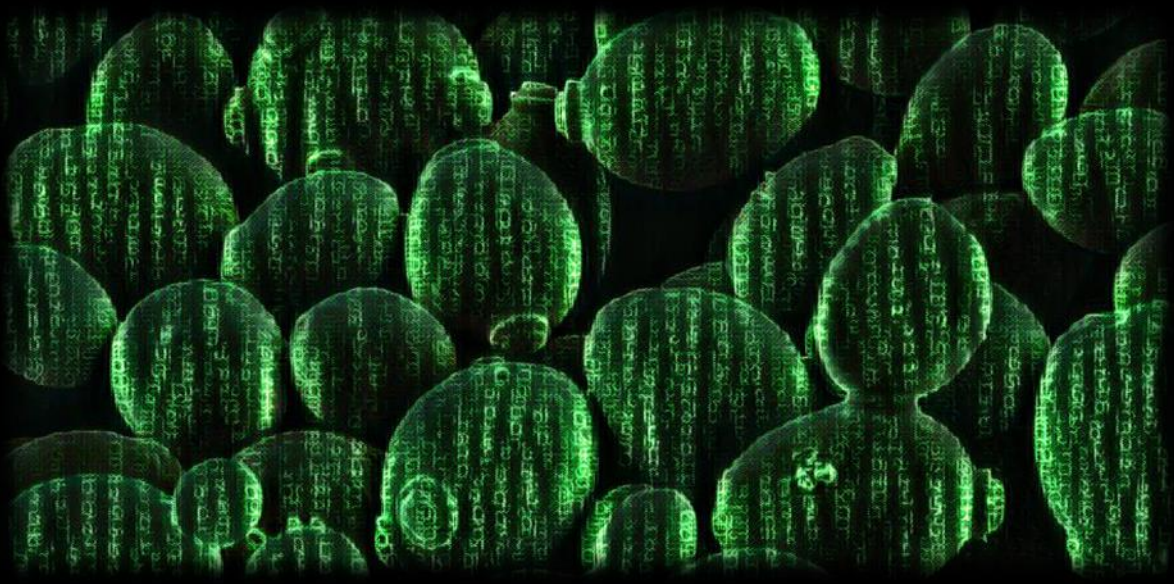
Global analysis of the interaction between *Saccharomyces cerevisiae* and other *Saccharomyces* species of enological interest

Javier Alonso del Real Arias
PhD Thesis

Valencia, 2019



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Dto. de Bioquímica y Bilogía Molecular

Doctorado en Biomedicina y Biotecnología

La Dra. Amparo Querol Simón, Profesora de investigación del Consejo Superior de Investigaciones Científicas (CSIC) en el Instituto de Agroquímica y Tecnología de los Alimentos (IATA) y el Dr. Eladio Barrio Esparducer, Profesor Titular del Departamento de Genética de la Universitat de València.

CERTIFICAN

Que Don. Javier Alonso del Real Arias, Licenciado en Biotecnología por la Universidad Pablo de Olavide de Sevilla, ha realizado bajo su dirección el trabajo titulado: "Global analysis of the interaction between *Saccharomyces cerevisiae* and other *Saccharomyces* species of enological interest", que presenta para optar al grado de Doctor en el programa de Biomedicina y Biotecnología por la Universitat de València. Asimismo, certifica haber dirigido y supervisado tanto los distintos aspectos del trabajo como su redacción.

Y para que así conste a los efectos oportunos, en cumplimiento de la legislación vigente, firman el presente certificado en

Valencia, a 2 de diciembre de 2018

Fdo. Dra. Amparo Querol Simón Fdo. Dr. Eladio Barrio Esparducer

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"Yo soy yo y mi circunstancia" (Ortega y Gasset), es una de las frases más famosas de la filosofía del siglo XX. Normalmente se podría entender que esta frase pretende quitar al individuo parte de su responsabilidad en lo que hace. En realidad, el filósofo quiere resaltar la importancia del medio que nos rodea, el cual tenemos que entender y explicar para entendernos a nosotros mismos, por así decirlo. En este momento, en el que la cultura del *Just do it* está más en auge que nunca, en el que nos hacen creer que todo lo bueno o malo que nos pase depende sólo de nuestro esfuerzo y nuestras decisiones, y que no hay más barreras que las de tus propias capacidades, creando así individuos frustrados y solitarios, me parece muy necesario recordar esta frase. Y es que, puestos a escribir unos agradecimientos por todo el apoyo recibido durante la realización de esta tesis, uno echa la vista atrás y se da cuenta de que nada de esto hubiera sido posible si no se hubiera dado determinada circunstancia, si tanta gente y, sí, tantas instituciones, no hubieran puesto de su parte a lo largo de mi formación. Por ello, quiero empezar resaltando lo agradecido y lo afortunado que me siento por haber tenido una buena educación, por haber nacido en un país próspero, y por todas las decisiones tomadas por personas más cercanas y más lejanas que me han permitido desarrollarme hasta aquí. Creo que es de justicia reconocer esto, sobre todo, con todas aquellas personas que no tienen la suerte que he tenido yo.

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Luz en la mañana,

y en la noche, quejío y quiebro.

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RESUMEN EN ESPAÑOL

Se cree que la aparición de los primeros procesos biotecnológicos que dieron lugar a alimentos fermentados vino de la mano de la agricultura, es decir, cuando tuvieron lugar las primeras poblaciones de humanos sedentarios. Estos procesos ocurrían de manera espontánea cuando la cosecha de determinado fruto (la uva) o cereal (el trigo o la cebada) se almacenaba en condiciones en las que no estaban aisladas de fenómenos como la humedad o la presencia de microorganismos, haciendo posible la transformación por procesos bioquímicos de estos alimentos (Katz and Voigt, 1986). De hecho, se han encontrado restos arqueológicos de alimentos fermentados en el Neolítico (8.500 – 4.000 AC) en China, Irán y Egipto (Cavalieri et al., 2003). A partir de ese momento, el vino fue desarrollándose y extendiéndose a múltiples regiones debido a su percepción como una bebida con propiedades farmacológicas, nutricionales y sensoriales (McGovern, 2003). En el año 500 DC ya se producía en toda el área Mediterránea. A partir del siglo XVI, los colonizadores europeos implantaron viñedos y bodegas en distintos lugares de Sudamérica y Norteamérica, Sudáfrica y Australia. Hoy en día, el volumen que supone el mercado del vino es enorme, con una producción anual de alrededor de 270 millones de hectolitros. En España, el sector del vino es un sector económico clave, con un 15% de la producción mundial, siendo uno de los mayores productores a nivel mundial (Organisation Internationale de la Vigne et du Vin, 2017).

