de novo, this capacity is reduced under certain environmental conditions, such as those occurring during wine fermentations; therefore, yeast require sufficient exogenous thiamine to achieve maximum growth and fermentation rates (Bataillon et al., 1996; Hohmann and Meacock, 1998; Nosaka, 2006). Zinc is an essential microelement for yeasts that influences yeast physiology and fermentation performance (de Nicola et al., 2009). As a cofactor of many enzymes, such as alcohol dehydrogenase (Adh1), its deficiency can result in slow or incomplete fermentations (Walker, 2004). In addition to acting as a cofactor for many enzymes, zinc is also required for the structural stability of zinc finger proteins, and its supplementation has been shown to improve yeast stress and ethanol tolerance (Zhao and Bai, 2012).

Similar to our results, Maisonnave et al. (2013) showed that the joint addition of vitamins, minerals and nitrogen was required to restart stuck

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

5. Conclusions

Our study confirmed clear nutrient competition between *T. delbrueckii* and *S. cerevisiae* during mixed fermentations, which may be especially relevant and problematic during sequential fermentations. In our hands, *S. cerevisiae* was able to complete fermentations without cell-to-cell contact between yeasts only if it was inoculated at higher populations or if thiamine, zinc and amino acids were added when *S. cerevisiae* was inoculated. However, this nutrient addition had a lower effect on the imposition of *S. cerevisiae* over *T. delbrueckii* in mixed fermentations. Therefore, this work highlighted the importance of controlling nutrient availability and inoculum size in sequential fermentations to avoid problems, such as stuck or sluggish fermentations.

Declaration of competing interest

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

Author Contributions

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

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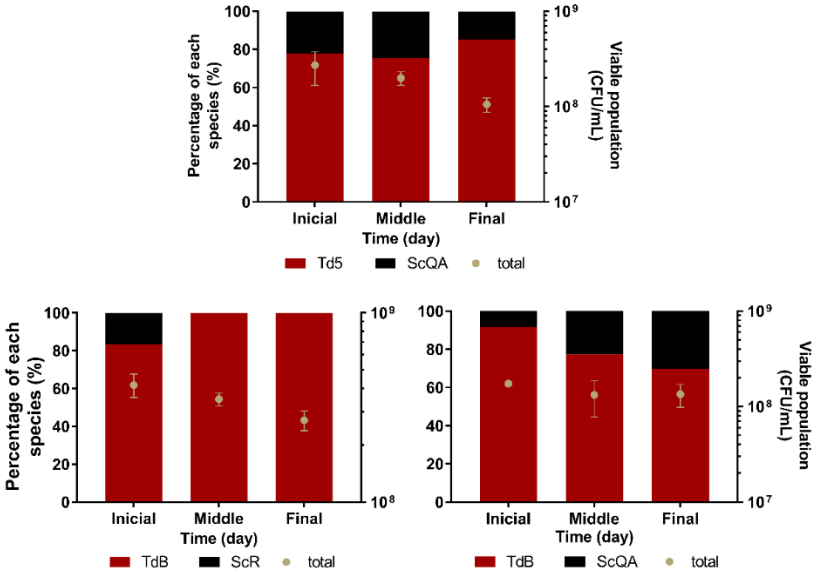
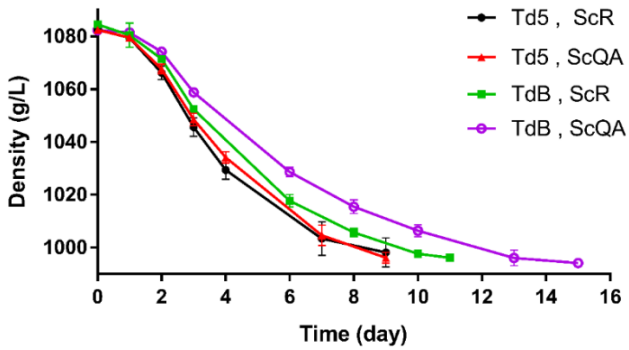
Supplementary Material

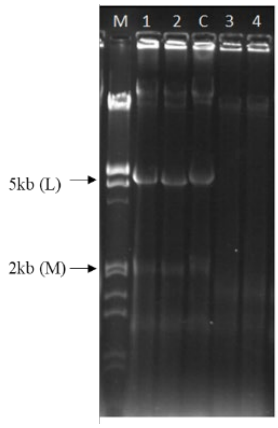
All supplementary material is attached below except for Supplementary Table S2 and Supplementary Tablee S3 that are not included in the present thesis due to format incompatibility, and are available online at: https://drive.google.com/drive/folders/1dd0-2fdjqn8ulcawWxGc5uchNeEmp0ed?usp=sharing. **Supplementary Table S2:** Screening of the addition of each amino acid in microfermentations. Means of the rate, generation time (GT), high OD and the area under the curve (AUC), and **Supplementary Table S3**: Screening of the addition of each vitamin with each oligoelement in microfermentations. Means of the rate, generation time (GT), high OD and the area under the curve (AUC).



Supplementary Figure S1. Evolution of total and specific population dynamics throughout sequential fermentations performed with *T. delbrueckii* and *S. cerevisiae* strains (a) Td5 and ScQA. (b) TdB and ScR and (c) TdB and ScQA.



Supplementary Figure S2. Density evolution of sequential fermentations between *T. delbrueckii* and *S. cerevisiae* strains in synthetic must at 16 °C.



Supplementary Figure S3. RNA electrophoresis of genomes of dsRNA killer viruses L (5 kbp) and M (2 kbp) in *S. cerevisiae* strains: ScQA (1), ScR (2) and F166 used as positive control with $K1^+$ phenotype strain; and *T. delbrueckii* Td5 (3) and TdB (4) strains. M as DNA molecular weight marker III (Roche) with size range from 0.12 to 21.2 kbp.

Supplementary Table S1. Nitrogen content (mg/L) present in the medium after 48 h of single fermentations with the *T. delbrueckii* (TdB and Td5) or *S. cerevisiae* (ScQA and ScR) strains in standard synthetic must (300 mg N/L). as well as after 48h of Td5 fermentation in high nitrogen content must (600 mg N/L; Td5_600N).

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

Lowercase letters indicate significant differences between fermentations in each compound.

Supplementary Table S4. Thiamine concentration (mg/L) in the medium after 24 and 48 h of single fermentations with *T. delbrueckii* (Td5 and TdB) and *S. cerevisiae* (ScQA and ScR) strains.

	Td5	**TdB**	**ScQA**	**ScR**
24h	< 0.08 ± 0.001	< 0.08 ± 0.001	< 0.08 ± 0.000	< 0.08 ± 0.000
48h	< 0.08 ± 0.000	< 0.08 ± 0.000	< 0.08 ± 0.000	< 0.08 ± 0.000

Supplementary Table S5. Oenological interesting compounds at the end of fermentation.

	Citric acid (g/L)	Tartaric acid (g/L)	Malic acid (g/L)	Succinic acid (g/L)	Lactic acid (g/L)	Acetic acid (g/L)	Glycerol (g/L)	Ethanol (%. v/v)
ScQA	0.39±0.13	1.94±0.01^{b}	3.77±0.28	0.36±0.09^{b}	0.34±0.03^{b}	0.49±0.01^{b}	7.83±0.00^{a}	11.10±0.15
ScR	0.45±0.02	2.40±0.14^{a}	3.38±0.25	0.59±0.12^{a}	0.72±0.20^{ab}	0.84±0.05^{a}	7.74±0.15^{a}	11.43±0.47
Td5	0.45±0.03	2.03±0.11^{b}	3.68±0.24	0.10±0.03^{c}	0.93±0.08^{a}	0.27±0.01^{c}	7.19±0.56^{a}	10.59±0.61
TdB	0.42±0.09	2.40±0.13^{a}	3.51±0.19	0.16±0.01^{bc}	1.02±0.28^{a}	0.22±0.02^{c}	5.04±0.27^{b}	11.10±0.51
ScR·Td (1:1)	0.18±0.05	1.57±0.20	4.12±0.36	0.96±0.19	0.23±0.05	0.28±0.03	6.41±0.23	11.02±0.07
ScR·Td (1:9)	0.12±0.03	1.43±0.02	4.21±0.39	1.09±0.12	0.22±0.06	0.28±0.03	7.94±0.62*	11.17±0.25
ScR·Td (9:1)	0.19±0.05	1.42±0.05	3.89±0.09	1.09±0.10	0.21±0.04	0.27±0.03	6.12±0.30	10.98±0.06
Td5 , ScR	0.40±0.03^{BC}	2.48±0.42	4.57±0.26	0.71±0.03^{AB}	0.23±0.10^{AB}	0.46±0.05^{AB}	6.61±1.43	10.93±0.92^{ABC}
Td5 , ScR 600N	0.37±0.03^{C}	2.08±0.44	4.57±0.41	0.55±0.04^{ABCD}	0.23±0.02^{AB}	0.25±0.04^{B}	5.78±0.34	11.45±0.17^{AB}
Td5 , ScR +Thi+Zn+aas	0.53±0.05^{ABC}	2.48±0.14	4.22±0.16	0.96±0.04^{A}	0.37±0.09^{AB}	0.74±0.14^{A}	6.06±0.31	11.03±0.09^{ABC}
Td5 , ScR + IDY	0.49±0.10^{ABC}	2.47±0.08	4.26±0.16	0.65±0.41^{ABC}	0.41±0.16^{A}	0.44±0.32^{AB}	6.12±0.13	11.37±0.16^{AB}
Td5 / ScR + all nutrients	0.70±0.14^{A}	2.13±0.22	4.95±0.42	0.32±0.05^{BCD}	0.28±0.08^{AB}	0.33±0.02^{B}	6.03±0.27	10.42±0.22^{BC}
Td5 / ScR								
+ Thi + Zn + aas	0.64±0.14^{AB}	2.12±0.15	4.48±0.13	0.13±0.02^{D}	0.17±0.03^{B}	0.26±0.04^{B}	7.02±0.51	11.73±0.25^{A}
Td5 / ScR (10^{8})	0.73±0.07^{A}	2.03±0.34	4.22±0.11	0.22±0.04^{CD}	0.15±0.02^{B}	0.33±0.02^{B}	5.43±0.41	10.09±0.64^{C}

	Citric acid (g/L)	Tartaric acid (g/L)	Malic acid (g/L)	Succinic acid (g/L)	Lactic acid (g/L)	Acetic acid (g/L)	Glycerol (g/L)	Ethanol (%. v/v)
Td5 / ScR (10^8)	0.73±0.07^{A}	2.03±0.34	4.22±0.11	0.22±0.04^{CD}	0.15±0.02^{B}	0.33±0.02^{B}	5.43±0.41	10.09±0.64^{C}
Td5 , ScQA	0.36±0.05^{B}	2.66±0.34	4.73±0.10^{A}	0.75±0.07^{B}	0.22±0.05^{AB}	0.51±0.04^{AB}	8.01±0.80^{A}	11.42±0.72
Td5 , ScQA +Thi+Zn+aas	0.52±0.01^{AB}	2.30±0.25	4.20±0.04^{B}	0.92±0.15^{AB}	0.32±0.06^{AB}	0.58±0.05^{A}	6.12±0.13^{B}	11.08±0.25
Td5 , ScQA + IDY	0.48±0.02^{AB}	2.40±0.11	4.52±0.04^{AB}	0.99±0.09^{A}	0.39±0.09^{A}	0.58±0.03^{A}	6.03±0.15^{B}	11.01±0.22
Td5 / ScQA + Thi + Zn + aas	0.45±0.17^{AB}	2.74±0.08	4.91±0.38^{A}	0.23±0.03^{C}	0.19±0.02^{B}	0.35±0.02^{C}	6.48±0.54^{B}	11.18±0.11
Td5 / ScQA (10^8)	0.73±0.24^{A}	2.67±0.35	4.80±0.11^{A}	0.32±0.05^{C}	0.27±0.07^{AB}	0.46±0.01^{B}	6.60±0.38^{B}	10.56±0.08
TdB , ScR	0.49±0.01^{A}	1.98±0.07^{B}	4.41±0.71	0.28±0.10	0.15±0.04	0.22±0.01^{B}	5.38±0.42	10.77±0.08^{B}
TdB / ScR + Thi + Zn + aas	0.34±0.03^{B}	2.50±0.14^{A}	3.99±0.28	0.49±0.10	0.22±0.07	0.46±0.07^{A}	6.05±0.72	11.17±0.24^{A}
TdB , ScQA	0.34±0.03^{B}	2.24±0.03^{B}	4.10±0.14^{B}	0.23±0.03^{B}	0.20±0.04	0.18±0.03^{B}	6.61±0.57	10.63±0.11
TdB / ScQA + Thi + Zn + aas	0.49±0.03^{A}	2.66±0.05^{A}	4.76±0.19^{A}	0.57±0.20^{A}	0.14±0.03	0.32±0.05^{A}	6.07±0.24	10.83±0.29

Lowercase letters indicate significant differences between single fermentations. * Indicates significant differences in coinoculated fermentations. Capital letters indicate significant differences between each strain combination in sequential fermentations.

UNIVERSITAT ROVIRA I VIRGILI
OPTIMIZACIÓN DEL USO DE LEVADURAS NO SACCHAROMYCES EN FERMENTACIONES MIXTAS: REQUERIMIENTOS NUTRICIONALES E INTERACCIONES MICROBIANAS
Elena Roca Mesa

Capítulo II. 2

The impact of nutrient supplementation and inoculum size in sequential fermentations with *Lachancea thermotolerans* and *Saccharomyces cerevisiae*

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Manuscrito en preparación

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Abstract:

The use of non-*Saccharomyces* yeasts has increased in winemaking in recent years due to their positive characteristics that can contribute to the final quality of wines. One of the most interesting non-*Saccharomyces* yeast species is *Lachancea thermotolerans,* which is used in mixed fermentations with *S. cerevisiae*. However, little is known about interactions between both species during alcoholic fermentation. The aim of this work was to analyze the performance of sequential fermentations with *L. thermotolerans* and *S. cerevisiae* and determine the effect of nutrient supplementation and inoculum size on the ability of *S. cerevisiae* to properly grow and ferment mixed fermentations. For this purpose, fermentation kinetics and yeast growth were measured over time during single and sequential fermentations, using synthetic must. Nutrient supplementation, inoculation size and cell-to-cell contact mechanism were also studied. Our results showed that *S. cerevisiae* barely dominated the sequential fermentations and experiments without yeast contact (in which *L. thermotolerans* cells were removed from the media before inoculating *S. cerevisiae* at 48 h) resulted in stuck fermentations except when the inoculum size was increased or the medium was supplemented with thiamine, zinc and amino acids before the inoculation of *S. cerevisiae*. This nutrient supplementation also had a positive effect on sequential fermentations with and without contact between both species. Further investigation is needed to understand these behaviours.

Keywords: non-*Saccharomyces*; nutrients; cell-to-cell contact; sequential fermentation.

1. Introduction

Non-*Saccharomyces* are indigenous yeasts present in grapes and distributed differently around the world. They are usually present in higher concentrations than *Saccharomyces cerevisiae* on the grape surface and in must at the beginning of alcoholic fermentation. The popularity and perception of non-*Saccharomyces* yeasts in winemaking has been changing over the last few years. Originally, they were considered spoilage microorganisms (Fleet and Heard, 1993) but it is currently known that some of these species can endow special traits to wines (Jolly et al., 2014). As a result, the interest aroused by these species as well as their use in the wine industry, mainly in mixed fermentations, has increased considerably, as have the studies surrounding their microbial interactions (Curiel et al., 2017; Mencher et al., 2020; Muñoz-Redondo et al., 2021; Petitgonnet et al., 2019; Taillandier et al., 2014; Tronchoni et al., 2017; Wang et al., 2016).

Yeast interactions are mainly based on nutrient competition, secretion of toxic compounds and cell-to-cell contact mechanisms. Several of these interactions have already been evidenced between *Saccharomyces* and non-*Saccharomyces* yeast under winemaking conditions (Zilelidou and Nisiotou, 2021). Transcriptomic analysis showed that a wine yeast modified its gene expression in response to the presence of other yeast species in mixed fermentations, mainly in genes related to nutrient competition. For example, the presence of non-*Saccharomyces* species stimulated glucose and nitrogen metabolism (by upregulating the related genes) (Curiel et al., 2017; Tronchoni et al., 2017) and genes related to iron and copper uptake (Alonso-del-Real et al., 2019; Shekhawat et al., 2019) in *S. cerevisiae*. Conversely, the secretion of toxic compounds has also been observed in

mixed cultures. For example, the early death of *Hanseniaspora* species was induced by killer-like toxins secreted by *S. cerevisiae* (Pérez-Nevado et al., 2006), and Albergaria et al. (2010) and Branco et al. (2014) also observed that *S. cerevisiae* secreted antimicrobial peptides into the medium during the late exponential phase of alcoholic fermentation and that these protein fractions induced the death of several non-*Saccharomyces* yeasts. Nissen and Arneborg (2003) demonstrated that the cell-to-cell contact mechanism induced the cell death of some *non-Saccharomyces* yeasts, such as *L. thermotolerans,* during mixed fermentations at high cell densities of *S. cerevisiae*. These observations were also confirmed by Renault et al. (2013). One of the most popular non-*Saccharomyces* species is *Lachancea thermotolerans*. Currently, there are seven strains of *L. thermotolerans* accessible on the market as active dry yeast (Vejarano and Gil-Calderón, 2021). This species has been reported to provide desirable oenological characteristics to wines, such as an increase in the concentration of glycerol (Kapsopoulou et al., 2007), aromatic molecules such as ethyl esters and terpenes (Balikci et al., 2016; Benito et al., 2016, 2015), and L-lactic acid, which consequently enhance the overall wine acidity (Gobbi et al., 2013; Mora et al., 1990). However, although some studies have shown this positive effect of *L. thermotolerans* on wine characteristics in mixed fermentations (Beckner Whitener et al., 2015; Benito et al., 2016; Domizio et al., 2014; Gobbi et al., 2013), in others, this improvement has not been evidenced (Belda et al., 2016b; Hranilovic et al., 2018). This divergence of results could be explained by the differences in the strain used, must composition and other fermentative conditions. Therefore, further studies

are needed to understand the interactions between *Saccharomyces* and non-*Saccharomyces,* also *L. thermotolerans,* to improve their implantation in winemaking at a high scale.