En cuanto al estudio científico de aspectos relacionados con la fermentación de vino, el descubrimiento de las levaduras tuvo lugar cuando el padre de la microbiología, el holandés Antoni van Leeuwenhoek, las observó bajo el microscopio y las llamó “animálculos”. Más tarde, en 1890, otra figura clave

de la microbiología, el científico francés Louis Pasteur, descubrió el papel de los microorganismos en el proceso de fermentación. Demostró que las bebidas fermentadas son el producto de la transformación de los azúcares en alcohol etílico por parte de las levaduras en ausencia de oxígeno, distinguiéndolo además de fermentaciones "malas" que ocurrían en aerobiosis. También hipotetizó que las levaduras de vino venían de la superficie de la piel de la uva, entrando en contacto con el mosto de esta manera (Barnett, 2000; Pasteur, 1857). El origen del término levadura va ligado a los procesos en los que participan dependiendo de la región geográfica. Así, los franceses la denominaron "levure" por su papel en el aumento de volumen de la masa de pan. De esta palabra surgió la voz en castellano "levadura". Por otro lado, los holandeses la llamaron "gist", la misma palabra que utilizaban para denominar la espuma que se formaba en la superficie del mosto de cerveza durante la fermentación. De "gist" derivó la palabra inglesa "yeast".

Al final del siglo XIX el proceso de fermentación del vino aún se veía como un proceso espontáneo en el que las levaduras se transferían inconscientemente a través de los recipientes de fermentación, o a través de los posos residuales que quedaban tras una fermentación. Poco a poco la bioquímica y la fisiología de la levadura fue describiéndose, y se hicieron las primeras identificaciones y descripciones de la levadura *Saccharomyces cerevisiae*, que a partir de ahí empezó a considerarse el microorganismo responsable de la fermentación de vino. También se introdujo el concepto de competición por el medio fermentativo, en el que *S. cerevisiae* excluía competitivamente a otros organismos naturalmente presentes en el mosto (Boulton et al., 1999).

S. cerevisiae es probablemente el organismo más relevante desde el punto de vista de la biotecnología. Además de los alimentos fermentados, se utiliza para producir bioetanol, así como enzimas de importancia para la industria y otros productos, como suplementos alimentarios u hormonas. Fue el primer organismo eucarionte cuyo genoma fue secuenciado (Goffeau et al., 1996). Alrededor del 60% de los genes de *S. cerevisiae* tienen ortólogos en el genoma humano. Por todo ello, se desarrollaron múltiples herramientas que promovieron su uso como organismo modelo en ciencia médica. Ha sido fundamental para el desarrollo en el conocimiento de algunas enfermedades como Alzheimer o Parkinson (Tenreiro et al., 2013).

La producción de vinos ha conservado aproximadamente las mismas fases a lo largo de toda la historia. Comienza con la recolección de la uva madura y su prensado. Posteriormente ocurre la fermentación alcohólica, etapa en la que, al principio, existe una gran variedad de microorganismos con una capacidad fermentativa débil que conforman la compleja microbiota natural del mosto y que son responsables del inicio de la fermentación (Jolly et al., 2003b). En esta etapa *S. cerevisiae* se encuentra en una proporción minoritaria con respecto a otros géneros de levadura, sin embargo, en pocas horas es capaz de crecer más rápido que el resto, excluyendo de la fermentación al resto de organismos, principalmente debido a su vigorosa capacidad fermentativa, que le permite producir grandes cantidades de etanol que resulta tóxico para el resto de microorganismos. Por tanto, *S. cerevisiae* va a ser responsable de la fermentación, que durará hasta que todos los azúcares del mosto son consumidos. Posteriormente, tiene lugar la proliferación de las bacterias del

ácido láctico, que son responsables de la fermentación maloláctica o segunda fermentación, que añade complejidad aromática a los vinos y reduce la acidez.

Tradicionalmente, se dejaba que el vino recorriera estas etapas de forma natural, con la microbiota indígena de cada producción. Esto tiene la desventaja de que el producto es poco reproducible entre botas y entre añadas, debido en parte a lo impredecible del desarrollo de la microbiota según las condiciones bióticas y abióticas en cada caso. De hecho, se pueden dar casos en que organismos poco deseables que estropean el vino proliferen, haciendo perder parte de la producción. Hoy en día, esto solo ocurre en pequeñas producciones, prácticamente la totalidad del vino que hay en el mercado es producido por compañías que no pueden permitirse dejar de controlar este factor. De manera que las bodegas realizan la inoculación de determinadas levaduras, casi siempre *S. cerevisiae*, en gran cantidad en el mosto para asegurar que la fermentación se complete con éxito.

Pese a lo controlado del proceso, la industria del vino se está enfrentando a distintos retos en los últimos años. En primer lugar, el cambio climático que provoca un mayor contenido de azúcares en la uva, y por lo tanto, mayor concentración de alcohol en el producto final. Además, el calentamiento global también está afectando a la acidez del vino, lo cual repercute en la percepción aromática de los vinos. Por otra parte, el mercado cada vez demanda productos más sofisticados, con mayor complejidad o diversidad aromática. Para conseguir conservar una mayor riqueza aromática en el vino, muchas bodegas llevan a cabo fermentaciones a baja temperatura en el caso de los vinos blancos y rosados, debido a que los compuestos aromáticos, por definición, son altamente volátiles, de manera que se conservan en mayor proporción en el

vino bajo estas condiciones. El problema de esta estrategia es que el tiempo de fermentación se alarga, haciendo que repercuta en la capacidad productiva de las bodegas, y llegando incluso a paradas de fermentación, debido a deficiencias en la adaptación de *S. cerevisiae* a estas temperaturas.

Como hemos dicho, la gran mayoría de cepas de levadura comerciales para su uso en fermentación vínica son de la especie *S. cerevisiae*, Sin embargo, para superar los retos que se plantean en la industria, una de las estrategias que están siendo más impulsadas en los últimos tiempos consiste en el uso de levaduras de otras especies. Algunas de las levaduras no pertenecientes al género *Saccharomyces* presentes en la microbiota natural de los mostos han sido probadas en fermentaciones como buenas opciones para reducir el nivel de etanol y variar el perfil aromático en los vinos (Cordero Otero et al., 2003; Gonzalez et al., 2013; González et al., 2018; Jolly et al., 2014; Nissen et al., 2003; Sadoudi et al., 2012). Sin embargo, el mayor problema es que son fácilmente desplazadas por las poblaciones de *S. cerevisiae* que se encuentran naturalmente en los mostos, y a menudo tendrían problemas para cumplir con su cometido de disminuir el nivel de etanol y cambiar el perfil aromático debido a no tener suficiente protagonismo durante el proceso (Albergaria et al., 2010; Arneborg et al., 2005; Ciani et al., 2016a; Granchi et al., 1998; Nissen et al., 2003; Pérez-Nevado et al., 2006; Torija et al., 2001; Vendramini et al., 2017).