In this work, interactions between *S. cerevisiae* and *L. thermotolerans* were analyzed to better control their use in mixed starters. For this reason, single and sequential fermentations were performed in synthetic must. Nutrient supplementation and cell-to-cell contact mechanisms were also studied.

2. Materials and Methods

2.1. Yeast strains and media

A strain of each species was used in this study: Viniferm Revelación (ScR) belonging to *Saccharomyces cerevisiae* and Lt2 to *Lachancea thermotolerans*, both provided by Agrovin S.L. (Spain). Both strains were preserved in YPD liquid medium (2% (w/v) glucose, 2% (w/v) bacto peptone, and 1% (w/v) yeast extract; Cultimed, Barcelona, Spain) with 40% (v/v) glycerol, at –80 °C until their use.

2.2. Fermentation conditions and sampling

Single and sequential fermentations were performed in synthetic must, as described by Beltran et al. (2004). The initial nitrogen content was 300 mg N/L (Roca-Mesa et al., 2020).

Fermentations were performed in triplicate in 250 mL borosilicate glass bottles containing 220 mL of medium and covered with closures that allowed carbon dioxide to escape and samples to be extracted and incubated at 16 °C with shaking (120 rpm). In general, yeasts were inoculated at an initial concentration of 2 x 10^6 cells/mL, except in one experiment where ScR was inoculated at 1 x 10^8 cells/mL. In sequential fermentations without

cell contact, prior to the inoculation of *S. cerevisiae*, the medium was centrifuged and filtered to eliminate the cells of *L. thermotolerans*. Moreover, in nutrient-supplemented fermentations, thiamine (Thi) (Sigma-Aldrich, Darmstadt, Germany), zinc (Zn) (Fluka Chemica, New York, USA) and amino acids (aas) were added when the *S. cerevisiae* strain was inoculated, at the same concentration as initially found in the synthetic must.

To monitor fermentation kinetics, must density was measured daily with an electronic densimeter (Densito 30PX Portable Density Meter (Mettler Toledo, Spain)) and viable yeast population was analyzed by plating serial dilutions of fermentation samples at different times on Wallerstein laboratory nutrient medium (WLN, Difco S. A., Saint-Ferréol, France). The endpoint of fermentation was considered when the sugar content was less than 2 g/L or when density became stable for more than two days, being in the latter stuck fermentations. For nitrogen and metabolite analysis, 1.5 mL of the supernatant was collected at 48 h and at the end of fermentation respectively, and stored at −20 °C, until analysis.

2.3. Nitrogen and metabolite analysis

The nitrogen content was analyzed by a multi analyzer Y-15 (BioSystems, Barcelona, Spain), using enzymatic kits for ammonium and alpha-amino nitrogen (TDI, Barcelona, Spain).

Some organic metabolites, relevant from an oenological point of view, such as ethanol, glycerol and acetic acid, were determined at the end of fermentation, following the protocol described by Quirós et al. (2010). For this analysis, we used an Agilent 1100 HPLC (Agilent Technologies,

Germany) equipped with a Hi-Plex H, 300 mm x 7.7 mm column inside a 1260 MCT (Infinity II Multicolumn Thermostat) connected to both an MWC and a RID (G1365B multi-wavelength detector and 1260 Infinity II refractive index detector) (Agilent Technologies, Germany). The column was maintained at 60 °C, and 5 mM H_2SO_4 was used as the mobile phase at a flow rate of 0.6 mL/min. Before analysis, samples were filtered through 0.22 μm pore size filters (Dominique Dutscher, Brumath, France).

2.4. Statistical analysis

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

3. Results

3.1. Single and sequential fermentations

Single and sequential fermentations using ScR and Lt2 strains were performed. In both types of fermentations, strains were able to deplete sugars, with single ScR fermentation being the fastest (12 days). In contrast, Lt2 in single and mixed inoculum needed 15 days to complete the fermentations (Figure 1a). Both strains reached similar populations when used in single inoculations, although ScR growth was slower than that of Lt2. Surprisingly, *S. cerevisiae* was not able to dominate fermentation,

barely reaching the 50% of the population by the end of fermentation (Figure 1b).

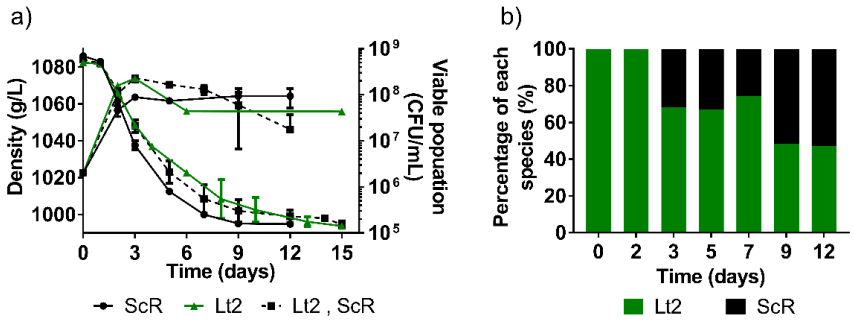

Figure 1. Single and sequential fermentations with ScR and Lt2 strains a) Must density and viable population of single (solid lines) and sequential (dropped lines) fermentations; b) population dynamics of each strain during sequential fermentation, expressed as a percentage of each species.

3.2. Cell-to-cell contact mechanism effect

To determine the impact of cell-to-cell contact on the microbial interactions between ScR and Lt2, we performed sequential fermentations without contact between strains. To achieve this goal, fermentations were inoculated first with the Lt2 strain, and after 48 h, Lt2 cells were removed from the medium by centrifugation and filtration, after which the ScR strain was inoculated. After removing Lt2 cells, ScR was not able to finish the sugars present in the must, and the fermentation process was halted (Figure 2), with only a small portion of the sugars consumed. We observed that even if *S. cerevisiae* was able to grow after its inoculation at 48 h, its growth occurred at a much lower concentration than when inoculated at time 0 as a single inoculum. Indeed, we found similarities in *S. cerevisiae* growth and the populations reached in both sequential fermentation systems (with and without contact), indicating that the populations of Lt2 might be mainly

responsible for the full consumption of sugars and consequently for the completion of the fermentations in the sequential inoculations.

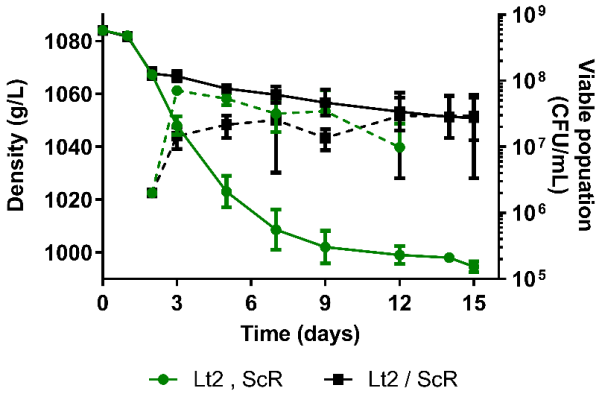

Figure 2. Must density (solid lines) and ScR viable population (dropped lines) of sequential fermentations with (green colour) and without (black colour) cell contact between ScR and Lt2 strains.

3.3. Nutrient supplementation and inoculum size effects

One of the causes of poor growth of *S. cerevisiae* in sequential inoculations is nutrient limitation generated by the previous growth of non-*Saccharomyces* yeast in the must, mainly nitrogen depletion. Therefore, the nitrogen content of the medium was analized at 48 h. In the Lt2 strain fermented medium, the total YAN concentration at that time was 38.75 ± 2.69 mg/L, with the organic fraction (amino acids) being clearly lower (3.30 ± 1.86 mg/L) than the inorganic fraction (ammonium) (35.44 ± 3.00 mg/L). This nitrogen content was lower compared with the ScR strain, which led to 89.22 ± 7.22 mg/L of total YAN in the medium, with similar values in the organic (45.41 ± 6.87 mg/L) and inorganic (43.81 ± 10.10 mg/L) fractions. Additionally, in our previous work (Chapter II.1), we found that thiamine and zinc were important nutrients for supplementation in sequential

fermentations for the proper development and performance of *S. cerevisiae*. Therefore, we also tested the effect of supplementing must with thiamine, zinc and amino acids, in sequential fermentations with (*Lt2 , ScR*) and without (*Lt2/ScR*) cell contact, at the precise time of ScR inoculation.

Our results showed that the addition of thiamine, zinc and amino acids (Thi+Zn+aas) had a positive effect on both fermentation procedures, with and without yeast species contact (Figure 3a).

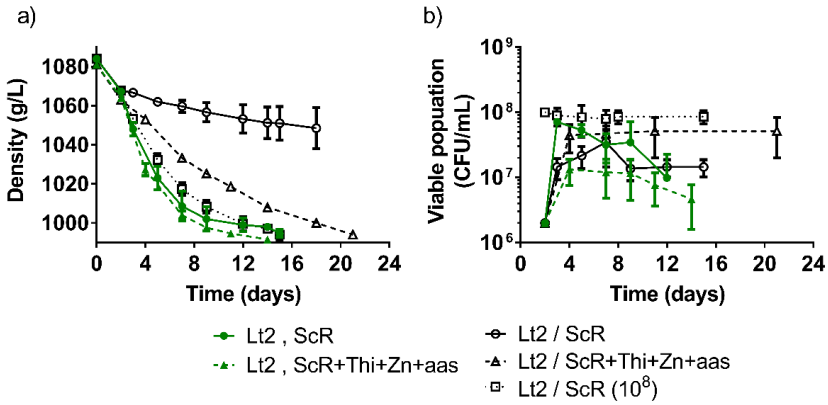

Figure 3. Must density (a) and ScR viable population (b) during sequential fermentations with (green colour) and without (black colour) cell contact between the Lt2 and ScR strains. Triangles with dropped lines represent fermentations with nutrient supplementation (Thi, thiamine, Zn, zinc and aas, amino acids). Squares with dropped lines represent fermentations with a higher inoculum.

The sequential fermentation with cell contact between strains, and nutrient addition was slightly faster (it finished in 14 days) than the same condition without Thi+Zn+aas supplementation, which was completed in 15 days (Figure 3a). However, Thi+Zn+aas supplementation had a greater effect on the fermentation without contact, significantly improving the performance of *S. cerevisiae* inoculated at 48 h (after removing Lt2 cells), which was able to finish the fermentation in 21 days, in comparison to the

nonsupplemented condition, which was stuck, and only a small portion of the sugar was consumed by *S. cerevisiae*. ScR growth was analyzed in the sequential fermentation without cell species contact (Figure 3b), and we observed a higher population of ScR in the condition with nutrient supplementation than without this addition (reaching a maximum population of $5.15x10^7 \pm 3.17x10^7$ cells/mL). Unexpectedly, ScR grew less in sequential fermentation with cell species contact and with nutrient supplementation. Most likely, as Lt2 was not removed from the medium, and increased its growth, it was able to consume these nutrients quickly and ScR had no option to benefit from all of them. Nevertheless, *S. cerevisiae* dominance at the end of sequential fermentations with the presence of both species was slightly higher than that without nutrient supplementation, increasing from 52 to 62% (data not shown).

To decipher if a higher population of *S. cerevisiae* would allow the completion of fermentation without nutrient addition, we also tested a higher *S. cerevisiae* inoculum size (10^8 cell/mL) in the sequential fermentation without cell contact between yeast strains. In the latter case, the fermentation finished in 15 days, which was even faster than the one with nutrient addition (which finished in 21 days). Under this condition, no growth of ScR was observed from the initial inoculated population, which remained stable and higher than that under the other conditions throughout the process (Figure 3b).

Some interesting oenological compounds were analyzed at the end of all the fermentation processes (Table 1). The presence of Lt2 increased the lactic acid content in wines, especially in the sequential fermentation (*Lt2 , ScR*).

Moreover, sequential fermentation with yeast contact and nutrient addition (*Lt2 , ScR* +Thi+Zn+aas) had the lowest glycerol content, while when the inoculum size was increased it reached the maximum value (*Lt2/ScR* 10^8), followed by sequential fermentation *(Lt2 , ScR).* ScR single fermentation presented the highest acetic acid content while it was reduced when the Lt2 strain was applied. In general, all fermentations with *L. thermotolerans* finished with lower ethanol values than the single fermentation of the ScR strain.

Table 1. Oenological interesting compounds at the end of fermentation.

	ScR	Lt2	Lt2 , ScR	Lt2 / ScR	Lt2 / ScR (10^8)	Lt2 , ScR +TiZiaas	Lt2 / ScR +TiZiaas
citric acid (g/L)	0.45 ± 0.02^{bcd}	0.26 ± 0.04^{d}	0.33 ± 0.04^{cd}	0.84 ± 0.34^{a}	0.63 ± 0.03^{abc}	0.48 ± 0.05^{abcd}	0.70 ± 0.03^{ab}
tartaric acid (g/L)	2.40 ± 0.14^{abc}	2.06 ± 0.02^{c}	2.54 ± 0.16^{ab}	2.26 ± 0.17^{bc}	2.54 ± 0.14^{ab}	1.51 ± 0.11^{d}	2.67 ± 0.05^{a}
malic acid (g/L)	3.38 ± 0.25^{d}	3.63 ± 0.20^{cd}	3.86 ± 0.49^{abcd}	4.46 ± 0.14^{a}	4.37 ± 0.12^{ab}	3.77 ± 0.03^{bcd}	4.17 ± 0.10^{abc}
succinic acid (g/L)	0.59 ± 0.12	0.57 ± 0.43	0.66 ± 0.25	0.23 ± 0.08	0.59 ± 0.73	0.56 ± 0.04	0.64 ± 0.02
lactic acid (g/L)	0.62 ± 0.14^{d}	0.85 ± 0.05^{cd}	3.14 ± 0.14^{a}	0.37 ± 0.05^{e}	1.06 ± 0.13^{c}	1.46 ± 0.25^{b}	1.42 ± 0.03^{b}
acetic acid (g/L)	0.84 ± 0.05^{a}	0.36 ± 0.09^{c}	0.39 ± 0.02^{bc}	0.41 ± 0.02^{bc}	0.49 ± 0.12^{bc}	0.33 ± 0.02^{c}	0.59 ± 0.11^{b}
glycerol (g/L)	7.74 ± 0.15^{ab}	7.07 ± 0.17^{b}	8.07 ± 0.14^{a}	5.30 ± 0.59^{c}	8.33 ± 0.17^{a}	5.05 ± 0.14^{c}	5.52 ± 0.24^{c}
Ethanol (%, v/v)	11.43 ± 0.47^{a}	10.62 ± 0.19^{a}	10.16 ± 1.05^{a}	2.96 ± 0.79^{b}	10.11 ± 0.58^{a}	10.27 ± 0.43^{a}	11.06 ± 0.19^{a}
Residual sugars (g/L)	1.22 ± 0.15^{b}	1.27 ± 0.11^{b}	1.02 ± 0.86^{b}	127.96 ± 0.36^{a}	1.82 ± 1.06^{b}	0.87 ± 0.34^{b}	0.55 ± 0.37^{b}

Lowercase letters indicate statistically significant differences between fermentations in each compound.

4. Discussion

For many years, non-*Saccharomyces* species have been considered spoilage microorganisms in winemaking, and most research has focused on *S. cerevisiae*. However, interest in the use of some non-*Saccharomyces* species in wine production has increased in recent decades (Padilla et al., 2016) and, consequently, further research on these yeasts and their microbial interactions is needed for the optimal use of these starters.

In this work, we examined the effect of using a sequential inoculation of *L. thermotolerans and S. cerevisiae,* with and without cell contact, and with and without nutrient supplementation, on fermentation performance and population dynamics. The Lt2 strain, which belongs to a non-*Saccharomyces* species with a strong fermentative capacity, was able to complete alcoholic fermentation even when used as single inoculum, as previously reported (Gobbi et al., 2013; Petitgonnet et al., 2019). In contrast to the initial statements indicating the low fermentative capacity of non-*Saccharomyces* species (Albergaria and Arneborg, 2016), some of these non-*Saccharomyces* yeasts have been demonstrated to be rather good fermenters, even capable of finishing wine fermentation when used as single inoculum, such as *T. delbrueckii* (Lin et al., 2020; Renault et al., 2015; Taillandier et al., 2014; Tronchoni et al., 2017; van Breda et al., 2018), *L. thermotolerans* (Balikci et al., 2016; Gobbi et al., 2013; Petitgonnet et al., 2019; Shekhawat et al., 2019), *Schizosaccharomyces pombe* (Benito et al., 2017), and *Hanseniaspora vineae* (Lleixà et al., 2019). Some strains of those species are currently commercialized as active dry yeast starters, although the recommendation is to use them in a mixed inoculum with *S. cerevisiae*

(Vejarano and Gil-Calderón, 2021). The good performance of some non-*Saccharomyces* strains could also be related to clonal selection for the best performing strains, or to their domestication (and adaptive evolution) to winemaking conditions in recent decades (Steensels et al., 2014; Williams et al., 2015).