Por ello, la utilización de levaduras del género *Saccharomyces* distintas de *S. cerevisiae* es uno de los mayores focos de investigación del campo de las levaduras alternativas para vinificación. Dadas las similares características con *S. cerevisiae*, son consideradas buenas fermentadoras, organismos, *a priori*,

seguros para la salud humana, y quizá de más fácil implantación debido a una mejor capacidad competitiva en comparación con levaduras de otros géneros, sobre todo, gracias a su alta tolerancia al etanol. De hecho, algunas de estas especies, como la criotolerante *S. uvarum*, han sido encontradas en vinos producidos en regiones con climas fríos. Esta levadura ha formado parte de estudios recientes para su caracterización y uso como levadura de interés en enología (Giudici et al., 1995; González Flores et al., 2017; Pérez-Torrado et al., 2016; Rodríguez et al., 2014). También se han aislado híbridos interespecíficos de esta especie y *S. cerevisiae*, e híbridos de tres especies diferentes en los que también se incluye *S. kudriavzevii*, otra levadura muy bien adaptada a bajas temperaturas (Salvadó et al., 2011b), que sólo ha sido encontrada en vinos en esta forma híbrida, pero nunca como organismo puro.

S. kudriavzevii, además de ser capaz de fermentar a bajas temperaturas donde *S. cerevisiae* presenta los problemas que hemos mencionado anteriormente, también ha resultado ser una buena especie candidata para conseguir reducir los niveles de etanol en vinos, aumentando además los niveles de glicerol debido a su metabolismo del carbono alternativo (Gonzalez et al., 2007; Pérez-Torrado et al., 2016, 2017a; Peris et al., 2016), lo que es considerado positivo desde el punto de vista organoléptico (Goold et al., 2017), y explica en parte la alta tolerancia de *S. kudriavzevii* a las bajas temperaturas (Oliveira et al., 2014). Además, posee un perfil aromático de potencial interés para la industria (Stribny et al., 2015).

Para comprobar la utilidad de estas especies en fermentación, es necesario caracterizar su comportamiento en una fermentación en la que *S. cerevisiae* está presente. Por ello, nuestro primer objetivo en la presente tesis fue analizar

fermentaciones llevadas a cabo por cada una de las especies alternativas consideradas de interés del género *Saccharomyces*; *S. paradoxus, S. eubayanus*, *S. uvarum,* y *S. kudriavzevii*, cuando compiten con una levadura *S. cerevisiae* vínica de referencia a diferentes temperaturas (12 °C, 20 °C y 25 °C). Preparamos botellas con 200 mL de mosto sintético (Riou et al., 1997) en las que inoculamos una mezcla de ambas cepas de levadura en proporción 1:1 con una densidad óptica inicial de 2×10^6 células/mL. A partir de ahí realizamos conteos de células a lo largo de la fermentación, y medimos la proporción de cada una de las cepas en cada punto por medio del uso de cebadores específicos de cada cepa en una reacción de polimerasa en cadena cuantitativa (QPCR, por sus siglas en inglés). Esto nos permitía calcular el desempeño de estas cepas en competición comparativamente con el cultivo simple, mediante el concepto de tasas de crecimiento intrínseco que utilizaron Williams y colaboradores (2015). La principal conclusión de este estudio fue que la temperatura afecta de manera notable a este desempeño. Para todos los casos, cuanto más alta era la temperatura, más facilidad tenía *S. cerevisiae* para imponerse en el cultivo. Cabe destacar que una cepa vínica de *S. uvarum* era capaz de imponerse sobre *S. cerevisiae* a 12 °C, lo que concuerda con el hecho de haber sido aislada de fermentaciones vínicas naturales de regiones frías. En el caso de las fermentaciones con *S. kudriavzevii*, *S. cerevisiae* fue capaz de desplazarla del medio incluso a temperaturas donde cultivos simples de *S. kudriavzevii* presentan mejores parámetros de crecimiento que *S. cerevisiae* (Salvadó et al., 2011b).

Además, en este ensayo utilizamos una cepa vínica y otra de ambientes no fermentativos de *S. cerevisiae*, al igual que de *S. uvarum*, para estudiar si la

capacidad competitiva de estas cepas dependía de su origen. Los resultados mostraron que efectivamente las cepas que provenían de aislados de vino mostraban una mayor capacidad para competir que las cepas no vínicas para todas las condiciones de temperatura ensayadas.

Todas estas fermentaciones fueron también monitorizadas mediante medidas de pérdida de peso hasta que este se mantenía constante, momento en el que se consideraban terminadas. Estos datos, junto con medidas de la concentración de azúcares que quedaban en el medio en el punto final de la fermentación obtenidas mediante cromatografía líquida de alta eficacia (HPLC, por sus siglas en inglés), nos permitieron obtener una curva de consumo de azúcares a lo largo del tiempo ajustable a la ecuación de Gompertz (Zwietering et al., 1990). De esta manera observamos que, en algunos casos, en fermentaciones llevadas a cabo por *S. cerevisiae* y, o bien *S. kudriavzevii*, o bien *S. uvarum*, la cinética era más rápida que para cualquiera de las dos especies en cultivo simple. Estos resultados son de potencial interés para la industria vitivinícola, para la que una reducción en los tiempos de fermentación, especialmente en fermentaciones a baja temperatura, sería de gran ventaja para mejorar el rendimiento económico de la producción.

Además, las citadas medidas de HPLC del sobrenadante de la fermentación en su punto final nos daban las concentraciones de glucosa, fructosa y glicerol. Como hemos comentado antes, la reducción de los niveles de etanol mediante la utilización de estas cepas era uno de los principales objetivos. Esto se consiguió en algunas de las competiciones en las que *S. kudriavzevii* o *S. uvarum* estaban implicadas. En concreto, el resultado más positivo se obtuvo para las competiciones de *S. kudriavzevii* a 12 °C, donde se redujo la

concentración de etanol hasta en dos grados y se aumentó la concentración de glicerol en 2 g/L con respecto a la cepa vínica de referencia en cultivo simple. Por el otra parte, en las fermentaciones mixtas en las que participaban *S. paradoxus* o *S. eubayanus*, tanto la concentración de metabolitos de interés como la cinética de fermentación resultaron contrarias a un posible interés industrial.

Una vez que obtuvimos estos buenos resultados para *S. kudriavzevii* a baja temperatura, condición que en la industria sólo se aplica a vinos blancos o rosados, decidimos intentar mejorar el desempeño de esta levadura a una temperatura de fermentación más común en vinos tintos: 25 °C. Así podríamos abrir una puerta a su utilización para reducir el grado alcohólico también en estos vinos. La primera de las estrategias que seguimos para ello fue basada en el hecho de que, si inoculábamos una cantidad mucho mayor de *S. kudriavzevii* que de *S. cerevisiae*, a esta última le costaría más desplazar a su competidor. Por ello, en nuestras fermentaciones de mosto sintético inoculamos *S. cerevisiae* y *S. kudriavzevii* en proporciones 1:1, 1:3, y 1:9. En este caso, las muestras de los inóculos en proporción 1:9 mostraron una mayor prevalencia de *S. kudriavzevii* a lo largo del cultivo, que estuvo en una proporción cercana al 50% al menos hasta los 6 primeros días de fermentación, en fermentaciones de 10 días de duración total.

También testamos la estrategia de inocular *S. kudriavzevii* pura al comienzo de la fermentación para dejar que la iniciara y alcanzara una densidad alta en el cultivo, y posteriormente, a las 24 horas, inoculamos *S. cerevisiae*, de manera que se encontrara en el cultivo en una proporción aproximada de 1:100. En

esta inoculación secuencial se consiguió que *S kudriavzevii* dominara el cultivo hasta el final de la fermentación.

Por último, diseñamos un sistema de oxigenación controlada de nuestras fermentaciones. El hecho de administrar oxígeno en el medio facilitaría la proliferación de *S. kudriavzevii*, con peor capacidad para fermentar en anaerobiosis que *S. cerevisiae*, ya que podrían utilizar este oxígeno para respirar y obtener energía de forma más eficiente. Nuestro sistema consiste en un caudal de aire modulado por una válvula para obtener el flujo deseado, que posteriormente se hace pasar por un filtro de 0,22 µm para evitar contaminaciones, antes de entrar en el sistema, cuya salida se encuentra en el fondo de la botella de fermentación, lo que permite una mejor difusión del oxígeno. Con este sistema podíamos trabajar en paralelo con hasta 12 fermentaciones. En cuanto al caudal adecuado, hicimos pruebas de la prevalencia de *S. kudriavzevii* en fermentaciones inoculadas con proporción 1:1 a caudales de 1, 5, 10, y 20 VVH (10 volúmenes de gas que pasan por volumen de fermentación en una hora), y se vio que el caudal de 20 VVH era el más adecuado para garantizar un mayor desempeño de nuestra levadura de interés, posiblemente debido a la mayor facilidad que tiene esta levadura para respirar y crecer más eficientemente. Posteriormente, se combinó esta estrategia con la primera descrita en la que se aumentaba la proporción de *S. kudriavzevii* en el inóculo. Los resultados mostraron un claro efecto sinérgico de la oxigenación controlada y la inoculación en proporción 1:9 sobre la prevalencia de *S. kudriavzevii* en la fermentación. En este caso, esta levadura dominó el cultivo hasta el final de la fermentación por encima de cualquiera

de las otras estrategias llevadas a cabo, con un 75% de la población total de levaduras al final de la fermentación.

De la misma manera que en experimentos anteriores, monitorizamos el estado de la fermentación mediante el pesado de las botellas en distintos puntos durante la fermentación. Además, también determinamos la composición final del mosto fermentado mediante análisis por HPLC. En primer lugar, observamos como el tiempo de fermentación se reducía considerablemente en las condiciones en las que aplicamos aireación a las botellas, debido al consumo más rápido de azúcares de forma aeróbica por respiración. Además, se consiguió la reducción de la concentración de etanol en aproximadamente 2 puntos porcentuales en la condición de proporción de inóculo 1:9 más oxigenación. Sin embargo, el aumento de glicerol observado en anteriores experimentos sin oxigenación, en la que el balance redox venía equilibrado por la síntesis de este compuesto, no se dio en este caso. En cambio, sí vimos un aumento dramático de la concentración de ácido acético, que nos indica que una mayor tasa de respiración fue la forma de obtención de energía, y que explicaría el nuevo equilibrio oxido-reductivo de la célula.

En conclusión, aunque conseguimos mejorar la cinética de la fermentación, así como reducir el grado alcohólico gracias a la oxigenación y mayor presencia de *S. kudriavzevii*, vemos que esta estrategia tiene el riesgo de producir una cantidad excesiva de ácido acético, lo que está relacionado con un sabor astringente en vinos.

Como ya hemos visto, la presencia de *S. cerevisiae* en una fermentación puede hacer que *S. kudriavzevii* vea mermada su capacidad para crecer dentro del

cultivo, incluso a bajas temperaturas a las que está mejor adaptada. Sin embargo, los mecanismos moleculares que tienen que ver con este fenómeno no han sido descritos. A pesar de ello, hay que destacar que se han descrito mecanismos en *S. cerevisiae* que afectan a la viabilidad de otras levaduras no *Saccharomyces* e incluso bacterias. Los factores clásicos son la producción de etanol y la reducción del pH del medio, que resultan tóxicos para la mayoría de microorganismos, pero no para otras levaduras del género *Saccharomyces*. Otros mecanismos tienen que ver con la producción de compuestos tóxicos de diversa índole como péptidos *asesinos* o péptidos tóxicos derivados de la enzima gliceraldehido-3-phosphato-deshidrogenasa que se acumulan en la pared celular de *S. cerevisiae* de forma constitutiva (Branco et al., 2014; Kemsawasd et al., 2015). También se ha hipotetizado que la pérdida de viabilidad de los organismos competidores de *S. cerevisiae* durante la fermentación pueda deberse a un gran aumento de la temperatura localizado en microambientes dentro del cultivo debido al vigoroso metabolismo de *S. cerevisiae* (Goddard, 2008).

Sea como fuere, es importante, desde el punto de vista biotecnológico, conocer de qué manera *S. cerevisiae* excluye de un cultivo a una especie tan cercana como *S. kudriavzevii*. Este conocimiento puede ayudar a avanzar hacia una mejor implantación de esta especie en condiciones industriales. Por ello nos planteamos el objetivo de investigar las interacciones a nivel molecular de estas especies durante la fermentación.