Indeed, in sequential fermentation with *S. cerevisiae*, Lt2 remained the dominant strain for most of the process, with ScR barely above 50% of the total population even at the end points. This result is consistent with previous studies (Gobbi et al., 2013; Petitgonnet et al., 2019; Shekhawat et al., 2017; Zhu et al., 2021), where *L. thermotolerans* showed a similar behaviour, being the dominant species in mixed fermentations with *S. cerevisiae*. Moreover, Petitgonnet et al. (2019) reported that the decrease in viability of *S. cerevisiae* in mixed fermentations with *L. thermotolerans* was more pronounced when there was cell-to-cell contact between both species. Therefore, we performed sequential fermentations without cell-to-cell contact between both yeast species, in which the first strain (Lt2) was removed immediately before inoculation of the second strain (ScR), to evaluate the ability of the *S. cerevisiae* strain to ferment in this media. This sequential inoculation resulted in stuck fermentations, indicating that the *S. cerevisiae* strain might face some impairments to grow and ferment properly, even in the absence of the *L. thermotolerans* strain. Therefore, we discarded the possibility that the main reason for the decreased viability of *S. cerevisiae* was a direct inhibition due to a cell-to-cell mechanism between these species. In the previous chapter (Ch. II.1), similar results were obtained in sequential fermentations with *T. delbrueckii* and *S. cerevisiae,*

also ruling out cell-to-cell inhibition as the main reason for *S. cerevisiae* growth impairment. After a thorough screening to determine the reason for these fermentation and growth problems, the low availability of some nutrients previously consumed by *T. delbrueckii* strains was finally the main factor responsible for the improper development of *S. cerevisiae* strains. It is well known that different factors could cause stuck or sluggish fermentations, such as nutrient limitation, ethanol toxicity, the presence of killer or other toxins, extreme temperatures, residual concentrations of pesticides or fungicides, microbial competition and poor oenological practices (Bisson, 1999). In our study, if *S. cerevisiae* was added at a higher inoculation rate ($1x10^8$ cell/mL instead of $2x10^6$ cell/mL) in the sequential fermentations without cell-to-cell contact, this strain was able to complete fermentation, pinpointing a problem for the growth of *S. cerevisiae* in this medium. Therefore, nutrient availability might be responsible for the growth impairment of the second inoculated strain. Previous studies have shown that variations in the size of the non-*Saccharomyces* inoculum can compromise *Saccharomyces* growth either in simultaneous or sequential inoculation, since non-*Saccharomyces* species consume nutrients that are essential for the growth of *S. cerevisiae,* as well as for the synthesis of some desirable aromatic compounds (Medina et al., 2012; Zhu et al., 2021). It is well known that nutrients that are usually limiting during alcoholic fermentation are nitrogen compounds, phosphates, vitamins and minerals (Bisson, 1999; Malherbe and du Toit, 2007). In the previous chapter (Ch. II.1), we found that supplementation of the medium after *T. delbrueckii* fermentation for 48 h with thiamine, zinc and amino acids had a positive

effect on the ability of *S. cerevisiae* to grow and ferment properly. Therefore, we tested the effect of these nutrients in mixed fermentations of *L. thermotolerans* and *S. cerevisiae* (with and without cell-to-cell contact between yeast species). Our results showed a positive effect of this nutrient addition in both cases, with a slight increase in *S. cerevisiae* dominance at the end of sequential fermentations with cell-to-cell contact and complete sugar depletion in sequential fermentations without cell-to-cell contact between strains. Previous studies have also demonstrated the importance of correct nutrition during mixed fermentations between non-*Saccharomyces* and *Saccharomyces* species. Medina et al. (2012) proposed the addition of diammonium phosphate and a vitamin mix as the most effective strategy to prevent stuck fermentations. Moreover, Petitgonnet et al. (2019) results showed competition for nitrogen compounds, oxygen, and must sterols between *L. thermotolerans* and *S. cerevisiae* in sequential fermentations. In fact, Maisonnave et al. (2013) studied the key nutritional factors to effectively restart stuck fermentation with *S. cerevisiae*, and found that minerals, vitamins and amino acids were absolutely required to restart and complete arrested fermentations, but oligoelements were not necessary under such conditions. However, in our hands, the presence of zinc was essential for *S. cerevisiae* to complete fermentation after the growth of both *T. delbrueckii* and *L. thermotolerans* in the medium.

Moreover, we observed differences in interesting oenological compounds at the end of fermentation. Wines with *L. thermotolerans* presented a slight increase in lactic acid concentration respect ScR wine, and a decrease in acetic acid and ethanol. On the other hand, glycerol was only increased in

some conditions. These results agree with other studies where wines produced with *L. thermotolerans* showed an increase in lactic acid (Beckner Whitener et al., 2016; Benito et al., 2015, 2016, 2017; Comitini et al., 2011; Gobbi et al., 2013), glycerol (Comitini et al., 2011; Gobbi et al., 2013) and a decrease in ethanol content (Balikci et al., 2016; Comitini et al., 2011; Cordero-Bueso et al., 2013; Gobbi et al., 2013).

In this work, we performed sequential fermentations between *L. thermotolerans* and *S. cerevisiae* strains with and without cell-to-cell contact to better understand the interactions between these species and improve their use as mixed starters in winemaking. As expected, competition for nutrients between these species was observed, which negatively affected the correct development of *S. cerevisiae* strains in sequential fermentations. The use of a larger inoculum of *S. cerevisiae* or the addition of some nutrients (thiamine, zinc and amino acids) immediately before the inoculation of *S. cerevisiae* prevented stuck fermentation when *L. thermotolerans* was removed from the process. This work highlights the importance of controlling nutrient availability and inoculum size in sequential fermentations. Further studies are needed to improve knowledge about interactions between *L. thermotolerans* and *S. cerevisiae* to ensure the correct performance of mixed fermentations with these yeast species.

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Capítulo II. 3

Effect of the combined use of *Torulaspora delbrueckii* and *Lachancea thermotolerans* as yeast starters on fermentation performance and wine aroma composition

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Manuscrito en preparación

UNIVERSITAT ROVIRA I VIRGILI
OPTIMIZACIÓN DEL USO DE LEVADURAS NO SACCHAROMYCES EN FERMENTACIONES MIXTAS: REQUERIMIENTOS NUTRICIONALES E INTERACCIONES MICROBIANAS
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Abstract:

The use of non-*Saccharomyces* species in mixed cultures in winemaking has increased in recent years. These yeasts increase the aroma complexity and could improve the final quality of wines. Some nonconventional yeasts have poor fermentative ability and are usually applied in mixed inoculations with *Saccharomyces cerevisiae* to ensure the end of fermentation. However, some non-*Saccharomyces* yeasts, selected for their good fermentative properties, are able to finish the fermentation as a single inoculum. The aim of this work was to investigate the use of some non-*Saccharomyces* species with high fermentative capacity, such as *Torulaspora delbrueckii* and *Lachancea thermotolerans*, as single and mixed starters in winemaking, without the use of *S. cerevisiae*. For this purpose, single, coculture and sequential fermentations with and without cell-to-cell contact between the non-*Saccharomyces* species were performed. Fermentation kinetics and yeast growth were measured over time, as well as nutrient requirements and volatile compounds. Our results showed that the fermentation kinetics, population dynamics and aromatic profile depended on the strain combination and the inoculation strategy applied. Some special traits over fermentations performed only with *S. cerevisiae* have been exposed, which demonstrated the potential use of non-*Saccharomyces* yeasts as single inocula in winemaking.

Keywords: alcoholic fermentation*;* nitrogen; volatile compounds, cell-to-cell contact; sequential fermentation; cocultures.

1. Introduction

In recent decades, the study and application of non-*Saccharomyces* yeasts in winemaking have increased due to their positive contributions to the aroma and final quality of wines (Jolly et al., 2014; Morata et al., 2020; Padilla et al., 2016; van Wyk et al., 2020). Moreover, the use of some non-*Saccharomyces* species can be used as a strategy to reduce the content of ethanol in wines, one of the current problems to solve in winemaking because of climate change (Canonico et al., 2019; Contreras et al., 2015; Morales et al., 2015; Puškaš et al., 2020; Quirós et al., 2014; Zhu et al., 2020). Two of these non-*Sacchaormyces* species with positive fermentative characteristics and currently commercialized as wine starters are *Torulaspora delbrueckii* and *Lachancea thermotolerans. T. delbrueckii* is one of the most studied non-*Saccharomyces* species. This yeast species improves complexity and can contribute to desirable aromatic characteristics (Benito, 2018a; Fernandes et al., 2021). Wines obtained with *T. delbrueckii* exhibit higher concentrations of some volatile compounds and glycerol, and lower levels of acetic acid and ethanol (Azzolini et al., 2012; Bely et al., 2008; Contreras et al., 2015; Lu et al., 2015; Medina-Trujillo et al., 2017; Renault et al., 2015). Moreover, most strains of this species show a very good fermentative capacity and are able to complete the fermentation of grape must even in monoculture (Vejarano and Gil-Calderón, 2021). Interest in *L. thermotolerans* has also increased in oenology due to its capacity to contribute to aromatic complexity, but mainly due to its ability to increase the acidity of wines (Vejarano and Gil-Calderón, 2021). Thus, this species can increase the concentration of glycerol (Kapsopoulou et al.,

2007) and interesting aromatic molecules, such as ethyl esters and terpenes (Balikci et al., 2016; Benito et al., 2016b, 2015), and produce large amounts of L-lactic acid, enhancing the overall wine acidity (Gobbi et al., 2013; Mora et al., 1990). However, although some studies have reported that *L. thermotolerans* can provide positive organoleptic characteristics in wines produced by mixed fermentations (Beckner Whitener et al., 2015; Benito et al., 2016b; Domizio et al., 2014; Gobbi et al., 2013), not all the results showed the same improvement (Hranilovic et al., 2018; Vilela, 2018), being dependent on the strain, the must composition and other fermentative conditions. Thus, the oenological potential of *L. thermotolerans* requires further investigation (Porter et al., 2019).

Grape must is a competitive environment for microorganisms, and the effect of microbial interactions must be taken into account when mixed fermentations are used, as they might affect fermentation development and the final product (Fleet, 2003). There are different types of yeast interactions, mainly based on nutrient competition, secretion of toxic compounds, and cell-to-cell contact mechanisms (Torres-Guardado et al., 2021). Interactions between *Saccharomyces* and non-*Saccharomyces* yeasts have been demonstrated by several authors (Zilelidou and Nisiotou, 2021). One type of microbial interaction is the cell-to-cell contact mechanism, which is when the presence of a certain yeast species may regulate the presence and persistence of another species. For example, cell death of some *T. delbrueckii* and *L. thermotolerans* strains during mixed fermentations was induced by a cell-to-cell contact mechanism at high cell density of *S. cerevisiae* (Nissen et al., 2003; Renault et al., 2013). Other microbial

interactions are related to the competition for nutrients, such as glucose or nitrogen. Transcriptomic analysis revealed that the presence of some non-*Saccharomyces* species, such as *T. delbrueckii*, stimulates glucose and nitrogen metabolism in *S. cerevisiae* (Curiel et al., 2017; Tronchoni et al., 2017) and could also stimulate competition for trace elements (Alonso-del-Real et al., 2019; Shekhawat et al., 2019). Finally, there are interactions involving the release of some inhibitory compounds, which could affect the growth of other yeasts. For example, *S. cerevisiae* and non-*Saccharomyces* species can secrete different killer toxins (Pérez-Nevado et al., 2006; Ramírez et al., 2015; Velázquez et al., 2015; Woods and Bevan, 1968; Yap et al., 2000), and other antimicrobial peptides (Albergaria et al., 2010; Bely et al., 2008; Branco et al., 2017, 2014). Moreover, the possible involvement of extracellular vesicles in fungal interactions during wine fermentation has recently been reported (Mencher et al., 2020). Although interactions between *Saccharomyces* and non-*Saccharomyces* yeasts have been studied, less is known about interactions between non-*Saccharomyces* species.

In the current study, we propose the use of yeast starters composed only of non-*Saccharomyces* species, specifically commercial non-*Saccharomyces* yeasts with good fermentative capacity, such as *T. delbrueckii* and *L. thermotolerans*, as single or mixed wine starters, instead of *S. cerevisiae*. Thus, in this work the contribution of non-*Saccharomyces* species to wine and the interactions between these yeast species were investigated. For this reason, single, coculture and sequential fermentations with and without cell-to-cell contact between the non-*Saccharomyces* species were

performed in synthetic must. Cell viability, nutrient supplementation, nitrogen consumption and aroma composition were analyzed.

2. Materials and Methods

2.1. Yeast strains and media

Two strains of each non-*Saccharomyces* species were used in this study. The *Torulaspora delbrueckii* strains were Viniferm NS-TD (Agrovin S.A., Spain) (Td5) and *Torulaspora delbrueckii* Biodiva™ TD291 (Lallemand, Canada) (TdB). The *Lachancea thermotolerans* strains were Laktia (Lallemand, Canada) (LtL) and Lt2 (Agrovin S.A., Spain). Moreover, *Saccharomyces cerevisiae* QA23 (Lallemand, Canada) (ScQA) was used as a control.

Yeast strains were taken from stocks preserved in YPD liquid medium (2% (w/v) glucose, 2% (w/v) bacto peptone, and 1% (w/v) yeast extract; Cultimed, Barcelona, Spain) with 40% (v/v) glycerol, at –80 °C, and grown on YPD agar plates (YPD liquid with 2% (w/v) agar). Then, isolated colonies were cultured in YPD at 28 °C and 120 rpm for 24 h and used as a preculture for inoculating the fermentations.

2.2. Fermentation conditions and sampling

Single and sequential fermentations were performed in synthetic must, as described by Beltran et al. (2004). The initial nitrogen content was 300 mg N/L.

Fermentations were carried out in triplicate at 16 °C with continuous shaking (120 rpm) in 250 mL borosilicate glass bottles containing 220 mL of medium and capped with closures that enabled carbon dioxide to escape and samples to be removed. Fermentations were conducted under

semianaerobic conditions since some aeration was necessary for harvesting samples for subsequent analysis. Synthetic must was inoculated to a final population of 2×10^6 cells/mL (Table 1). In coculture fermentations, each strain was inoculated to a concentration of 1×10^6 cells/mL. In sequential fermentations without cell contact, prior to inoculation of the second species, the medium was centrifuged and filtered to discard cells from the first inoculated yeast. Nutrient supplementation (+nutrient) was performed, when necessary, by adding vitamins, amino acids, anaerobic growth factors and oligoelements at the same concentration as in the initial synthetic must.

Fermentation kinetics were monitored by measuring daily the must density with an electronic densimeter (Densito 30PX Portable Density Metre (Mettler Toledo, Spain)) and the viable population of each species by plating serial dilutions of samples on Wallerstein laboratory nutrient medium (WLN, Difco TM, Sparks, NV, USA), since this medium allows us to distinguish colonies of *T. delbrueckii* (white colonies) from those of *L. thermotolerans* (green colonies). Fermentation was considered finished when the sugar content was below 2 g/L, or stuck when the density remained stable for more than two days. For analysis of the nitrogen compounds or organic metabolites, 1.5 mL of the supernatant was collected during fermentation and stored at −20 °C, until analysis.

Table 1. Inoculation strategies.

Inoculum Procedures	Name	Inoculum ratios (Td:Lt)	*Torulaspora delbrueckii* (Td)	*Lachancea thermotolerans* (Lt)	*Saccharomyces cerevisiae* (Sc)
Control single inoculation	ScQA	-	-	-	2×10^6
Single inoculation	Td5 TdB	1:0	2×10^6	-	-
	LtL Lt2	0:1	-	2×10^6	
Coinoculation	$Td_x + Lt_x$ $Lt_x + Td_x$	1:1	1×10^6	1×10^6	-
Sequential inoculation	Td_x , Lt_x Lt_x , Td_x	1:1	2×10^6	2×10^6	-
Sequential without contact*	Td_x / Lt_x Lt_x / Td_x	1:1	2×10^6	2×10^6	-

*In this procedure, the must fermented by the first yeast species was centrifuged and filtered before the inoculation of the second yeast species.

2.3. Nitrogen analysis

The nitrogen content was analyzed by HPLC (high-performance liquid chromatography), according to the method of Gómez-Alonso et al. (2007). The HPLC (Agilent 1100, Agilent Technologies, Germany) was equipped with two detectors, a DAD ultraviolet detector and a fluorescence detector (Agilent Technologies, Germany) and a Hypersil ODS C18 column (Agilent Technologies, Germany) with a particle size of 5 μm (250 mm × 4.6 mm) and temperature of 20 °C. The mobile phases were prepared as follow: the mobile phase (A), 2.05 g/L of sodium acetate anhydrous and 0.2 g/L of sodium azide dissolved in Milli-Q water (Millipore Q-PODTM Advantage A10) and adjusted to pH 5.8 with glacial acetic acid and the mobile phase (B), 80% (v/v) acetonitrile and 20% (v/v) methanol. Agilent ChemStation

Plus software (Agilent Technologies, Germany) was used to analyze the chromatograms. Yeast assimilable nitrogen (YAN, expressed as mg N/L) was calculated according to the nitrogen atoms of each amino acid.

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

2.4. Metabolite analysis

The concentration of some organic metabolites (was determined at the end of fermentation as described by Quirós et al. (2010). An Agilent 1100 HPLC (Agilent Technologies, Germany) equipped with a Hi-Plex H, 300 mm x 7.7 mm column inside a 1260 Infinity II Multicolumn Thermostat and connected to two detectors, a G1365B multiwavelength detector and a 1260 Infinity II refractive index detector (Agilent Technologies, Germany), was used. The column was maintained at 60 °C, and 5 mM H_2SO_4 was used as the mobile phase at a flow rate of 0.6 mL/min. Before analysis, samples were filtered through 0.22 μm pore size filters (Dominique Dutscher, Brumath, France). Chromatograms were analyzed using Agilent ChemStation Plus software (Agilent Technologies, Germany) and quantified by comparison with standards.

To determine the volatile compounds, 10 mL of wine was liquid/liquid extracted with 0.4 mL dichloromethane and 2.5 g $(NH_4)_2SO_4$ using heptanoic acid (0.7 g/L) and 4- methyl-2-penthanol (0.8 g/L) as internal standards (Ortega et al., 2001). Briefly, after 90 min of stirring at room temperature, samples were centrifuged (6,000 rpm, 5 min) and 2 μL of the organic phase was injected in split mode (10:1, 30 mL/min) into a gas

chromatograph (Agilent Technologies, Böbligen, Germany) with an FFAP column (30 m × 0.25 mm × 0.25 μm). The temperature program was as follows: 35 °C for 5 min, then raised at 3 °C/min up to 200 °C, and after 8 °C/min up to 220 °C. The injector and detector temperatures were 180 °C and 280 °C, respectively. The carrier gas was He at 3 mL/min. Volatile compounds were identified using Agilent ChemStation Plus software (Agilent Technologies, Germany) and quantified by comparison with standards.