En primer lugar, hicimos un análisis transcriptómico de nuestra cepa vínica de *S. cerevisiae* y de *S. kudriavzevii* cuando se encontraban en co-fermentación, para compararlo con la expresión génica en cultivos simples. Para ello se

secuenció el RNA extraído de muestras tomadas de la fermentación en tres diferentes puntos; Fase Exponencial Temprana (FETe), Fase Exponencial Tardía (FETa), y Fase Estacionaria (FE), por medio de un secuenciador ILLUMINA. Las lecturas de cada muestra se alinearon contra una referencia consistente en las secuencias genómicas completas concatenados de ambas cepas mediante el alineador *bowtie2* (Langmead and Salzberg, 2012). Tras ello, se obtuvo el conteo de lecturas en cada gen usando el programa *HTSeq-Count* (Anders et al., 2015). Estas fueron analizadas estadísticamente con el paquete de *R* (Team, 2018) *DESeq2* (Love et al., 2014) para obtener aquellos genes que presentaban una expresión diferencial entre las condiciones de interés, así como la variabilidad de las muestras en cuanto a su expresión génica. Los principales resultados de este análisis mostraron que *S. cerevisiae* mostraba una respuesta transcriptómica mucho más fuerte que *S. kudriavzevii* en el primero de los puntos analizados (FETe), con más de mil genes expresados de forma diferencial, frente a unas pocas decenas o cientos de *S. kudriavzevii*. En las siguientes fases, el nivel de genes identificados en *S. cerevisiae* se reduce prácticamente hasta 0, mientras que en *S. kudriavzevii* aumentan dramáticamente hasta alcanzar más de 2.000 genes en FE. Esto concuerda con las curvas de crecimiento de este experimento y con los resultados que hemos reportado previamente en este resumen, en los que observamos que *S. cerevisiae* prácticamente no ve afectado su crecimiento en el cocultivo con respecto al cultivo simple, mientras que *S. kudriavzevii* si tiene su crecimiento muy por debajo del cultivo simple, con lo que a medida que avanza la fermentación va a presentar una respuesta transcriptómica muy relacionada con estrés y falta de nutrientes que refleja su estado. Con lo cual, nuestra hipótesis es que *S. cerevisiae* hace algo al principio del cocultivo que

le permite desarrollarse posteriormente en condiciones muy cercanas a su estado óptimo, y por eso apaga toda esa respuesta en las fases posteriores. Además, *S. kudriavzevii* no es capaz de hacer esto de forma igual de eficiente que *S. cerevisiae*, y por ello le ocurre exactamente lo opuesto.

En este momento cabe hacerse la pregunta de qué es lo que está haciendo *S. cerevisiae* en FETe para que esto ocurriera así. Pues bien, aunque la respuesta es realmente compleja por la gran cantidad de genes obtenidos, llaman la atención algunos elementos. En primer lugar, existen numerosos genes que están involucrados en la regulación de la propia expresión a diferentes niveles; transcriptómico, traduccional y pos-traduccional. En concreto, encontramos diversos genes de modificación de histonas, así como el factor de transcripción *CDC39*, lo cual es indicativo de una profunda regulación transcripcional. Además, observamos una gran cantidad de genes relacionados con el aumento de la proliferación celular, como genes del ciclo celular, mitosis, síntesis de ribosomas o disminución de la protección de los telómeros de los cromosomas. Por último, también encontramos genes relacionados con la regulación de la composición de la membrana plasmática, como genes involucrados en la síntesis de esterol, y de ácidos grasos de cadena larga. También genes involucrados en la activación de la ruta de señalización TOR (diana de la rapamicina, por sus siglas en inglés), transportadores de aminoácidos (*GAP*, *APG1*), y genes involucrados en la captación de sideróforos, todos ellos involucrados en la captación de nutrientes en un momento de la fermentación en la que todavía no ha ocurrido el agotamiento de ninguno de ellos.

Precisamente, el consumo de nutrientes fue la respuesta más clara que pudimos encontrar relacionada con un posible mecanismo de competición, que

consistiría en impedir que los competidores accedan a los nutrientes del medio y que se desarrollen normalmente. Para comprobar si esto podría estar ocurriendo realmente en nuestras competencias, en primer lugar, medimos la concentración de las principales fuentes de nitrógeno en el medio (amonio y aminoácidos) a tiempo inicial, a las 12 y a las 24 horas mediante HPLC. A las 12 horas, no se observaban prácticamente diferencias entre *S. cerevisiae*, *S. kudriavzevii*, o el cocultivo. Sin embargo, se observaron diferencias claras a las 24 horas, que dividían nuestras muestras en dos grupos; por un lado, las de *S. cerevisiae* y las del cocultivo, que habían consumido una cantidad similar de recursos, mucho mayor en general que la cantidad que había consumido el cultivo simple de *S. kudriavzevii* por su parte. Es llamativo el caso del triptófano. En cultivo simple, *S. kudriavzevii* consumió más cantidad de este aminoácido que *S. cerevisiae*, sin embargo, en la competencia la cantidad consumida era similar a la del cultivo de *S. cerevisiae*, y por tanto menor que la del cultivo de *S. kudriavzevii*. Estos resultados son indicativos de que *S. cerevisiae* está liderando de alguna manera el consumo de fuentes de nitrógeno, y concordaría con nuestra hipótesis sobre el papel del consumo de nutrientes para el fenómeno de la competición.

Además de las fuentes de nitrógeno, realizamos medidas de concentración de azúcares a lo largo de la fermentación en varios puntos mediante HPLC. Esto nos permitió construir curvas de consumo de glucosa y de fructosa mediante el ajuste de estos datos por el método de mínimos cuadrados de la misma manera que lo hicieron Tronchoni y colaboradores (2009). Estos resultados nos mostraron una aceleración del consumo de azúcares en el cocultivo con respecto a ambas especies en cultivos simples a 12 °C, que se observó tanto en

las curvas de consumo como en el parámetro t_{90} (tiempo para consumir el 90% del compuesto) de la fructosa. De esta manera, confirmamos que se estaba dando un consumo de azúcares más rápido en competición. Este podría ser, además, el caso de otros nutrientes de importancia, y por tanto constituir un mecanismo de exclusión competitiva de *S. cerevisiae* contra *S. kudriavzevii*. De hecho, en un trabajo reciente de Curiel y colaboradores (2017) en el que estudiaban la respuesta transcriptómica de *S. cerevisiae* a la presencia de levaduras no-*Saccharomyces* en fermentación, se observó la expresión de genes que tenían que ver con el consumo de nutrientes.

Una vez estudiado la importancia de la captación de nutrientes para la competición, nos preguntamos si la activación de este mecanismo dependía de un reconocimiento de la cepa competidora mediante contacto directo entre células. Para resolver esta cuestión preparamos un experimento en el que utilizamos membranas de diálisis para obtener fermentaciones con dos compartimentos separados, pero con intercambio de moléculas de masa menor a 12000-14000 Da. De esta manera podríamos separar físicamente una cepa de la otra aun estando dentro de un cultivo homogéneo (Wang et al., 2015b). Los resultados mostraron cómo la pérdida de viabilidad de las levaduras sólo ocurría en el caso de las fermentaciones en que ambos tipos de levaduras se encontraban en el mismo compartimento. En las fermentaciones en que estaban separadas por nuestra membrana de diálisis, el crecimiento fue prácticamente idéntico al de los cultivos simples. Esto demuestra que para que se active el mecanismo que permite a *S. cerevisiae* desplazar a *S. kudriavzevii* debe haber algún tipo de reconocimiento mediado por el contacto físico.

Sin embargo, al repetir el mismo ensayo sustituyendo la cepa vínica por una cepa aislada de un ambiente no fermentativo, no identificamos un defecto de crecimiento para ninguna de las dos cepas incluso en el caso de las fermentaciones que permitían el contacto entre especies. Es más, al realizar el mismo análisis transcriptómico de las competiciones entre estas dos cepas, no observamos la fuerte respuesta del primer punto en *S. cerevisiae* como en el caso anterior, sino todo lo contrario, prácticamente ningún gen diferencialmente expresado en ninguna de las etapas de la fermentación estudiadas. Por todo ello, concluimos que el mecanismo de competencia para la exclusión de otras especies del género *Saccharomyces* es dependiente de cepa, y posiblemente, esté asociado al origen de las mismas. De esta manera, la capacidad de dar esta respuesta ante la presencia de otro organismo, habría evolucionado recientemente en las cepas de *S. cerevisiae* asociadas a ambientes manipulados por el ser humano, entrando dentro del concepto de evolución de un organismo dirigida por el hombre.

Por último, realizamos el análisis transcriptómico del tiempo FETe de fermentaciones llevadas a cabo por nuestra representante vínica *S. cerevisiae* T73 y dos cepas de *S. uvarum*, la vínica BMV58 y la no fermentativa CECT12600 a 20 ºC. Cabe destacar que la respuesta de *S. cerevisiae* compartía una gran parte de genes diferencialmente expresados debido a la presencia de ambas cepas de *S. uvarum*. Como en el caso de las anteriores competiciones con *S. kudriavzevii*, *S. cerevisiae* mostró una respuesta más intensa que sus competidores. Además, en esta respuesta también encontramos numerosos genes relacionados con la modificación de la membrana plasmática y la captación de nutrientes, especialmente hierro y azufre. Además, fue muy

interesante comprobar que la cepa vínica de *S. uvarum* también parecía tenida orientada su expresión a la captación de recursos del medio, mientras que en la cepa no vínica destacaban los procesos de degradación de proteínas y parada del crecimiento y ciclo celular. Como en el caso de las distintas cepas de *S. cerevisiae*, estos resultados nos indican que la respuesta a la presencia de un competidor es dependiente de cepa, y probablemente relacionada con su origen.

Aunque nuestros resultados constituyen un importante avance en el conocimiento de las relaciones entre levaduras del género *Saccharomyces* en fermentación, aún queda mucho trabajo por hacer. Probablemente, lo más relevante sea identificar las moléculas que son responsables del reconocimiento entre especies que desataría el mecanismo de competición, y que aún no han sido identificadas. Cabe mencionar, sin embargo, que Rossouw y colaboradores (2015) descubrieron que las proteínas de la familia *FLO*, involucradas en la floculación de las levaduras, eran determinantes para que ocurriera el fenómeno de dominación de un cultivo por parte de *S. cerevisiae* sobre levaduras no-*Saccharomyces*. Por su parte, Pérez-Torrado y colaboradores (2017b) probaron que el mecanismo de dominación de una determinada cepa de *S. cerevisiae* sobre otra, tenía que ver con la resistencia y secreción de sulfito al medio. De cualquier forma, este reconocimiento, así como la posible cascada de señalización que da lugar a la respuesta, debe ser elucidado en el futuro para el caso de los organismos implicados en nuestro estudio. Así tendríamos una base más sólida de conocimiento de la ecología y relaciones de las especies de potencial utilidad en enología para afrontar los retos de la industria.

PREFACE

This thesis was developed in Amparo Querol's and Eladio Barrio's labs, with the support of the Ministry of Economy of the Spanish Government through financed project (AGL2012-39937-C02-01), which also included the funding (BES-2013-066434) for hiring a graduate student for accomplishing a PhD, in this case myself. Concretely, my work corresponded to the Subproject 1, which was the continuation of a strong research line on the study of the alternative species of the *Saccharomyces* with potential interest to winemaking industry. In fact, the only commercialized strain from this species until now, Velluto BMV58^{TM} (Lallemand), was isolated and initially characterized in our lab.

Looking at traditional food biotechnology, and given that GMO are not allowed for food production in Europe, one may think that there is room left only for the mere physiological characterization of organisms for its selection. And that if you are lucky, you might even get some trait improvement by "legal" genetic manipulation such as random mutagenesis and evolution. I ought to say that this is something absolutely mastered by my supervisor Dr. Amparo Querol, who has been doing a lot of yeast isolation, identification, directed evolution and hybridization of loads of different strains from different sources of isolation in the last decades. Evidence of that is easily found when you do a quick search in *Scopus*, typing "yeast AND wine" and looking into the top 2 authors.

But there is a lot more to it than simply looking at how yeasts ferment, or if they produce good aroma, and in Amparo and Eladio's team they have pioneered a lot of different aspects of research on *Saccharomyces*.

Following the main interest and expertise of Dr. Eladio Barrio, one of the main topics is the reconstruction of the history of evolution and spreading of the different lineages of *Saccharomyces*. In the last years, important progress has been made by our lab in the knowledge of the mechanisms of adaptation of the *Saccharomyces* species to the fermentative environment. Those traits which gave the ability to the wine strains to colonize their medium also revealed some footprints for the understanding of how and when yeast got to certain places around the world, which constitutes important pieces of the puzzle of yeast evolution.

Within this frame, is that Amparo and Eladio focused on the understanding of the interactions among the yeast of the *Saccharomyces* genus during the fermentation of grape must. This had double interest; one, the application of strains that had been previously evaluated as good candidates for improving industrial processes, to look how they behave when they have to face natural competitors, and how we can improve their performance; and second, to gather knowledge from this interactions that could explain why certain yeasts are able to adapt to the fermentative environment and other are not, because that can feed the history of yeast adaptation.