2.5. Statistical analysis

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

3. Results

3.1. Single non-Saccharomyces fermentations

First, single fermentations were carried out with the *T. delbrueckii* and *L. thermotolerans* strains using *S. cerevisiae* as a control to determine the fermentative capacity of these non-*Saccharomyces* strains. As expected, fermentations with non-*Saccharomyces* strains were slower than those with *S. cerevisiae*, but unexpectedly, all of them were able to deplete sugars in 14-15 days (vs. 9 days for *S. cerevisiae*) (Figure 1a). The evolution of non-*Saccharomyces* viability was similar to that observed in the *S. cerevisiae*

strain, except for the LtL strain, which presented the highest viable population but also a rapid decrease in viability, resulting in a final viable population one order of magnitude lower than that of the rest of the strains (Figure 1b). Regarding the main chemical compounds produced during the alcoholic fermentations, significant differences were detected between the non-*Saccharomyces* strains and the control (Table 2). All fermentations performed with non-*Saccharomyces* strains exhibited higher contents of lactic acid and lower contents of glycerol and succinic acid than those with the *S. cerevisiae* strain. In the case of acetic acid, the result was strain dependent, since an opposite behaviour was observed in the final concentration of this compound between strains of the same species, in relation to the *S. cerevisiae* strain. However, no significant differences were detected in the ethanol content. Among the non-*Saccharomyces* strains, TdB and Lt2 produced the lowest and highest contents of glycerol and acetic acid, respectively.

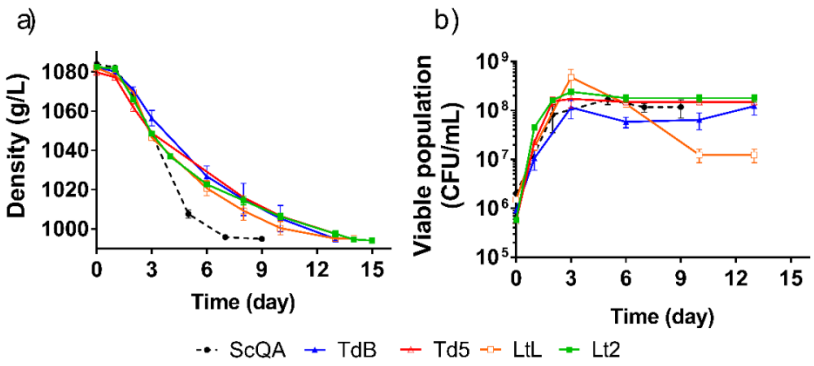

Figure 1. Monitoring of single alcoholic fermentations at 16 °C. Must density (a) and viable population of each species (b) over time.

Table 2. Concentrations (g/L) of interesting oenological compounds at the end of different fermentations performed in this study.

	Citric acid	Tartaric acid	Malic acid	Succinic acid	Lactic acid	Acetic acid	Glycerol	Ethanol (%, v/v)
Single Fermentations								
ScQA	0.39±0.09	1.94±0.01	3.77±0.20	0.36±0.06	0.34±0.02	0.49±0.01	7.83±0.00	11.10±0.11
Lt2	0.26±0.04*	2.06±0.02*	3.00±1.10	0.32±0.01	0.85±0.05*	0.87±0.03*	7.66±0.03*	11.15±0.05
LtL	0.30±0.02	2.09±0.27*	3.56±0.07	0.15±*0.06	0.52±0.08	0.27±0.04*	6.63±0.67*	11.45±0.23
Td5	0.45±*0.03	2.03±0.11*	3.68±0.24	0.10±*0.03	0.93±0.08*	0.58±0.01*	7.19±0.56*	10.90±0.00
TdB	0.42±0.09	2.40±0.13*	3.51±0.19	0.16±*0.01	0.86±0.08*	0.22±0.02*	5.04±0.27*	11.10±0.51
Coinoculated Fermentations								
TdB + LtL	0.41±0.04^{A}	2.39±0.12^{B}	4.07±0.51	0.18±0.01	0.69±0.19	0.22±0.04^{B}	6.13±0.70^{C}	11.29±0.20^{A}
Td5 + LtL	0.33±0.02^{B}	2.93±0.09^{A}	4.28±0.07	0.14±0.03	0.50±0.01	0.19±0.02^{B}	7.66±0.12^{AB}	11.59±0.29^{A}
Td5 + Lt2	0.37±0.02^{AB}	3.10±0.01^{A}	4.57±0.16	0.18±0.05	0.54±0.08	0.36±0.05^{A}	8.99±0.78^{A}	10.55±0.14^{B}
TdB + Lt2	0.38±0.01^{AB}	3.10±0.01^{A}	4.24±0.22	0.18±0.03	0.64±0.16	0.23±0.04^{B}	7.14±0.44^{BC}	10.640.12±^{B}
Sequential Fermentations								
Td5 , Lt2	0.40±0.04^{ab}	2.41±0.10^{ab}	4.28±0.14^{ab}	0.14±0.04^{cd}	1.16±^{abc}	0.52±0.02^{a}	5.33±0.16^{b}	10.09±0.11^{d}
Lt2 , Td5	0.30±0.14^{b}	1.60±1.33^{b}	4.09±0.79^{ab}	0.09±0.00^{d}	0.70±0.04^{c}	0.28±0.07^{c}	5.97±0.35^{b}	10.20±0.19^{cd}
Td5 , LtL	0.54±0.03^{a}	2.23±0.08^{b}	3.75±0.12^{b}	0.46±0.05^{bc}	1.20±0.05^{abc}	0.44±0.06^{ab}	6.45±1.13^{b}	11.08±0.11^{ab}
LtL , Td5	0.49±0.00^{a}	2.27±0.24^{b}	3.69±0.12^{b}	0.35±0.04^{cd}	0.77±0.27^{bc}	0.43±0.06^{abc}	8.06±0.34^{a}	11.17±0.32^{ab}
TdB , Lt2	0.56±0.06^{a}	2.38±0.06^{b}	3.66±0.14^{b}	0.48±0.01^{bc}	1.15±0.16^{abc}	0.47±0.01^{ab}	5.36±0.17^{b}	11.34±0.18^{a}
Lt2 , TdB	0.52±0.02^{a}	2.26±0.22^{b}	3.64±0.13^{b}	0.97±0.02^{a}	1.70±0.88^{ab}	0.43±0.03^{abc}	8.11±0.76^{a}	10.97±0.04^{ab}
TdB , LtL	0.51±0.01^{a}	2.49±0.06^{ab}	3.99±0.22^{ab}	0.73±0.23^{ab}	0.99±0.27^{bc}	0.43±0.01^{abc}	6.32±0.15^{b}	11.16±0.22^{ab}
LtL , TdB	0.51±0.01^{a}	2.47±0.26^{ab}	3.73±0.24^{b}	0.54±0.07^{abc}	0.76±0.14^{bc}	0.46±0.02^{ab}	8.13±0.35^{a}	10.94±0.08^{ab}
Sequential Fermentation without contact #								
Lt2 / Td5	0.49±0.06^{a}	2.89±0.23^{a}	4.72±0.05^{a}	0.21±0.02^{cd}	1.22±0.02^{abc}	0.37±0.05^{bc}	3.28±0.07^{c}	10.70±0.17^{bc}
TdB / LtL + nutrients	0.29±0.01^{b}	2.07±0.04^{b}	3.48±0.11^{a}	0.53±0.24^{abc}	2.16±0.09^{a}	0.42±0.05^{abc}	5.72±0.24^{b}	10.92±0.20^{ab}

Results are only presented for fermentations that exhausted sugars. * Indicates significant differences between single fermentations regarding ScQA. Capital letters indicate significant differences between coinoculated fermentations. Lowercase letters indicate significant differences between sequential fermentations.

Differences in nitrogen consumption between non-*Saccharomyces* species and the control strain were studied (Figure 2, Supplementary Table S1). TdB and LtL were the strains that consumed less nitrogen during the first 48 h. The LtL strain left the highest ammonium concentration in the medium after 48 h of fermentation. Indeed, both Lt strains showed a clear preference for organic nitrogen. TdB, showed a slower consumption pattern than Td5. Indeed, TdB was the strain with the lowest consumption of total organic nitrogen at 48 h, followed by the LtL strain. Td5 and Lt2 were the non-*Saccharomyces* strains that consumed more nitrogen, leaving less nitrogen in the medium than *S. cerevisiae*.

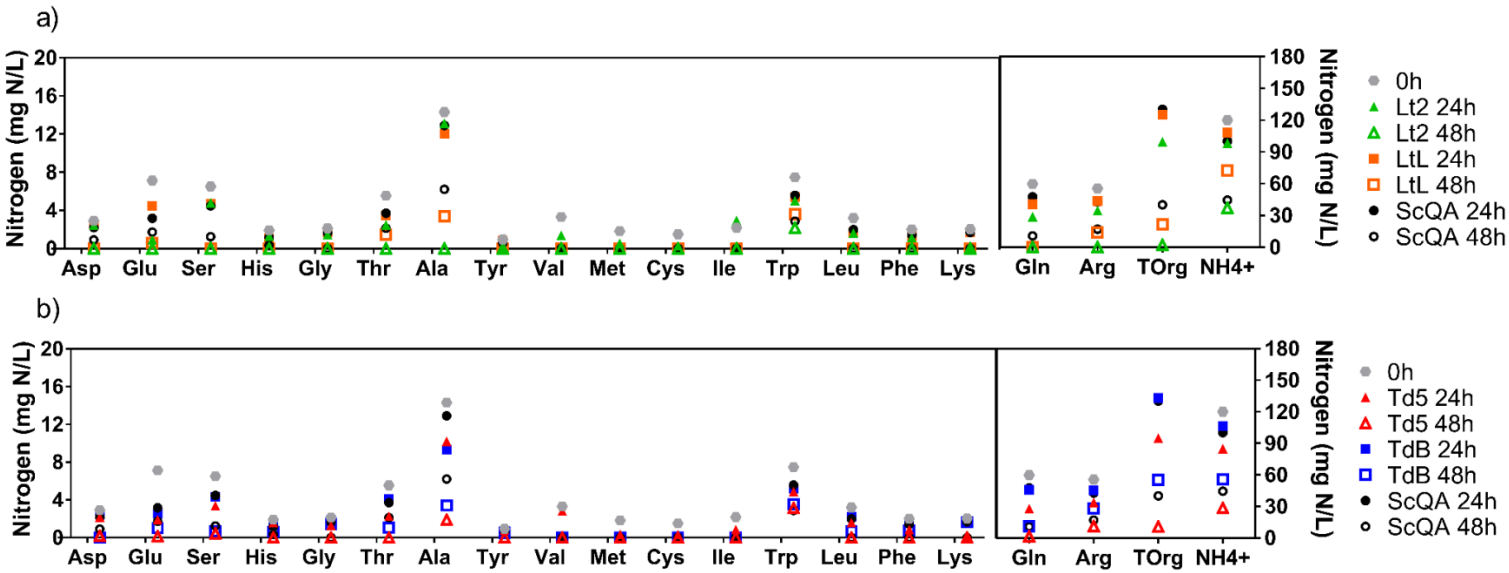

Figure 2. Nitrogen composition of the medium (mg N/L) after 24 and 48 h of fermentation by (a) two strains of *L. thermotolerans*, Lt2 and LtL, and (b) two strains of *T. delbrueckii*, Td5 and TdB, using ScQA as a control fermentation. The initial nitrogen composition of the medium was also indicated. TOrg refers to total organic assimilable nitrogen.

3.2. Coinoculated fermentations with non-Saccharomyces yeasts

After individual fermentations, coinoculations of these two non-*Saccharomyces* species, combining one strain of each species, were tested. All coinoculated fermentations were able to deplete sugars in 22 days, except *TdB+LtL*, which did so in 17 days (Figure 3).

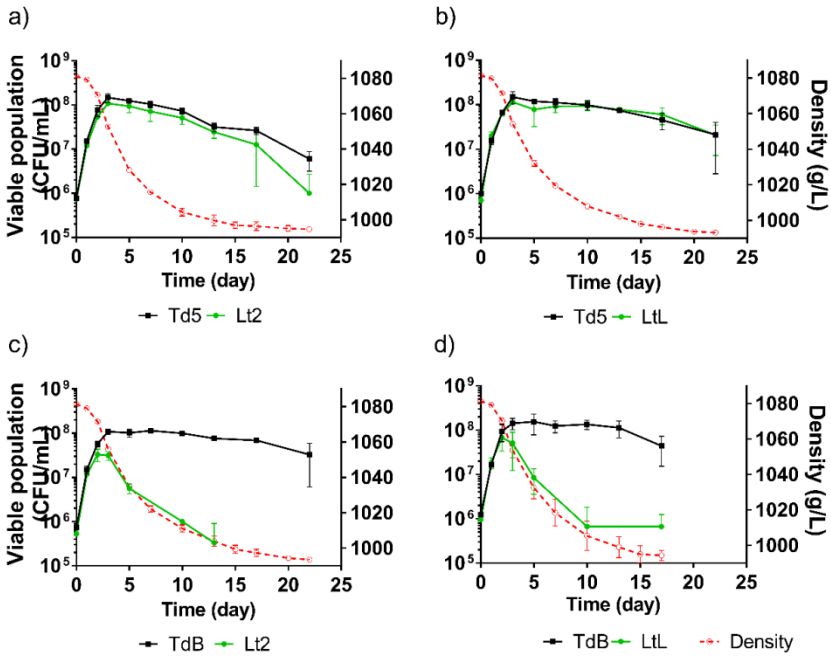

Figure 3. Must density and viable population of *T. delbrueckii* and *L. thermotolerans* strains during coinoculated fermentations: a) *Td5+Lt2*, b) *Td5+LtL*, c) *TdB+Lt2* and d) *TdB+LtL*.

Differences in growth and viability were observed depending on the strain combination. In fact, both parameters were seriously compromised in *L. thermotolerans* strains when inoculated together with the TdB strain but were practically unaffected by the presence of the Td5 strain (Figure 3). The Lt strain most affected by TdB was Lt2, which lost its cultivability in WLN plates by mid-fermentation. The effect of these strain combinations on the

main fermentation compounds was also analyzed. Fermentations performed with the Lt2 strain exhibited significantly lower ethanol concentration (a decrease between 0.6-1% (v/v)) than wines coinoculated with LtL (Table 2). The highest values of glycerol were detected in wines with the Td5 strain *(Td5+LtL and Td5+Lt2).* Indeed, the latter was the fermentation with the lowest ethanol and highest glycerol and acetic acid contents. Conversely, the wine fermented with *TdB+LtL* presented the lowest content of tartaric acid.

Nitrogen consumption during the first 48 h of fermentation was studied, and similar consumption patterns were observed in all coinoculated fermentations (Supplementary Figure S1). After 48 h, approximately 80 mg/L of nitrogen remained in the medium, mainly inorganic nitrogen. Practically all the organic nitrogen was consumed. Therefore, although coinoculation initially seemed to accelerate nitrogen consumption, after 24 h, only half of the initial assimilable nitrogen remained available for yeast growth, surprisingly, after 48 h more nitrogen remained unconsumed in these fermentations than in some individual fermentations. All these data were used in a principal component analysis (PCA) (Figure 4) to find correlations between nitrogen consumption, strains and fermentation types.

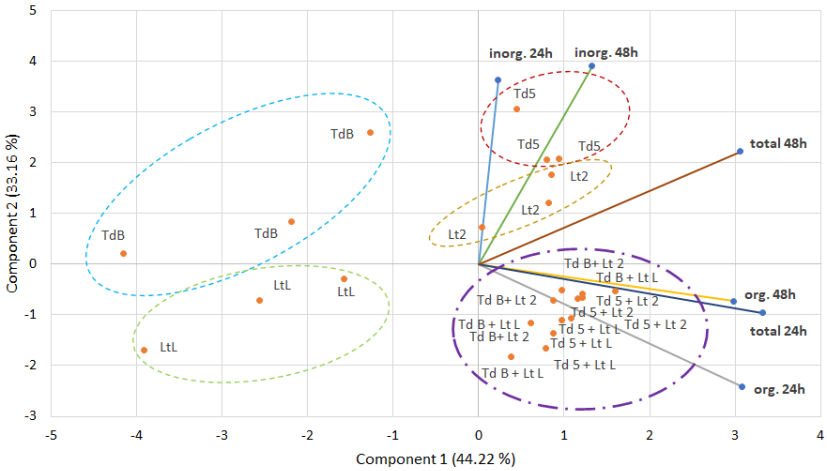

Figure 4. Biplot of principal component analysis (PCA) using the consumption of organic and inorganic nitrogen sources in single and coinoculated fermentations at 24 and 48 h as variables. The explicative variables were distributed along the PCA axes as follows: Component 1 (+): total and organic nitrogen consumed at 24 h and 48 h. Component 2 (+): inorganic nitrogen consumed at 24 and 48 h.

The PCs explained 77.38% of the variance, and the variables correlated in each component are listed in Supplementary Table S2. The eight conditions analyzed clustered into five groups, since all coinoculated fermentations grouped together, due to their preference for organic nitrogen (Component 1). Conversely, strains of the same species were grouped separately due to their different nitrogen consumption, since TdB and LtL used less nitrogen during the first 48 h than Td5 and Lt2.

3.3. Sequential fermentations with non-Saccharomyces yeasts

Finally, sequential fermentations with those strains were performed. We tested the same combinations of strains as in the coinoculated fermentations, but introduced a new variable, the order of species inoculation. The fermentations needed between 8 and 17 days to exhaust sugars (Figure 5a), with those carried out with the Lt2 and Td5 strains being

the fastest fermentations. In all cases, the first inoculated strain was the dominant strain at the end of the process, making it impossible to detect the second species in the WLN medium throughout the fermentation in most cases (Supplementary Figure S2). Only in the fermentation performed by Lt2 and TdB was the second strain, TdB, detected in percentages close to 20%. Fermentations carried out with both combinations of Td5 and Lt2 resulted in a reduction of approximately 1 % (v/v) in the ethanol content of the final wines (Table 2). Moreover, the fermentations initially inoculated with a strain of *L. thermotolerans* synthesized a higher concentration of glycerol (approximately 8 g/L), except for *Lt2 , Td5*, which was also the one that presented the least amount of acetic acid. In contrast, the fermentations initiated by a strain of *T. delbrueckii* exhibited a higher concentration of lactic acid with the exception of *Lt2 , TdB* fermentation.