One of the main factors regarding domination phenomenon in wine fermentations seemed to be the temperature at which the process takes place. It was interesting to notice how the only wine fermentations that were not led by *S. cerevisiae* were situated in European regions with cold climates. That motivated the study by Salvadó *et al.* (2011b) who showed that indeed these other species were better adapted to low temperatures than *S. cerevisiae*. In addition low temperature also plays an important role in the winemaking

industry nowadays, since it is a common approach for keeping the volatile aroma compounds in the final product. Those facts motivated our first published work, where we studied the implantation of yeast of the different *Saccharomyces* species at different temperatures when they had to compete against a *S. cerevisiae* wine strain (Alonso-del-Real et al., 2017b).

Furthermore, there was a special interest with the species *S. kudriavzevii*. In the formerly cited work, it was reported to be the best adapted to cold among all the *Saccharomyces* species. Moreover, under laboratory conditions, it had been shown to be able to perform a complete fermentation on different natural grape musts, with the benefits of having a promising aroma profile, and one of the most pursued characteristics for winemakers nowadays; notably reducing ethanol yield. And on top of that, it also produced increased levels of the taste fosterer compound glycerol (Peris et al., 2016; Stribny et al., 2015). Strikingly, it has never been isolated from any wine production around the world to our knowledge; however, it has been found as part of several different interspecific hybrids with *S. cerevisiae*. That raises the question of whether the hybrid was formed in an external to fermentation niche and then arrived to it somehow, or if *S. kudriavzevii* was present in the fermentation but it only has been able to survive there in form of hybrids. So of course, the main line that can be followed in the present thesis is the study of how *S. kudriavzevii* behaves in fermentations where *S. cerevisiae* is present, and also what *S. cerevisiae* does in response to this competitor. Thus, we studied *S. kudriavzevii* performance at different temperatures. The outcome of this can be learn from **Chapter I** of the present document and is also reflected in the already mentioned first paper (Alonso-del-Real et al., 2017b). Later on, after the promising results of *S.*

kudriavzevii at low temperature, we wandered if we could make this yeast to work at regular red wine production temperatures. That is why we proposed different approaches that would be possible to implement at industrial level. Eventually, these techniques definitely improved its performance as it is reported in **Chapter II**, as well as in our second published work (Alonso-del-Real et al., 2017a).

During this path we realized that temperature was not the only factor influencing *S. kudriavzevii* and the other species prevalence in wines, since *S. cerevisiae* was able to outcompete the cryotolerant species even at low temperatures. Moreover, this phenomenon was strain dependent. Although several works focused on unraveling the competition mechanisms of *S. cerevisiae* to face non-*Saccharomyces* yeast, none of them seemed to fit our case with the two closely related species. Thus, we tried to get deeper knowledge at the molecular level by performing one of the first transcriptomics analyses of the competition between two yeast, which plays a central role in **Chapter III**. Most of this part of the work was recently sent for publication and accepted by the journal *Environmental Microbiology* (Alonso-del-Real et al., 2019).

From that moment on, most of the effort I put into my thesis went in the direction of learning RNAseq analysis basics. Although I already had some background on bioinformatics and programming, that was the first time I was facing a real scientific dataset to solve on my own. At the beginning it was really harsh, and we even had doubts whether the mixed transcriptomes of our close species would be easily distinguishable when the time of the analysis came. This is why we decided that a good place to do a short fellowship abroad

would be Justin Fay lab, which was formerly placed in the Medical School of Washington University in St. Louis (MO, USA). Prof. Fay had worked on transcriptome analysis of *Saccharomyces* interspecific hybrids, which share in common with my experiment that different species transcriptomes are mixed in the samples. Of course, one of the main things he taught me was how to analyze this data. Still, every experiment is different and you need to know how to ask the right questions to your dataset, so it took me a while after I came back from the US to fully understand what I was doing, and how to get the maximum from this experiment, but finally we were able to get a real picture of what was going on in the competition, prove some facts by wet lab experiments, and write the paper.

In parallel, we also performed RNAseq experiments of two strains of *S. uvarum*; a wine one and a wild one, both in competition with *S. cerevisiae*. The idea was to see whether the response observed for the *S. kudriavzevii* case, was also present here. In addition, making use of our dataset, we also want to check for correlation of phenotypic characteristics and gene depression among the genus. Unfortunately, I did not have time enough to finish this work.

Nevertheless, it is important to mention in this point that my supervisors being referent in the field of the *Saccharomyces* species related to fermentation has allowed me to collaborate in different projects. One of them was about the improvement of osmotic resistance in *S. kudriavzevii* in collaboration with Prof. Sychrova from Praha University that was published recently (Dibalova-Culakova et al., 2018). In addition, as part of the project lead by Dr. Querol, Dr. Barrio and Dr. Balsa from IIM (Vigo, Spain), I participated by providing the data and by the interpretation of the results in the predictive modelling of

S. cerevisiae and *S. kudriavzevii* performance in wine fermentation (Henriques et al., 2018). This project is specially promising as we were able to predict the inoculum size and temperature of fermentation needed to optimize ethanol and glycerol content of wines, and could constitute an approach for the prediction of many other parameters. Currently, Dr. Balsa is working in modelling competitions data in order to model this kind of fermentation as a tool for winemaking industry.

Moreover, given the importance the powerful technique RNAseq is gaining in our field, and the numerous collaborators our lab holds among the community, I had the opportunity to engage several projects working with the transcriptomics part, to which I dedicated part of my time in the lab. One of them, in collaboration with Dr. Rial from the University of Ourense (Spain), in which they want to study the effect of certain antifungal compounds used in agriculture on *S. cerevisiae*. Secondly, the collaboration with Prof. Combina from University of Mendoza (Argentina), in which we want to identify marker genes for stuck fermentations after heat shock. Third, a very similar project to our competitions, in which Dr. Camarasa from INRA (Montpellier, France) wants to study yeast interactions in the sequential inoculation of *S. cerevisiae* after an alternative yeast. Finally, with the Dr. Guillamón from our institution and Prof. Liti from IRCAN (Nice, France), experiments related to temperature adaptation in industrial wine yeast.

Finally, I also have been given the opportunity to participate in many other activities of relevance, such as receiving bioinformatics courses, attending and presenting my work in several national and international conferences and symposia, or writing a book chapter for “Advances in Food and Nutrition

Research" (Querol et al., 2018). All and all, this almost five-year period have given me the opportunity to learn a range of different techniques, get involved in many projects and made me up as scientist to have the tools to continue developing in my future career.