Moreover, volatile compounds were analyzed in wines obtained by single, coinoculated and sequential fermentations (Figure 5b, Supplementary Tables S3, S4). In general, in single fermentations, wines elaborated with non-*Saccharomyces* strains presented different aromatic profiles than wines produced with *S. cerevisiae*. These profiles were strain dependent, with Td5 being the one that synthesized less volatile compounds, since it presented the lowest content of fusel alcohols and an absence of acids. In contrast, wines produced with the LtL strain presented the highest amount of acetate esters and acids among all the individual fermentations, mainly due to the high concentration of hexyl acetate and isobutyric acid, respectively. Wines elaborated by coinoculation of non-*Saccharomyces* were the least aromatic compared with both single and sequential

fermentations. In coinoculated wines, no ethyl ester was detected, and the concentration of acetate esters was very low, with hexyl acetate only being detected in the combination of *TdB+LtL*, which was also the fermentation that produced more 2-phenylethanol acetate.

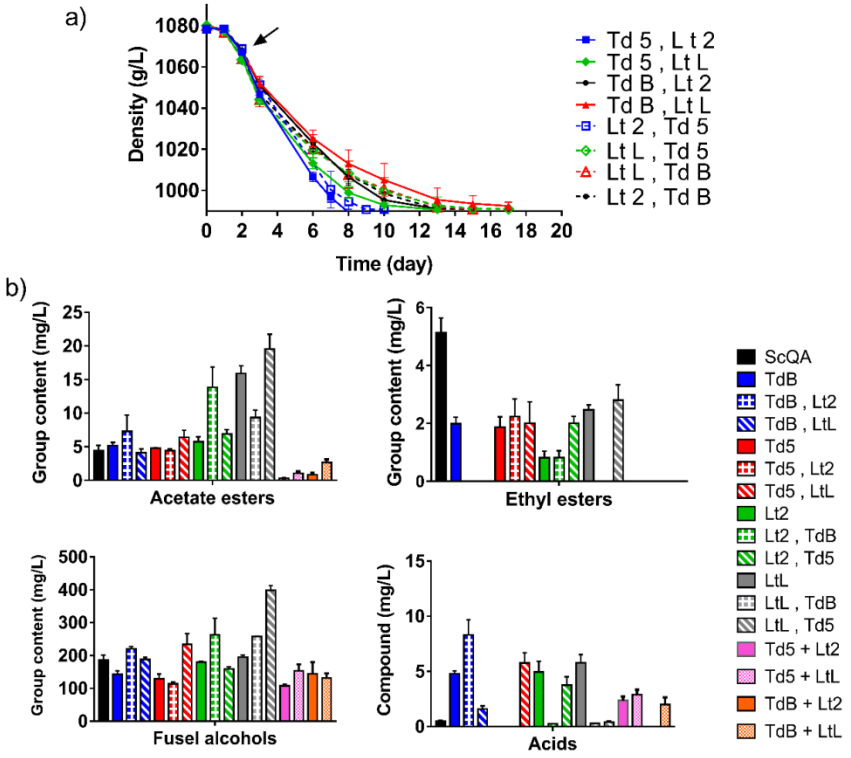

Figure 5. a) Must density in sequential fermentations using combinations of two strains (one strain of each species: *T. delbrueckii* and *L. thermotolerans*). The arrow indicates the time of inoculation of the second yeast species. b) Concentration of the main aromatic families (mg/L) at the end of single, sequential and coinoculated fermentations of *T. delbrueckii* and *L. thermotolerans*, using fermentation with the ScQA strain as a control.

In contrast, sequential fermentations starting with the *T. delbrueckii* species increased the concentration of fusel alcohols with respect to the single fermentations of this species, except in the combination of *Td5 , Lt2*. This

increase was especially important in the case of the Td5, LtL, because this combination of strains synthesized twice the amount of 3-methyl-1-butanol (isoamyl alcohol) and 2-methyl-propanol (isobutanol) and three times the amount of 2-phenylethanol produced by Td5 in the single fermentation (Figure 5, Supplementary Tables S3, S4). Additionally, the *Td5 , LtL* wine also exhibited an increase in acetate esters and acids in relation to Td5 single fermentation, with an increase in fatty acids due to the high content of isobutyric acid, a compound not detected in Td5 single fermentations but highly synthesized by LtL in single fermentations. In the case of the other combination of strains starting with Td5 (*Td5 , Lt2),* practically no effect due to the presence of the *L. thermotolerans* strain was observed, resulting in an aromatic profile very similar to that obtained in the Td5 single fermentation. The major difference was the presence of 2-butanol in the sequential fermentation, a compound not detected in Td5 single fermentations, but produced by Lt2 in single fermentations. In the case of fermentations starting with TdB, there was an increase in fusel alcohols mainly due to the increment of 3-methyl-1-butanol, 2-methyl-propanol and 2-phenyethanol. Remarkably, no ethyl ester was detected in these combinations even though all the strains were able to synthesize some of them when used in single fermentations. Similarly, isobutyric acid was not detected in the *TdB , LtL* combination, despite both strains being good producers in single fermentations.

Some similarities in the profile of volatile compounds were observed between combinations of strains regardless of the order used for the inoculation. Fermentations initiated with a *L. thermotolerans* strain, such

as those initiated with *T. delbrueckii*, increased the content of fusel alcohols, except in the combination of *Lt2 , Td5*, in which they even decreased. In this condition, as in the inverse, the volatile profile of the sequential fermentation practically coincided with that of the individual fermentation of the first strain inoculated. The main differences were the absence of 1-pentanol and butyric acid, and the presence of ethyl octanoate. Conversely, the combination that increased the fusel alcohol content the most (twofold higher than in the LtL single fermentation) was the *LtL , Td5*. However, in this case, this high concentration was not due to the increase in 2-phenylethanol, as in the case of *Td5 , LtL,* but rather to the significant increase in 3-methyl-1-butanol (isoamyl alcohol). Finally, the combination *LtL , Td5* significantly increased the content of acetate esters, since it was the strain combination that synthesized the highest concentration of hexyl acetate among all the combinations tested.

Considering all of these data, a PCA was constructed (Figure 6) to determine possible correlations between volatile composition and type of inoculation. The PCs explained 75.13% of the variance, and the variables that were correlated in each component are listed in Supplementary Table S5. The wines produced by coinoculation clustered together due to their low contents of fusel alcohols and acetate esters. In contrast, the wines produced by single strains were separated according to the content of ethyl esters and acids, with the ScQA wine clearly different from the non-*Saccharomyces* wines, mainly due to its high content of ethyl esters. Finally, the wines obtained after sequential fermentations were not grouped together, and little correlation was obtained due to the combination of

strains or order of inoculation. Among them, the *LtL , Td5* wine stood out for its high content of fusel alcohols and acetate esters, *TdB , Lt2*, for its high content of acids, and *Td5 , Lt2*, for being the least aromatic wine produced by sequential fermentation.

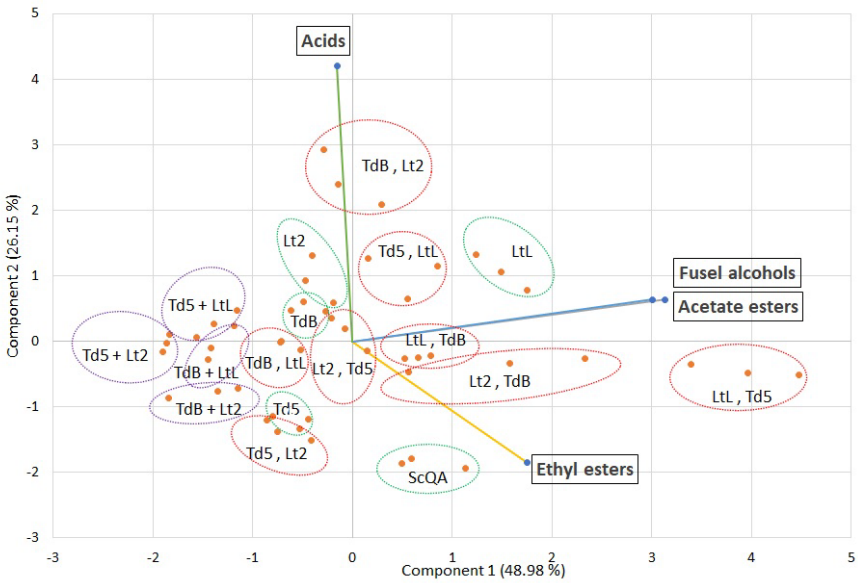

Figure 6. Principal components analysis (PCA) constructed using the total concentration (mg/L) of the main wine aromatic families at the end of single (green circles), sequential (red circles) and coinoculated (violet circles) fermentations as variables. *Acetate esters* (isoamyl acetate, hexyl acetate, 2-phenylethanol acetate), *Ethyl esters* (ethyl butanoate, ethyl dodecanoate, diethyl succinate and ethyl octanoate), *Fusel alcohols* (1-propanol, 2-methyl-propanol, cis-3-hexen-1-ol, 1-butanol, 1-pentanol, 3-methyl-1-butanol, 2-phenylethanol, 2-butanol and isopropanol) and *Acids* (isobutyric acid, butyric acid, valeric acid, decanoic acid and dodecanoic acid). The explicative variables were distributed along the PCA axes as follows: Component 1 (+): Acetate esters, Ethyl esters and Fusel alcohols. Component 2 (+): Acids.

3.4. Sequential fermentations with non-Saccharomyces yeasts without cell-to-cell contact

Due to the low imposition of the second inoculated strain, we decided to repeat these sequential fermentations, but removed the first inoculated strain by centrifugation and filtration before adding the second strain, to

determine if the lack of imposition was due to cell-to-cell inhibition. When a *T. delbrueckii* was the first inoculated strain, no strain of *L. thermotolerans* was able to finish the fermentation, with *TdB/Lt2* and *Td5/Lt2* being the combinations of strains that consumed more and less sugars, respectively, after 27 days (Figure 7a). In the case of fermentations initiated with a *L. thermotolerans* strain, only the *Lt2/Td5* combination depleted all sugars after 27 days (Figure 7b).

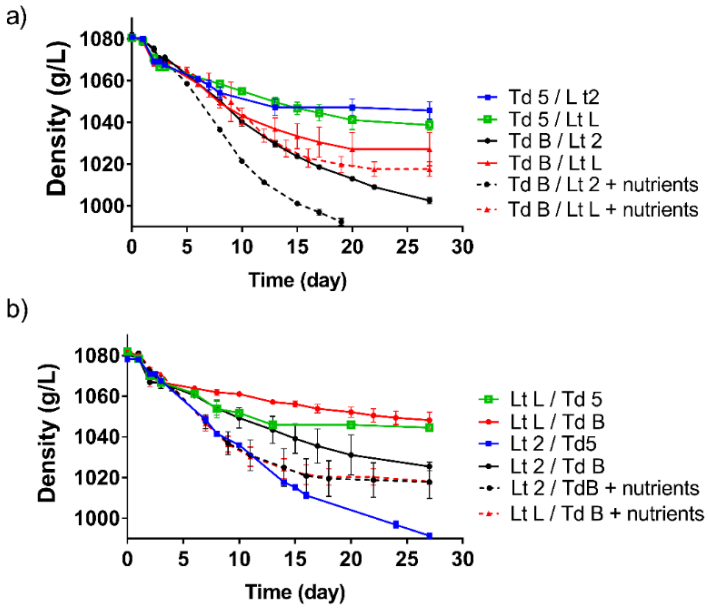

Figure 7. Must density in sequential fermentations without cell-to-cell contact. a) Fermentations initiated by the *T. delbrueckii* strain b) Fermentations initiated by the *L. thermotolerans* strain. The + nutrients refers to vitamins, amino acids, anaerobic growth factors and oligoelements mix.

Remarkably, Td5 presented a low growth in the medium previously fermented by Lt2, even exhibiting the lowest viability among all the tested combinations (Supplementary Figure S3), but it was the only one able to finish fermentation (Figure 7).

Next, to study whether stuck fermentations were due to nutrient depletion by the first inoculated strain, nutrient supplementation was tested in all the TdB combinations (*TdB/Lt2*, *TdB/LtL*, *Lt2/TdB*, *LtL/TdB*). For this test, the same concentrations of vitamins, amino acids, anaerobc growth factors and oligoelements as in the initial synthetic must were added before inoculation of the second strain (Figure 7a and 7b). In general, the nutrient-supplemented fermentations slightly improved their kinetics and cell viability, although they still resulted in stuck fermentations, except in the *TdB/Lt2* condition, which was able to finish all the sugars in 19 days (Figure 7a and Supplementary Figure S3).

In the two combinations in which sugar exhaustion occurred, the main oenological metabolites were determined in the final wines and, as expected, some differences were observed between these wines and those obtained with cell-to-cell contact between species (Table 2). When comparing *Lt2/Td5* with *Lt2 , Td5*, an increase in the concentration of all organic acids and ethanol, but a decrease in glycerol was observed. In contrast, in the comparison of *TdB/LtL* + nutrients with *TdB , LtL*, all parameters decreased, except for the concentration of lactic acid, which doubled.

4. Discussion

In this study, we performed fermentations using yeast combinations composed only of non-*Saccharomyces* species as starter cultures to determine which advantages could offer their use, as an alternative to the habitual use of *S. cerevisiae* strains. In recent decades, numerous studies have been conducted to assess the influence of several non-*Saccharomyces*

species on winemaking, and, as a result, the use of selected cultures of non-*Saccharomyces* and *S. cerevisiae* yeasts in mixed starters has been encouraged (Jolly et al., 2014; Morata et al., 2020; Padilla et al., 2016). Two of the main non-*Saccharomyces* species used for this purpose, and currently marketed by different yeast producers, are *T. delbrueckii* and *L. thermotolerans* strains. Both species have shown interesting oenological properties in mixed fermentations with *S. cerevisiae* (Benito, 2018a; Vicente et al., 2021). However, previous studies (Branco et al., 2017; Lleixà et al., 2016; Medina et al., 2012; Nissen and Arneborg, 2003; Petitgonnet et al., 2019) have revealed the potential competition between some non-*Saccharomyces* species (*Zygosaccharomyces fermentati*, *Schizosaccharomyces pombe*, *H. vineae, L. thermotolerans*, *T. delbrueckii,* etc) and *S. cerevisiae*, since the strong fermentation capacity of these non-*Saccharomyces* species (Benito et al., 2017; Jolly et al., 2014; Vejarano and Gil-Calderón, 2021) resulted in a delay in the implantation of *S. cerevisiae* in mixed fermentations in most cases. This phenomenon was observed in our previous studies (Chapters II.1 and II.2), where the use of mixed inocula of *T. delbrueckii* or *L. thermotolerans* with *S. cerevisiae* compromised the proper development of *S. cerevisiae* strains in both coinoculated and sequential fermentations. Indeed, some strains of these non-*Saccharomyces* strong fermenters have been reported to be able to complete alcoholic fermentation without the presence of a *S. cerevisiae* strain (Vejarano and Gil-Calderón, 2021), such as the strains of *T. delbrueckii* and *L. thermotolerans* used in this study, which were able to complete the alcoholic fermentation in single inoculation. Therefore, we performed

fermentations testing different inoculation modalities, such as coinoculation and sequential fermentations, the latter with and without cell-to-cell contact, combining in each of them one strain of each species, to investigate the fermentation kinetics and metabolic compounds produced by these mixed cultures and determine if they might have an oenological interest.

When used in coinoculated fermentations, the persistence of the species throughout the fermentation was dependent on the *T. delbrueckii* strain used, since in the combinations with Td5, both species remained in similar proportions during the entire process, but in the presence of TdB, the viability of both *L. thermotolerans* strains was seriously compromised. Moreover, use of the *TdB+LtL* combination resulted in a reduction of 5 days in the fermentation duration. In our hands, this is the first time that these two species have been used together without the presence of *S. cerevisiae*. Indeed, Escribano-Viana et al. (2021, 2018) used a combination of these two species but in different percentages (70:30; *T. delbrueckii: L. thermotolerans*) in sequential fermentations with *S. cerevisiae* (inoculated 72 h after non-*Saccharomyces*), and *S. cerevisiae* was the only yeast detected a week after the non-*Saccharomyces* inoculation. However, the population dynamics between these two species before *S. cerevisiae* inoculation were different depending on the medium used; in synthetic medium, the initial percentages remained similar until the inoculation of *S. cerevisiae*, whereas in Tempranillo must, there was an increase in *T. delbrueckii* (87% at Day 3). Instead, other studies have described that these species can survive until the end of fermentation despite the presence of *S. cerevisiae* (Bely et al.,

2008; Gobbi et al., 2013; Petitgonnet et al., 2019; Shekhawat et al., 2017; Taillandier et al., 2014; Zhu et al., 2021).

Sequential inoculation involving the combination of Td5 and Lt2 strains enhanced the fermentation performance, in comparison to single inoculation, reducing the time needed to complete the process. Therefore, although only the first inoculated strain was detected in WLN medium throughout the fermentation, some type of positive interaction between these strains might occur. In fact, in all the sequential fermentations, the first inoculated strain was responsible for the alcoholic fermentation, with a low presence and in most cases, even a total absence of the second inoculated species. Therefore, to assess whether this lack of imposition of the second species was due to cell-to-cell inhibition, we conducted sequential fermentations without cell-to-cell contact. To achieve this goal, cells of the first inoculated strain were eliminated from the must before inoculating the second strain. All sequential fermentations without cell-to-cell contact between species became stuck, except for the *Lt2/Td5* combination, which, albeit slowly, managed to complete the fermentation. In contrast to our results, Taillandier et al. (2014) did not observe kinetic differences between sequential fermentations performed with inoculation of *T. delbrueckii* and *S. cerevisiae* in the same and in separate containers, completing fermentations in both cases. However, the experimental design could account for these differences, since in our case, as the first species was eliminated from the medium, its participation was limited to the first 48 h, while in Taillandier et al. (2014), as a two-compartment membrane

bioreactor was used, both species participated throughout the process, although spatially separated.

Different factors could be responsible for these fermentation problems such as nutrient limitation, ethanol toxicity, the presence of killer or other toxins, extreme temperatures, the residual concentration of pesticides or fungicides, microbial competition, and poor oenological practices (Bisson, 1999). Among these factors, competition for nutrients, such as vitamins and nitrogen compounds, can be the earliest handicap during coinoculated and sequential fermentations. However, in these experiments, the low presence of the second species during sequential fermentations seemed not to be due to cellular inhibition or nutrient competition, as the addition of amino acids, vitamins, oligoelements and anaerobic growth factors did not prevent stuck fermentations. Similar results were obtained by Taillandier et al. (2014) in which the death of *T. delbrueckii* cells in sequential fermentation with *S. cerevisiae* could not be explained by a substrate competition and the authors proposed that *S. cerevisiae* may produce an unknown metabolite that affects *T. delbrueckii* viability. It is known that some yeasts can release certain metabolites that can compromise the growth of other yeast species or strains, such as killer toxins, which are not exclusive to *S. cerevisiae* (Ramírez et al., 2015; Sangorrín et al., 2008, 2007; Velázquez et al., 2015; Woods and Bevan, 1968), antimicrobial peptides (Albergaria et al., 2010; Branco et al., 2017, 2014; Kemsawasd et al., 2015b) or the recently described possible role of *T. delbrueckii* extracellular vesicles in fungal interactions (Mencher et al., 2020). In our previous study (Chapter II.1) we did not observe the secretion of inhibitory compounds (killer toxins or peptides) by

T. delbrueckii strains, with potential inhibitory activity against *S. cerevisiae*. However, further studies are needed to investigate the possible secretion of antimicrobial compounds by other non-*Saccharomyces* yeasts, and their inhibitory effect against other yeast species.

Both *T. delbrueckii* and *L. thermotolerans* species have been reported to reduce acetic acid in sequential fermentations with *S. cerevisiae* (Ciani et al., 2010; Jolly et al., 2014; van Wyk et al., 2020). In our study, all tested mixed inocula resulted in reduced volatile acidity compared with *S. cerevisiae* single fermentation, especially when the strains were coinoculated. However, it should be noted that in single fermentations, this characteristic was not always fulfilled, since two of the strains (one of each species) significantly increased acetic acid. Moreover, *L. thermotolerans* strains have stood out in oenology due to their outstanding ability to produce lactic acid, and therefore, to acidify wines, which usually leads to a reduction in ethanol (Porter et al., 2019; Sgouros et al., 2020). However, although in our single fermentations, all the non-*Saccharomyces* strains were able to increase lactic acid, the highest amounts were detected in *T. delbrueckii* strains, rather than in *L. thermotolerans*. Moreover, no decrease in ethanol was observed. In general, sequential fermentations were the inoculation strategy that most increased the concentration of lactic acid. In relation to ethanol reduction, all the combinations using Td5 and Lt2 achieved a higher reduction of one alcoholic degree.

Furthermore, the two non-*Saccharomyces* species used in this study have been described to have specific flavour-active characteristics that modulate wine flavour and aroma (Jolly et al., 2014; Porter et al., 2019; Zilelidou and

Nisiotou, 2021). The biosynthesis of fermentative aromatic compounds strongly depends on the yeast species but also on the strain used. Moreover, the positive or negative impact of these molecules on wine aroma is highly influenced by their concentrations (Tufariello et al., 2021). Therefore, we were interested in studying how the use of mixed inocula of these species in different inoculation strategies affected the wine volatile composition. Wines obtained by a single inoculation of *T. delbrueckii* or *L. thermotolerans* presented higher levels of acetate esters and acids than *S. cerevisiae*, mainly due to the higher synthesis of hexyl acetate and isobutyric acid, respectively, and lower levels of ethyl esters and fusel alcohols, with the exception of the LtL strain, which increased the concentration of fusel alcohols, mainly 1-propanol. Hexyl acetate is associated with red berry aroma, usually a pleasant aroma descriptor, and its synthesis during *S. cerevisiae* fermentation was reported to be dependent on the presence of its precursors in grape musts (C6 compounds, namely, hexan-1-ol, hexenal, (E)-2-hexen-1-ol, and (E)-2-hexenal)). However, a single *S. cerevisiae* strain was used in this study, and the ability of yeast to produce this compound might be strain dependent. Indeed, the presence of hexyl acetate in our wines produced from synthetic must, in the absence of those C6 precursors, indicates that some yeast strains, mainly non-*Saccharomyces*, might be able to synthetize this compound *de novo*, without the presence of the C6 precursors. The isobutyric acid, with a sweet, apple-like aroma, is produced from valine, and, in fact, higher concentrations of this compound in wines have been previously associated with *T. delbrueckii* (Herraiz et al., 1990, Sereni et al., 2020). However, in our study, the highest values were

synthesized by *L. thermotolerans* strains and only one strain of *T. delbrueckii* produced this compound, confirming that the aroma profile was highly dependent on the strain. Although isobutyric acid was not detected in our coinoculated wines, some wines obtained by sequential inoculation also presented higher concentrations of this compound, even higher than the ones previously reported in mixed inoculations with *T. delbrueckii* and *S. cerevisiae* (Herraiz et al. 1990). Surprisingly, sequential fermentations that started with *L. thermotolerans* strains (with the exception of Lt2, Td5), exhibited lower levels of total acids.

In contrast, all wines produced by *T. delbrueckii* and *L. thermotolerans* in single or mixed fermentation exhibited much lower concentrations of ethyl esters than *S. cerevisiae*, supporting previous studies that highlighted that non-*Saccharomyces* species are low producers of these compounds (Escribano et al., 2018; Rojas et al., 2003, 2001).

Regarding the fusel alcohols, even if single or coinoculations with non-*Saccharomyces* yeast generally decreased their levels compared with those obtained by *S. cerevisiae*, there was a general increase in these compounds in sequential inoculations using two non-*Saccharomyces* yeasts, as previously reported in mixed fermentations using non-*Saccharomyces/S. cerevisiae* inocula (Arslan et al., 2018; Escribano-Viana et al., 2021). Sequentially inoculated wines showed higher 2-methyl-propanol (except Td5-Lt2 combinations) and 1-propanol levels (in cases where *L. thermotolerans* was the first species inoculated) than *S. cerevisiae* wine. These results are consistent with those of Escribano-Viana et al. (2021), that showed an increase in the concentration of higher alcohols in wines

obtained by sequential inoculation with *T. delbrueckii* and *L. thermotolerans* compared with those fermented only with *S. cerevisiae*. Furthermore, as a characteristic of *L. thermotolerans*, Lt2 and LtL wines presented high 2-phenylethanol levels (Beckner Whitener et al., 2015), and this increase was also observed in most sequential fermentations (except for Td5 and Lt2 combinations). These results are in agreement with previous studies that showed higher levels of 2-phenylethanol in wines by mixed inoculation with *L. thermotolerans* and *S. cerevisiae* (Comitini et al., 2011; Gobbi et al., 2013). Fusel alcohols contribute to the wine aromatic complexity at concentrations below 300 mg/L (more than 400 mg/L is considered to have a negative impact on the aroma), and some compounds of this family also play an important role as ester precursors, associated with pleasant aromas (Larnbrechts and Pretorius, 2000). In fact, only the combination *LtL , Td5* produced an excessive amount of fusel alcohols, although a sensory analysis will be necessary to determine if this high concentration negatively impacts wine aroma.

In general, the wines obtained by coinoculation of *T. delbrueckii* and *L. thermotolerans* were the poorest in relation to their concentration of aromatic compounds, so, this inoculation strategy did not seem to be aromatically interesting.

Finally, it should be noted that this study highlights the relevance of the choice of the strains, since different combinations of strains belonging to the same species revealed different results, both at the fermentative and aromatic levels. In addition, the inoculation strategy chosen to carry out the mixed fermentation also had a strong impact on the alcoholic fermentation

performance and the aromatic profile of the wine, and, in the case of sequential fermentation, the order of strain/species inoculation must also be taken into account. This last parameter has not been considered thus far; in current sequential fermentations, *S. cerevisiae* is always inoculated in second place, but it becomes relevant when fermentations are performed without *S. cerevisiae*. In fact, in our study, the second species inoculated was hardly detected on plates throughout the fermentation and, in some cases, seemed to have very little impact on the volatile compound profile. These results support those of Bagheri et al. (2019), which suggested that the chemical profile of wines can be influenced by complex indirect or direct interactions between different non-*Saccharomyces* species, and highlighted the role of cell density in shaping yeast community structure and hence in the formation of different aroma signatures in wine.

In conclusion, in this study, wines were produced using starters composed exclusively of non-*Saccharomyces* species, without the participation of *S. cerevisiae*. The combination of strains and the inoculation strategy determined the fermentation kinetics, population dynamics and aromatic profile. Therefore, more studies are needed to assess whether these non-*Saccharomyces* inocula have real oenological potential to be used instead of the current mixed inocula with *S. cerevisiae*. For this purpose, essays on natural musts should be performed, and the obtained wines should be analyzed in depth, including analytical and sensory analyses. Moreover, the production and release of antimicrobial metabolites by these species should also be analyzed to evaluate whether they are the cause of the inhibitory

interactions observed among these species, which could compromise their correct development and fermentation.

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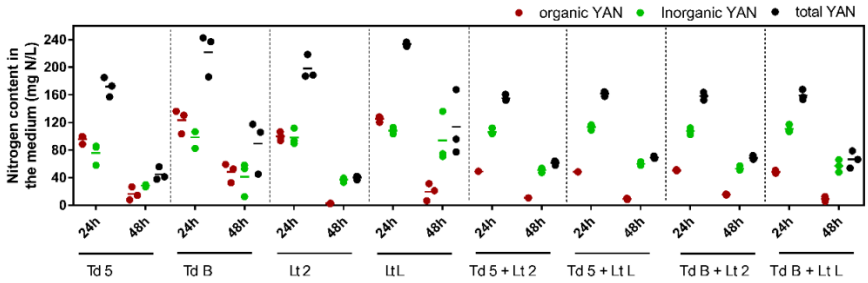
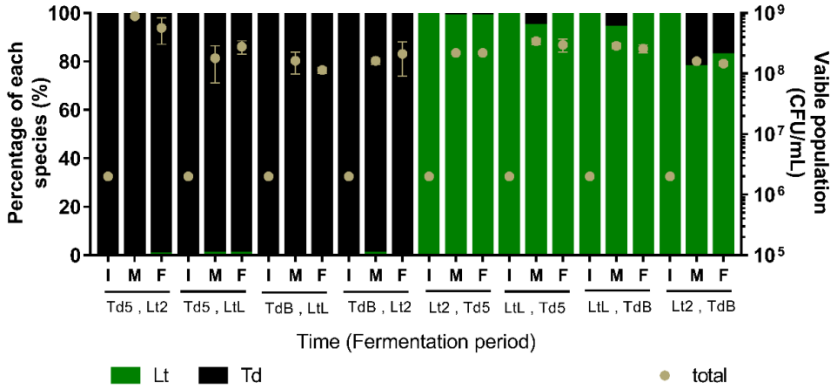
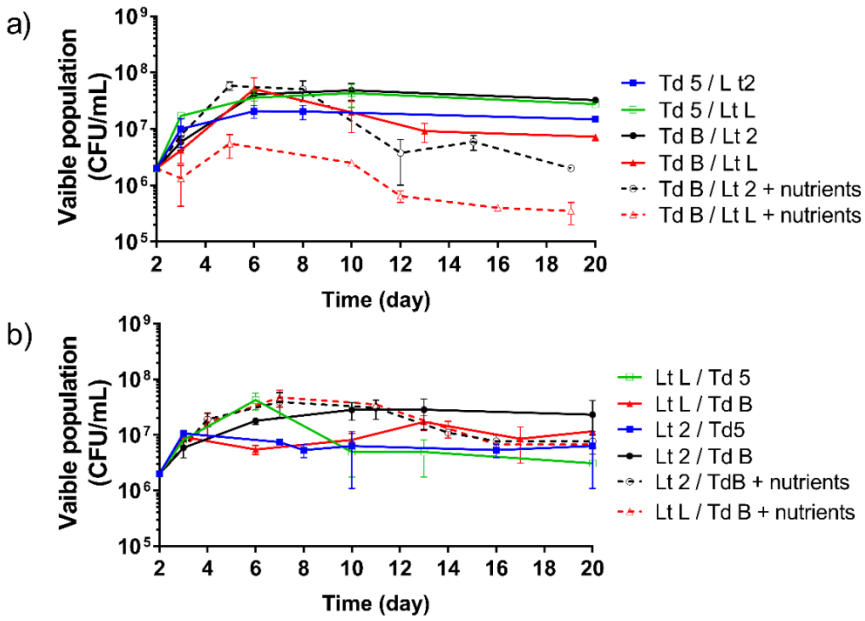
Supplementary Material



Supplementary Figure S1. Nitrogen present in the media at 24 and 48 h during single and coinoculated fermentations using different strains of *T. delbrueckii* (Td5 and TdB) and *L. thermotolerans* (Lt2 and LtL).



Supplementary Figure S2. Viable population and percentage of each species throughout the process during sequential fermentations using combinations of two strains (one strain of each species: *T. delbrueckii* and *L. thermotolerans*).



Supplementary Figure S3. a) Viable population of the *L. thermotolerans* strain during sequential fermentations without cell-to-cell contact after removing the cells of the *T. delbrueckii* strain (48 h). b) Viable population of the *T. delbrueckii* strain during sequential fermentations without cell-to-cell contact after removing the cells of the *L. thermotolerans* strain (48 h). The + nutrients refers to vitamins, amino acids, anaerobic growth factors and oligoelements mix.

Supplementary Table S1. Nitrogen (mg N/L) present in the single fermentation must at 24 and 48 h.

	Initial	ScQA		Lt2		LtL		Td5		TdB	
	0h	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h
Asp	2.91±0.00	2.19±0.14	0.92±0.22	2.46±0.27	nd	2.49±0.26	nd	2.15±0.24	0.19±0.02	2.44±0.15	nd
Glu	7.12±0.00	3.18±1.14	1.73±0.45	0.91±0.26	nd	4.49±0.07	0.57±0.03	1.89±0.05	0.14±0.02	2.54±0.21	1.00±0.06
Ser	6.5±0.00	4.47±0.29	1.24±0.16	4.81±0.25	nd	4.70±0.08	nd	3.38±0.30	0.44±0.03	4.31±0.83	0.67±0.08
His	1.91±0.00	1.17±0.04	0.32±0.16	1.31±0.06	nd	1.05±0.08	nd	1.62±0.35	nd	1.53±0.20	0.63±0.01
Gly	2.12±0.00	1.65±0.05	nd	1.47±0.17	nd	1.72±0.02	nd	1.30±0.06	nd	1.90±0.28	1.44±0.16
Thr	5.54±0.00	3.70±0.23	2.13±0.22	2.48±0.30	nd	3.46±0.26	1.46±0.47	2.27±0.19	nd	4.12±0.39	1.08±0.76
Ala	14.31±0.00	12.91±0.23	6.21±1.87	13.14±1.30	nd	12.04±0.84	3.38±0.06	10.21±0.70	1.89±0.15	9.30±1.07	3.43±1.33
Tyr	0.94±0.00	0.95±0.11	0.59±0.09	0.18±0.03	nd	0.84±0.02	nd	0.81±0.06	nd	0.74±0.15	0.51±0.09
Val	3.31±0.00	nd	nd	1.38±0.30	nd	nd	nd	2.83±0.99	nd	nd	nd
Met	1.83±0.00	nd	nd	0.54±0.29	nd	nd	nd	0.37±0.06	nd	nd	nd
Cys	1.50±0.00	nd	nd	0.28±0.13	nd	nd	nd	0.32±0.17	nd	nd	nd
Ile	2.17±0.00	nd	nd	2.95±1.49	nd	nd	nd	0.85±0.01	nd	nd	nd
Trp	7.47±0.00	5.58±0.01	2.87±0.05	5.01±0.51	2.18±0.08	5.39±0.43	3.59±0.76	4.87±0.51	3.15±0.33	5.19±0.85	3.53±0.24
Leu	3.21±0.00	1.97±0.22	nd	1.63±0.26	nd	1.70±0.06	nd	1.58±0.27	nd	2.21±0.34	0.64±0.04
Phe	2.00±0.00	1.42±0.15	nd	1.15±0.23	nd	1.27±0.05	nd	0.87±0.09	nd	1.52±0.22	0.77±0.02
Lys	2.03±0.00	1.69±0.04	nd	nd	nd	1.73±0.11	nd	0.20±0.12	nd	1.79±0.43	1.64±0.57
Gln	59.84±0.00	47.69±3.74	10.72±3.08	28.87±3.05	nd	40.65±1.28	nd	27.77±2.52	1.63±0.13	45.79±5.62	11.35±0.89
Arg	55.50±0.00	42.78±0.32	17.00±4.81	34.65±4.05	nd	43.58±2.33	14.22±3.13	33.68±1.00	11.08±6.17	45.18±0.34	28.27±2.65
TOrg	180.21±0.00	131.34±6.67	43.72±11.11	103.22±12.94	2.18±0.08	125.11±5.89	23.22±4.45	96.96±7.71	18.50±6.84	128.56±11.07	55.30±5.44
NH4+	120.00±0.00	100.16±7.53	44.53±5.23	98.34±11.95	37.19±3.49	108.26±4.65	72.71±2.71	84.93±0.94	28.55±1.62	106.54±0.09	55.73±3.51
Total	300.21±0.00	231.49±14.20	88.25±16.34	201.56±24.89	39.37±3.57	233.37±10.54	95.92±7.16	181.88±8.65	47.05±8.47	235.09±11.16	110.66±10.43

Supplementary Table S2. Correlated variables of nitrogen PCA.

	F1	F2
org. 24h	**0.786**	-0.535
org. 48h	**0.763**	-0.162
inorg. 24h	0.059	**0.804**
inorg. 48h	0.339	**0.863**
total 24h	**0.849**	-0.213
total 48h	**0.783**	0.491

Supplementary Table S3. Concentrations of wine volatile compounds (mg/L) at the end of single and coinoculated fermentations.

	ScQA	Td5	TdB	Lt2	LtL	Td5 + Lt2	Td5 + LtL	TdB + Lt2	TdB + LtL
Acetate esters									
Isoamyl acetate	nd	nd	nd	0.58±0.12	0.38±0.02	nd	nd	nd	nd
Hexyl acetate	2.45±0.08^{fg}	4.08±0.03^{defg}	4.44±0.28^{defg}	2.56±0.55^{fg}	12.76±1.12^{ab}	nd	nd	nd	1.09±0.47^{g}
2-phenilethanol acetate	2.01± 0.67^{bcd}	0.71±0.09^{ef}	0.76±0.17^{ef}	2.63±0.42^{bc}	2.77±0.04^{b}	0.34±0.02^{f}	1.05±0.28^{def}	0.84±0.30^{ef}	1.60±0.06^{cde}
Total	**4.47±0.75^{defg}**	**4.80±0.07^{def}**	**5.20±0.45^{de}**	**5.76±0.73^{cde}**	**15.91±1.14^{ab}**	**0.34±0.02^{h}**	**1.05±0.28^{fgh}**	**0.84±0.30^{gh}**	**2.69±0.45^{efgh}**
Ethyl esters									
Ethyl butanoate	nd	nd	0.71±0.06^{b}	nd	1.13±0.10^{a}	nd	nd	nd	nd
Ethyl dodecanoate	2.57±0.32^{a}	nd	nd	nd	nd	nd	nd	nd	nd
Diethyl succinate	nd	nd	nd	0.81±0.23^{a}	nd	nd	nd	nd	nd
Ethyl octanoate	2.57±0.19^{ab}	1.86±0.36^{bcd}	1.27±0.16^{cd}	nd	1.33±0.07^{d}	nd	nd	nd	nd
Total	**5.14±0.51^{a}**	**1.86±0.36^{b}**	**1.98±0.23^{b}**	**0.81±0.23^{c}**	**2.46±0.17^{b}**	**nd**	**nd**	**nd**	**nd**
Fusel alcohols									
1-propanol	21.32±2.99^{cdef}	23.33±3.57^{bcd}	20.73±0.36^{cdef}	22.51±0.78^{bcde}	31.13±2.09^{ab}	21.88±1.24^{bcdef}	13.07±0.79^{efg}	13.89±2.35^{defg}	6.35±0.52^{g}
2-methyl-propanol	40.16±3.42^{bcde}	27.91±4.66^{cdef}	32.50±1.01^{cdef}	26.73±0.65^{cdef}	30.78±1.05^{cdef}	6.34±3.10^{g}	31.28±0.66^{cdef}	22.62±6.87^{fg}	30.59±4.63^{cdef}
cis-3-hexen-1-ol	1.97±0.33^{def}	5.14±0.12^{a}	2.64±0.01^{cdef}	1.51±0.27^{f}	1.95±0.21^{def}	2.14±0.35^{def}	2.87±0.82^{cde}	1.75±0.28^{ef}	2.05±0.88^{def}
1-butanol	nd	1.84±0.01^{bc}	2.25±0.48^{bc}	3.62±0.50^{ab}	3.67±0.65^{ab}	5.14±2.48^{a}	3.36±0.00^{ab}	nd	3.46±0.00^{ab}
1-pentanol	nd	nd	0.91±0.24^{bc}	1.35±0.21^{a}	1.09±0.03^{ab}	nd	nd	nd	nd
3-methyl-1-butanol	95.60±7.59^{cd}	49.36±3.01^{fg}	62.04±4.12^{defg}	86.83±2.34^{de}	96.80±3.77^{cd}	54.50±5.46^{efg}	82.70±20.54^{def}	83.13±31.69^{def}	73.79±7.13^{defg}
2-phenylethanol	27.52±7.16^{def}	21.26±3.46^{ef}	21.37±4.76^{ef}	36.40±0.67^{cde}	28.71±2.47^{def}	17.32±5.87^{f}	19.52±0.00^{f}	21.89±1.21^{ef}	15.00±2.39^{f}
2-butanol	nd	nd	0.57±0.06^{bcd}	0.69±0.23^{bcd}	0.66±0.27^{bcd}	0.31±0.01^{cd}	0.61±0.00^{bcd}	0.66±0.40^{bcd}	0.25±0.07^{d}
Total	**186.57±14.67^{cdef}**	**128.83±14.56^{fg}**	**143.01±10.66^{defg}**	**179.66±2.08^{cdef}**	**194.80±6.12^{cd}**	**107.64±4.16^{g}**	**153.42±19.59^{defg}**	**143.93±35.76^{defg}**	**131.50±14.17^{efg}**

	ScQA	Td5	TdB	Lt2	LtL	Td5 + Lt2	Td5 + LtL	TdB + Lt2	TdB + LtL
Acids									
Isobutyric acid	nd	nd	4.10±0.23^{bc}	4.56±1.12^{bc}	5.49±0.74^{ab}	nd	nd	nd	nd
Butyric acid	nd	nd	nd	0.13±0.23^{d}	nd	0.96±0.19^{b}	1.64±0.10^{a}	nd	0.65±0.10^{c}
Valeric acid	0.49±0.10^{b}	nd	0.15±0.03^{d}	0.27±0.04^{cd}	0.29±0.01^{cd}	0.17±0.04^{d}	nd	nd	nd
Decanoic acid	nd	nd	0.55±0.22^{b}	nd	nd	0.62±0.41^{b}	nd	nd	nd
Dodecanoic acid	nd	nd	nd	nd	nd	0.61±0.09^{b}	1.26±0.35^{a}	nd	1.36±0.58^{a}
Total	**0.49±0.10^{fg}**	**nd**	**4.81±0.24^{bc}**	**4.96±0.96^{bc}**	**5.78±0.75^{b}**	**2.37±0.35^{de}**	**2.90±0.46^{de}**	**nd**	**2.01±0.64^{def}**

Lowercase letters indicate significant differences between single, coinoculated and sequential fermentations in each compound.

Supplementary Table S4. Concentrations of wine volatile compounds (mg/L) at the end of sequential fermentations.

	Td5 , LtL	Td5 , Lt2	TdB , LtL	TdB , Lt2	LtL , Td5	LtL , TdB	Lt2 , TdB	Lt2 , Td5
Acetate esters								
Isoamyl acetate	nd	nd	nd	nd	0.55±0.50	nd	nd	nd
Hexyl acetate	5.02±1.07^{def}	3.08±0.01^{def}	3.62±0.40^{efg}	6.74±2.38^{cde}	14.94±1.89^{a}	7.30±0.92^{cd}	9.85±2.59^{bc}	4.94±0.83^{def}
2-phenilethanol acetate	1.41±0.03	1.34±0.27	0.50±0.18	0.55±0.05	4.06±0.77	2.07±0.17	4.02±0.70	1.97±0.25
Total	**6.43±1.03^{cde}**	**4.43±0.28^{defg}**	**4.12±0.58^{defg}**	**7.30±2.42^{cd}**	**19.55±2.19^{a}**	**9.37±1.10^{c}**	**13.87±3.01^{b}**	**6.91±0.64^{cd}**
Ethyl esters								
Ethyl butanoate	nd	nd	nd	nd	nd	nd	nd	nd
Ethyl dodecanoate	0.50±0.27^{b}	nd	nd	nd	nd	nd	nd	nd
Diethyl succinate	nd	nd	nd	nd	nd	nd	0.82±0.24^{a}	0.56±0.03^{b}
Ethyl octanoate	1.50±0.47^{cd}	2.23±0.61^{abc}	nd	nd	2.80±0.53^{a}	nd	nd	1.45±0.22^{cd}
Total	**2.00±0.74^{b}**	**2.23±0.61^{b}**	**nd**	**nd**	**2.80±0.53^{b}**	**nd**	**0.82±0.24^{c}**	**2.01±0.24^{b}**
Fusel alcohols								
1-propanol	12.65±1.64^{fg}	14.62±1.67^{cdefg}	19.62±5.15^{cdef}	21.07±3.73^{cdef}	34.81±4.48^{a}	22.52±3.01^{bcde}	33.87±7.06^{a}	23.51±2.56^{bc}
2-methyl-propanol	56.92±7.18^{b}	23.95±2.13^{ef}	42.33±0.19^{bcd}	49.71±3.70^{b}	83.07±5.60^{a}	56.20±7.73^{b}	43.53±16.18^{bc}	25.97±2.39^{def}
cis-3-hexen-1-ol	1.87±0.49^{def}	4.24±0.59^{ab}	3.62±0.19^{bc}	3.11±0.33^{bcd}	2.26±0.28^{def}	2.52±0.00^{cdef}	3.05±0.10^{bcd}	1.74±0.55^{ef}
1-butanol	0.75±0.01^{c}	4.29±0.38^{ab}	nd	nd	3.50±1.08^{ab}	3.78±0.00^{ab}	5.02±1.73^{a}	4.03±0.31^{ab}
1-pentanol	0.72±0.22^{c}	nd	nd	nd	nd	nd	nd	nd
3-methyl-1-butanol	89.42±9.26^{de}	46.81±2.22^{g}	80.24±3.68^{defg}	92.25±3.36^{d}	217.82±3.88^{a}	136.48±9.99^{b}	128.83±22.10^{bc}	77.28±5.23^{defg}
2-phenylethanol	70.78±13.94^{a}	19.16±0.70^{f}	41.90±6.69^{bcd}	53.50±6.94^{b}	55.79±0.47^{ab}	35.42±1.76^{cde}	48.12±3.08^{bc}	25.71±1.10^{ef}
2-butanol	0.84±0.04^{ab}	0.74±0.00^{bc}	0.50±0.12^{bcd}	0.86±0.16^{ab}	1.24±0.12^{a}	0.96±0.13^{ab}	0.88±0.00^{ab}	0.53±0.16^{bcd}
Total	**233.96±32.69^{bc}**	**113.80±6.04^{g}**	**188.21±6.03^{cde}**	**220.50±6.60^{bc}**	**398.48±14.05^{a}**	**257.89±1.12^{b}**	**263.30±50.24^{b}**	**158.76±6.86^{defg}**

	Td5 , LtL	Td5 , Lt2	TdB , LtL	TdB , Lt2	LtL , Td5	LtL , TdB	Lt2 , TdB	Lt2 , Td5
Acids								
Isobutyric acid	5.56±0.89^{ab}	nd	nd	6.87±1.57^{a}	nd	nd	nd	3.38±0.86^{c}
Butyric acid	nd	nd	nd	nd	nd	nd	nd	nd
Valeric acid	nd	nd	0.54±0.07^{ab}	0.71±0.15^{a}	0.41±0.10^{bc}	0.29±0.00^{cd}	0.26±0.00^{cd}	0.37±0.11^{bc}
Decanoic acid	nd	nd	1.07±0.34^{a}	0.74±0.16^{ab}	nd	nd	nd	nd
Dodecanoic acid	0.21±0.05^{b}	nd	nd	nd	nd	nd	nd	nd
Total	**5.77±0.91^{b}**	**nd**	**1.60±0.28^{efg}**	**8.32±1.35^{a}**	**0.41±0.10^{fg}**	**0.29±0.00^{fg}**	**0.26±0.00^{fg}**	**3.75±0.77^{cd}**

Lowercase letters indicate significant differences between single, coinoculated and sequential fermentations in each compound.

Supplementary Table S5. Correlated variables of volatile compounds PCA.

	F1	F2
Acetate esters	**0.936**	0.139
Ethyl esters	**0.523**	-0.405
Fusel alcohols	**0.898**	0.139
Acids	-0.046	**0.918**

Discusión General

UNIVERSITAT ROVIRA I VIRGILI
OPTIMIZACIÓN DEL USO DE LEVADURAS NO SACCHAROMYCES EN FERMENTACIONES MIXTAS: REQUERIMIENTOS NUTRICIONALES E INTERACCIONES MICROBIANAS
Elena Roca Mesa

Las levaduras no-*Saccharomyces* se han considerado tradicionalmente microorganismos no deseables porque se asociaban a vinos con una elevada acidez volátil y a otros compuestos negativos (Jolly et al., 2014). Hoy en día sabemos que contribuyen de manera positiva en el vino (Ciani et al., 2012; Padilla et al., 2016). Por este motivo en esta tesis se ha estudiado la capacidad fermentativa y el efecto que tienen algunas levaduras no-*Saccharomyces*, tanto en fermentaciones individuales como mixtas, con y sin *S. cerevisiae*.

T. delbrueckii y *L. thermotolerans* son dos de las especies no-*Saccharomyces* que aportan aromas y otras características positivas al vino (Benito, 2018a; Benito, 2018b). En esta tesis hemos demostrado que los vinos obtenidos por fermentaciones mixtas con estas especies y *S. cerevisiae* pueden reducir la acidez volátil (Ciani et al., 2010; Jolly et al., 2014; van Wyk et al., 2020) y la concentración de etanol (Contreras et al., 2015; Belda et al., 2015; Zhu et al. 2020), y en el caso de *L. thermotolerans* puede haber también un incremento del ácido láctico (Porter et al., 2019; Sgouros et al., 2020). Además, en los últimos años se ha visto que algunas levaduras no-*Saccharomyces* tienen una buena capacidad fermentativa (Vejarano and Gil-Calderón, 2021; Vicente et al., 2021), y de hecho, en este trabajo, hemos visto como cepas de *T. delbrueckii* y *L. thermotolerans* eran capaces de acabar la fermentación por sí solas. Esto abre las puertas a la posibilidad de utilizar exclusivamente levaduras no-*Saccharomyces* para vinificar. Por ello, en el capítulo II.3 comprobamos que las cepas de *T. delbrueckii* y *L. thermotolerans* utilizadas eran capaces de terminar la fermentación en coinoculación y en inoculaciones secuenciales sin *S. cerevisiae*, dando lugar

a vinos con diferentes perfiles aromáticos. Observamos que había diferencias según el tipo de inoculación, así como el orden de las especies en la inoculación secuencial. Mediante la coinoculación se obtuvieron los vinos con perfiles aromáticos más pobres, por lo que no es una estrategia recomendable para incrementar los aromas. Comprobamos que las especies no-*Saccharomyces* son bajas productoras de ésteres etílicos (Escribano et al., 2018; Rojas et al., 2003, 2001), ya que observamos concentraciones bajas de estos compuestos en los vinos donde intervinieron *T. delbrueckii* y *L. thermotolerans*. También vimos que los vinos de fermentaciones secuenciales contenían más alcoholes superiores, tal y como se ha observado en vinos de fermentaciones mixtas utilizando levaduras no-*Saccharomyces* y *S. cerevisiae* (Arslan et al., 2018; Escribano-Viana et al., 2021). Además, en el capítulo I.2 quisimos ver la capacidad de dos cepas de *T. delbrueckii* para liberar aromas varietales tiólicos, ya que se había visto que algunas cepas de esta especie pueden incrementar los tioles volátiles (Belda et al., 2016, 2017; Renault et al., 2016; Zott et al., 2011). Pero en nuestro caso, debido a la elevada concentración de nitrógeno del mosto Verdejo utilizado, no pudimos ver ese aumento esperado, ya que la actividad enzimática relacionada con la liberación de tioles parece estar regulada por el mecanismo NCR (Subileau et al., 2008; Thibon et al., 2008). El próximo paso sería ver cómo son, tanto el perfil aromático, como el perfil fermentativo y la dinámica poblacional, cuando se utilizan diferentes mostos naturales con diferentes concentraciones de nitrógeno, así como cuando se va aumentando la escala de volumen de producción. De esta

manera sabríamos qué beneficios y qué limitaciones tendría la estrategia de utilizar levaduras no-*Saccharomyces*.

Uno de los posibles problemas de las fermentaciones mixtas es la limitación de nutrientes. En concreto, en el capítulo I.1. vimos que la capacidad de fermentar mejor estaba asociada a un consumo más rápido de nitrógeno, siendo las cepas de *T. delbrueckii* y *L. thermotolerans* capaces de consumir prácticamente todo el nitrógeno en 48 h. Por este motivo, estas cepas podrían comprometer el crecimiento y la capacidad fermentativa de *S. cerevisiae* en fermentaciones secuenciales (Rollero et al., 2018b). Es por ello que, cuando se utilizan levaduras no-*Saccharomyces* con buena capacidad fermentativa, sería recomendable inocular *S. cerevisiae* después de 24 h de inocular estas no-*Saccharomyces*. En cambio, las levaduras no-*Saccharomyces* que presentan un bajo perfil fermentativo, pueden estar más tiempo solas al inicio de la fermentación y, de esta manera, pueden tener un mayor impacto en el vino antes de inocular *S. cerevisiae* (a las 48 h). Además, el momento en que se inocula *S. cerevisiae* en las fermentaciones secuenciales va a tener un impacto tanto en el desarrollo de la fermentación como en el vino final (Ciani et al., 2010). Teniendo esto en cuenta, y viendo los resultados obtenidos en esta tesis, en los que tras inocular *S. cerevisiae* a las 48 h ésta ha tenido problemas para imponerse, en un futuro podrían realizarse de nuevo las fermentaciones secuenciales con estas levaduras no-*Saccharomyces,* pero inoculando especies *S. cerevisiae* a las 24 h, con la finalidad de observar qué diferencias hay a nivel de dinámica poblacional, de cinética de fermentación y de perfil aromático respecto a la inoculación a las 48 h.

En bodega, la suplementación con nitrógeno es una práctica habitual para el correcto desarrollo de la fermentación (Ribéreau-Gayon et al., 2006). La manera más común es la suplementación con sulfato de amonio, es decir, nitrógeno inorgánico (Gutiérrez et al., 2012; Martíez-Moreno et al., 2014; Medina et al., 2012; Ribéreau-Gayon et al., 2006). Actualmente existen diferentes tipos de preparados comerciales, algunos de ellos basados en levaduras secas inactivas y/o autolisadas, como los que hemos utilizado en los capítulos I.2 y II.1, que garantizan, entre otras cosas, un aporte nitrogenado al mosto. Este tipo de nutrientes son más completos que solamente añadir sulfato de amonio, ya que, a pesar de aportar menos nitrógeno, contienen nitrógeno orgánico y otros compuestos, como péptidos, polisacáridos, vitaminas, minerales y ácidos grasos que ayudan en la fermentación (Pozo-Bayón et al., 2009). Esto es un gran avance a la hora de evitar problemas de fermentación utilizando levaduras no-*Saccharomyces* ya que, en los últimos años, gracias al aumento de estudios sobre las preferencias nitrogenadas de estas levaduras, se ha evidenciado la importancia del nitrógeno orgánico para un mejor desarrollo y fermentación (Su et al., 2020; Prior et al, 2019). En el capítulo I.1. hemos comprobado que existen grandes similitudes entre *S. cerevisiae* y *T. delbrueckii* en lo que a preferencias de asimilación de nitrógeno se refiere, como se ha observado en otros estudios (de Koker, 2015; Kemsawasd et al, 2015; Su et al., 2020). Estas similitudes no son de extrañar ya que estas dos especies son muy próximas genéticamente; de hecho, anteriormente *T. delbrueckii* estaba incluida en el género *Saccharomyces* spp. como *Saccharomyces rosei* (Bely et al., 2008). Estos resultados sugieren que ambas

especies podrían compartir un sistema de regulación del nitrógeno similar (Crépin et al., 2012; García-Ríos et al., 2014), aunque se requieren más estudios para obtener más información acerca de los mecanismos de regulación del nitrógeno en levaduras no-*Saccharomyces*. Teniendo todo esto en cuenta, una línea de investigación podría dedicarse al desarrollo de preparados comerciales, a partir de lisados de diferentes especies de levaduras, para tener una mayor diversidad de nutrientes que se podrán utilizar según la estrategia o tipo de fermentación a desarrollar.

Como he mencionado antes, las levaduras inactivas contienen una diversidad de compuestos y nutrientes, que no son solamente nitrogenados. Esto también es beneficioso para la fermentación ya que no solamente la falta de nitrógeno es la responsable de paradas y fermentaciones lentas. De hecho, la presencia de levaduras no-*Saccharomyces* puede comprometer el crecimiento de *S. cerevisiae* porque limitan los nutrientes presentes en el medio (nitrógeno, vitaminas, minerales, etc.) (Bataillon et al., 1996; Guzzon et al., 2011; Wang et al., 2003). En este trabajo hemos demostrado la importancia de la tiamina y el zinc en fermentaciones secuenciales con *T. delbrueckii* y *S. cerevisiae* (capítulo II.1), y con *L. thermotolerans* y *S. cerevisiae* (capítulo II.2), donde su consumo por parte de las cepas no-*Saccharomyces* afectaba el crecimiento y el desarrollo de la fermentación por parte de *S. cerevisiae*, sobre todo cuando no había contacto celular entre las especies. Estos resultados concuerdan con estudios previos, donde ya se confirmó su importancia para evitar problemas de fermentación (Maisonnave et al., 2013; Medina et al., 2012; Rollero et al., 2018; Walker, 2004; Zhao y Bai, 2012). Esto tiene sentido teniendo en cuenta los papeles

que juegan estos compuestos en las levaduras. Por un lado, la tiamina está implicada en diferentes procesos metabólicos como la asimilación del carbono y la producción de lípidos, entre otros (Hohmann y Meacock, 1998; Labuschagne y Divol, 2021), y participa en la protección contra el estrés térmico, osmótico y oxidativo (Kartal et al., 2018; Kowalska et al., 2012; Li et al., 2019; Wolak et al., 2014). Aunque las levaduras son capaces de sintetizar tiamina *de novo*, esta capacidad se ve reducida en determinadas condiciones ambientales, como las que se dan durante la fermentación del vino; por lo tanto, la levadura requiere suficiente tiamina exógena para lograr tasas máximas de crecimiento y fermentación (Bataillon et al., 1996; Hohmann y Meacock, 1998; Nosaka, 2006). Por otro lado, el zinc actúa como cofactor de varias enzimas, entre las cuales está la alcohol deshidrogenasa, y es necesario para la estabilidad estructural de algunas proteínas, por lo que su deficiencia resulta en fermentaciones lentas o incompletas, y su suplementación mejora la resistencia al estrés y la tolerancia al etanol (de Nicola et al., 2009; Walker, 2004; Zhao y Bai, 2012). Además de la importancia de estos nutrientes, en las fermentaciones sin contacto celular vimos que *S. cerevisiae* sí que era capaz de acabar la fermentación cuando, aparte de suplementar con tiamina, zinc y aminoácidos, este inóculo se añadía a una concentración mayor (10^8 cel/mL). De esta manera, prácticamente no hay crecimiento celular y, por lo tanto, los requerimientos de nutrientes son bajos. En cambio, cuando el inóculo es menor (10^6 cel/mL), *S. cerevisiae* requiere más divisiones para poder llegar a una población que pueda fermentar todos los azúcares, con lo cual las necesidades nutricionales son más elevadas. Estos resultados nos

demuestran, por un lado, la importancia que tiene la concentración de inóculo que se vaya a utilizar, tal y como también se ha visto en otros estudios (Medina et al., 2012; Zhu et al., 2021) y, por otro lado, nos demuestra que tanto *T. delbrueckii* como *L. thermotolerans* compiten con *S. cerevisiae* por los nutrientes, y no sólo los nitrogenados. Esta competencia por los nutrientes es una de las interacciones que existe entre especies no-*Saccharomyces* y *S. cerevisiae,* la cual está menos estudiada entre levaduras no-*Saccharomyces* (Zilelidou and Nisioutou, 2021). De hecho, nuestros resultados muestran que el crecimiento previo de *T. delbrueckii* o *L. thermotolerans* en el mosto crea una limitación de nutrientes (principalmente aminoácidos, tiamina y zinc) que afecta al crecimiento y capacidad fermentativa de *S. cerevisiae* (capítulos II.1 y II.2), pero no tanto al de *T. delbrueckii* o *L. thermotolerans* (capítulo II.3). Por ello, en un futuro sería necesario investigar más acerca de los requerimientos nutricionales, no sólo nitrogenados, que presentan las cepas de especies no-*Saccharomyces* de interés enológico.

Hay que tener en cuenta que hemos visto que la limitación de nutrientes afectaba a *S. cerevisiae,* sobre todo, cuando descartábamos el efecto del contacto célula-célula. Para hacer esto, eliminamos del medio la especie que había empezado a fermentar (*T. delbrueckii* en el capítulo II.1 y *L. thermotolerans* en el capítulo II.2) mediante centrifugación y filtración, y a continuación inoculamos *S. cerevisiae* en este medio. Así comprobamos que la falta o baja imposición de *S. cerevisiae* en fermentaciones secuenciales no era consecuencia del contacto celular entre la especie no-*Saccharomyces* y *S. cerevisiae*, dado que sin la presencia de las no-

Saccharomyces, S. cerevisiae tampoco era capaz de completar las fermentaciones. Pese a que esta técnica de separación celular es una técnica sencilla para realizar estudios de interacciones celulares (Pérez-Nevado et al., 2006), no deja de ser agresiva y quizás se pierden compuestos importantes o interesantes durante su realización. Además, el desarrollo de la fermentación es llevado a cabo primero por la levadura no-*Saccharomyces*, y luego, por *S. cerevisiae*, por lo tanto, en ningún momento coexisten en el fermentador. Es por ello que, para estudiar mejor las interacciones entre levaduras (tanto entre no-*Saccharomyces* y *S. cerevisiae*, como ente especies no-*Saccharomyces*), podrían realizarse otro tipo de ensayos menos agresivos y donde las dos especies pudieran coexistir sin contacto celular a lo largo de toda la fermentación. Por ejemplo, podría utilizarse un biorreactor con dos compartimentos (Taillandier et al., 2014), membranas de diálisis (Branco et al., 2017; Kemsawasd et al., 2017; Petigonnet et al., 2019) o la encapsulación de una de las levaduras, por ejemplo, en alginato (Duarte et al., 2013; Kregiel et al., 2013). Durante la realización de esta tesis hicimos algunas pruebas con estos dos últimos métodos (resultados no mostrados en la tesis). El uso de membranas de diálisis tuvo como limitación la puesta a punto del sistema y la toma de muestra del compartimento interior; mientras que la técnica de la encapsulación de levaduras con alginato presentó ciertos problemas, como la liberación de levaduras encapsuladas al medio, o la falta de fermentabilidad de las levaduras encapsuladas, según el tratamiento utilizado. En un futuro podrían explorarse mejor estas metodologías y

estudiarse con ellas las interacciones que se dan tanto entre levaduras no-*Saccharomyces* y *S. cerevisiae*, como entre especies no-*Saccharomyces*.

La aplicación de estas metodologías de separación celular también nos permite estudiar otro tipo de interacciones entre levaduras, como la producción de metabolitos antimicrobianos (Branco et al., 2017). En el capítulo II.1. observamos que las cepas de *T. delbrueckii* utilizadas no secretaban toxinas killer contra *S. cerevisiae* (Ramírez et al., 2015; Velázquez et al., 2015). Además, estudiamos el efecto del sobrenadante de *T. delbrueckii* sobre *S. cerevisiae,* mediante la liofilización del medio fermentado por *T. delbrueckii* que posteriormente se añadió a fermentaciones de *S. cerevisiae,* sin observarse ningún efecto inhibitorio por parte de estos extractos. En caso de haber observado un efecto de este extracto sobre *S. cerevisiae*, se podría haber utilizado un separador de fracciones proteicas, y de esta manera detectar en qué rango de peso molecular se encontraría el/los compuestos antimicrobianos y así poderlos identificar y caracterizar.

La capacidad de fermentar de las levaduras no-*Saccharomyces*, así como las diferentes interacciones que hemos comprobado a lo largo de la realización de esta tesis, son cepa-dependientes. Es decir, los resultados obtenidos ponen de manifiesto que estas levaduras presentan una alta variabilidad entre cepas de una misma especie, tal y como sucede entre cepas de *S. cerevisiae* (Pretorius, 2000). De esta manera, la elección de las levaduras no-*Saccharomyces* que se utilicen para vinificar no tiene que depender únicamente de las características que nos aportará al vino a nivel de especie, sino de cepa. Es por ello que, en un futuro, se deberían realizar más estudios

para caracterizar diferentes cepas no-*Saccharomyces* teniendo en cuenta diferentes condiciones de fermentación que mimeticen el proceso de vinificación (con mostos naturales, más volumen, diferentes concentraciones de nitrógeno…). Para realizar esta caracterización, primero sería necesario optimizar técnicas moleculares que sirvan para poder tipificar las diferentes especies no-*Saccharomyces* de interés. Aunque actualmente existen múltiples técnicas moleculares para identificar a nivel de especie, hay más limitaciones para diferenciar a nivel de cepa (Loira et al., 2020). En este trabajo intentamos diferenciar cepas de las especies *T. delbrueckii*, *L. thermotolerans* y *M. pulcherrima* utilizando la técnica de PCR del ARNt (ARN transferente), pero no fuimos capaces de obtener perfiles diferentes entre las cepas (resultados no mostrados en la tesis). Pese a que pueden utilizarse otro tipo de técnicas moleculares para tipificar levaduras no-*Saccharomyces*, como por ejemplo la técnica RAPD-PCR (Renault et al., 2009), no son técnicas tan reproducibles y discriminantes como lo son las técnicas de tipificación de *S. cerevisiae*, como los elementos delta o el RFLP del mtDNA (Legras and Karst, 2003; Querol et al, 1992). En un futuro sería muy útil poder disponer de una técnica molecular semejante a estas para poder identificar cepas diferentes de especies no-*Saccharomyces*.

Adicionalmente, se podría optimizar las características de las levaduras no-*Saccharomyces* con interés comercial mediante evolución dirigida, tal y como se está haciendo con cepas de *S. cerevisiae* (Steensels et al., 2014). De esta manera, las levaduras evolucionadas sí que podrían utilizarse en la

industria, al contrario de lo que sucede con las levaduras modificadas genéticamente.

En conclusión, la hipótesis inicial de esta tesis "**El uso de levaduras no-*Saccharomyces* en fermentaciones mixtas afecta negativamente a las levaduras *S. cerevisiae*, debido a la competencia por los nutrientes del medio, así como a interacciones microbianas existentes entre ellas**" se cumple, pero no siempre. Dependerá de la cepa utilizada, así como de la concentración del inóculo y la disponibilidad de nutrientes, no sólo nitrogenados, en el medio. Además, el hecho de que el uso de especies no-*Saccharomyces* afecte negativamente a *S. cerevisiae* no implica que haya un efecto negativo sobre la fermentación y el vino obtenido. De hecho, si las levaduras no-*Saccharomyces* tienen buena capacidad fermentativa y contribuyen positivamente en el vino, no tiene por qué ser un problema que *S. cerevisiae* no se imponga. En un futuro hay diversas líneas de investigación para continuar con el estudio de las interacciones entre levaduras no-*Saccharomyces* y *S. cerevisiae*, y entre especies no-*Saccharomyces:* la puesta a punto de otras metodologías para estudiar interacciones sin contacto celular, así como de técnicas para tipificar cepas no-*Saccharomyces*, y técnicas para caracterizarlas; el estudio de los requerimientos nutricionales de cepas no-*Saccharomyces* de interés enológico; el desarrollo de preparados comerciales dirigidos a la cepa no-*Saccharomyces* que se vaya a utilizar; y el estudio del uso de levaduras no-*Saccharomyces* para vinificar sin presencia de *S. cerevisiae*. Así pues, actualmente el estudio de las levaduras no-*Saccharomyces*, así como su aplicación, es un amplio campo en el que queda mucho por investigar.

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OPTIMIZACIÓN DEL USO DE LEVADURAS NO SACCHAROMYCES EN FERMENTACIONES MIXTAS: REQUERIMIENTOS NUTRICIONALES E INTERACCIONES MICROBIANAS
Elena Roca Mesa

Conclusiones Generales

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1. Los requerimientos y las preferencias de nitrógeno de las levaduras no-*Saccharomyces* durante la fermentación dependen de la especie y la composición del medio.

   a. Las levaduras con buena capacidad fermentativa, como *T. delbrueckii* y *L. thermotolerans* presentan un perfil de consumo de nitrógeno similar al de *S. cerevisiae*, siendo el medio completo el más óptimo para su crecimiento.

   b. Algunas cepas de *L. thermotolerans* muestran problemas de crecimiento y fermentación en medios con sólo nitrógeno orgánico o inorgánico, tratándose de una característica cepa-dependiente.

   c. Las levaduras con baja capacidad fermentativa *M. pulcherrima, S. bacillaris* y *H. uvarum*, muestran preferencia por nitrógeno inorgánico, y en las dos últimas, el consumo de aminoácidos ser retrasa significativamente en presencia de amonio, pero se adelanta en ausencia de éste.

   d. La composición nitrogenada del mosto tiene un efecto directo sobre el perfil de fermentación y el crecimiento de las levaduras no-*Saccharomyces*.

   e. La suplementación del mosto con levadura seca inactiva mejora el crecimiento celular, sobre todo en medios con bajos niveles de nitrógeno. Esta mejora no es debida únicamente al aumento de nitrógeno asimilable obtenida por esta adicción.

2. La liberación de tioles varietales en mostos ricos en nitrógeno no se ve afectada ni por la presencia de *T. delbrueckii*, ni por la adición de levadura seca inactiva.

3. La imposición de *S. cerevisiae* en fermentaciones mixtas con levaduras con alta capacidad fermentativa, como *T. delbrueckii* y *L. thermotolerans*, se ve comprometida, principalmente en inoculaciones secuenciales.

a. La disponibilidad de nutrientes y el tamaño del inóculo tienen un efecto en el crecimiento y el desarrollo de la fermentación de *S. cerevisiae* en fermentaciones secuenciales.

b. En un medio parcialmente fermentado por *T. delbrueckii*, *S. cerevisiae* tiene problemas para crecer y consumir los azúcares, a causa de la limitación de nutrientes de ese medio, y no por presencia de compuestos antimicrobianos secretados por esta especie ni por una inhibición por contacto celular.

c. La adición de tiamina, zinc y aminoácidos tiene un efecto positivo en *S. cerevisiae* en fermentaciones secuenciales, con y sin contacto celular.

4. Es posible la utilización de únicamente levaduras no-*Saccharomyces* con buena capacidad fermentativa, como *T. delbrueckii* y *L. thermotolerans*, como inóculos para la fermentación alcohólica de vinos, ya sea a nivel individual o en combinaciones entre ellas.

a. En fermentaciones secuenciales, la primera especie inoculada es la que se impone a lo largo de la fermentación, siendo la principal responsable de las características del vino final.

b. La limitación de nutrientes no es la causa principal de la falta de crecimiento y fermentación de la segunda especie inoculada.

c. El perfil aromático de los vinos está influenciado por la estrategia y el orden de inoculación de las levaduras no-*Saccharomyces*.

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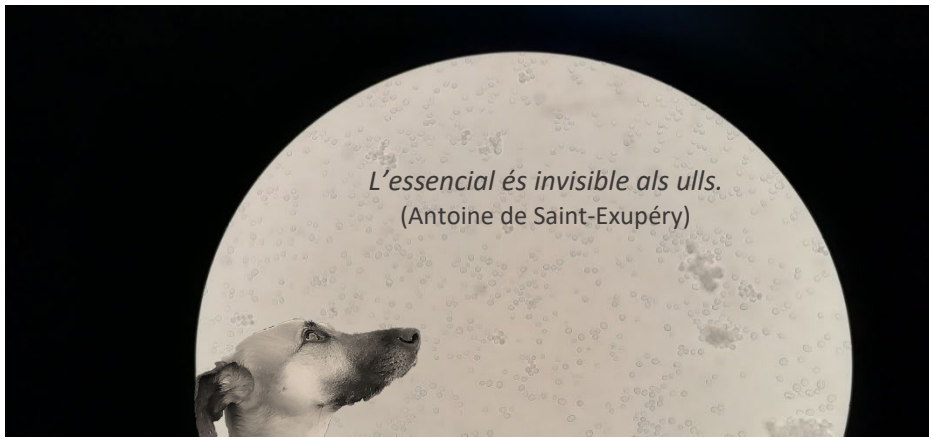

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