INTRODUCTION

1. Historical perspectives of wine and yeast fermentation

The ancient biotechnological processes originating fermented food and beverages have strongly determined human social advance since ancient times. Specially, the domestication of the yeast *Saccharomyces cerevisiae* is considered an essential milestone in Human History (Legras et al., 2007). Archeological evidence of fermented beverages in the Neolithic (8500 – 4000 BC) in China, Iran and also Egypt were found in form of paintings and bronze sculptures in which harvesting and milling of cereals were depicted (Cavalieri et al., 2003). It is thought that fermented products appeared together with agriculture, thus contributing to the deep transformation of societies from a nomad state to the agricultural conformation that allowed the development of the first great civilizations (Katz and Voigt, 1986). Moreover, wine and the other fermented beverages, due to their perceived pharmacological, nutritional, and sensory benefits, played key roles in the development of human culture and technology, with the advance and intensification of agriculture and food-processing techniques (McGovern, 2003; McGovern et al., 2004; Underhill, 2002). Also, wine combined analgesic, disinfectant, and mind-altering effects caused for it to have taken part in many aspects of human way of life, such as nutrition, religious rituals, distinction of high social stratum, social gathering, or in the military as safe hydrating beverage, or even as reward or motivation (Earle, 2002; McGovern, 2003).

By 500 BC wine was spread out through the Mediterranean, being produced in Italy, Sicily, France, Spain, Portugal and North Africa. Later, in the XVI Century, Spanish colonizers introduced wine into America, planting *Vitis vinifera* in

Argentina, Chile, Mexico and Peru. In the XVII Century, Dutch also planted vineyards in South Africa and shortly after, in California and Australia (Pretorius, 2000).

After these historical facts, the father of microbiology, Antoni van Leeuwenhoek, using his handcrafted microscopes, observed the first yeast and introduced to the world "animalcules", Latin word for "little animal". However, it was not until 1890, that Louis Pasteur (1822-1895) discovered the role of microorganism in the process of fermentation. He was the first to prove that fermented beverages are the product of alive yeast converting sugars into ethanol in the absence of oxygen (Barnett, 2000; Pasteur, 1857). That was especially controversial since the mainstream theories at that time considered yeast to be non-living organisms. In fact, the prestigious chemists Jöns Berzelius, Justus von Liebig and Friedich Wöhler considered yeast as lifeless decaying matter of fermentation (Soetaert et al., 2010). Those were Dutch people who called the foam formed during the fermentation of beer with the Dutch word "gist", which originated the word yeast. French people, on their side, used the word "levure" to refer to the role of yeast in causing bread dough to rise. This term originated the word for yeast in some other romance languages.

Furthermore, Pasteur introduced the concept of aseptic conditions and pure cultures to help to solve problems of "bad" fermentations, and distinguished between alcoholic fermentations and aerobic growth in yeast. Moreover, he hypothesized that wine yeast could come from grape surface, providing with a source of inoculation for musts.

The first pure culture used for alcoholic beverage production was obtained by Emil Christian Hansen (1842-1909) from the physiology department of the Carlsberg Foundation in Copenhagen in 1988. This popular beer fermenter yeast was named after the foundation; *Saccharomyces carlsbergensis*, and its economic impact still today make it one of the most important microorganisms at industrial level worldwide. In 1890, Müller-Thurgau obtained a wine yeast pure culture in Geisenheim, Germany and introduced the concept of inoculating wine fermentation (Dequin, 2001).

During most part of the XIX Century, wine fermentation was seen as a spontaneous process in which yeast unconscientiously transferred among infusions from one fermentation vessel to another, or from remains that were kept for later fermentations. In the latter XIX century, new discoveries about yeast physiology and metabolism allowed the change of paradigm. This came together with fundamental breakthrough in the field of cell biology and biochemistry (Lachance, 2003), which allowed to describe *S. cerevisiae* as the responsible organism to carry out the fermentation process in the must, outcompeting other microorganism in an environment characterized by the increasing presence of ethanol (Boulton et al., 1999).

2. Yeast, human best friend

Yeasts are involved in very different kind of activities that improve our daily life. The species *S. cerevisiae* and *Schizosaccharomyces pombe* present particularly suitable characteristics for their use in experimental biology, such as a short generation time (1.5-3 hours), reproducible growth and genetic stability. This have positioned them as a model system for studying multiple molecular

phenomena of relevance for human biology, especially after *S. cerevisiae* became the first eukaryotic genome to be sequenced in 1996 (Dolinski and Botstein, 2005; Goffeau et al., 1996). About 60% of *S. cerevisiae* genes have orthologues in the human genome, and important analog metabolic and cell signaling pathways are also present. This fostered the development of databases focused on its genome, proteome, metabolome or interactions networks, together with a complete set of wet-lab molecular tools for this organism, that has contributed to the development of entirely new biological research fields called "functional genomics" and "system biology" (Botstein and Fink, 2011). Probably, the most relevant breakthroughs derived from this knowledge has taken place in the study of neurodegenerative processes such as Alzheimer disease or Parkinson disease (Tenreiro et al., 2013). Similarly, *S. pombe* genome was sequenced in 2002 (Wood et al., 2002), and has been deeply applied as a model for cell-cycle and cancer research (Fantes and Hoffman, 2016).

Moreover, yeasts constitute the most relevant group of organisms for biotechnology. They participate in the production of the highest number of biotechnological products (**Table 1**), and surpass any other group of industrial microorganisms in economic terms. Ethanol as a combustible carbon source synthesized by *S. cerevisiae* constitutes the main biotechnological product. According to the World Bioenergy Association Annual report of 2017, the production of bioethanol increased dramatically in the last years from 13.2 billion of liters in the year 2000 to 78.0 billion liters in 2,014 and it will continue to do so. Nevertheless, there are other 100 yeast genera, some of which with potential to become human best friends too. Several physiological features make them a good platform for the production of chemicals or recombinant proteins: high

substrate uptake rates, potentially high metabolic rates and robustness again stressful process conditions. In fact, there are an important number of industrially produced chemicals, enzymes or proteins products derived from enzymes. Some of this products are naturally produced by yeasts such as astaxanthin by *Phaffia rhodozyma* or riboflavin by *Pichia guillermondii*, and other are the result of metabolic or genetic engineering, such as insulin by *P. pastoris* (Mattanovich et al., 2014).

Table 1: Industrial products from yeasts

Product	
Beverages	Beer, wine, sider, sake, distillate drinks (whiskey, rum, gin, vodka)
Human and animal food	Baker's yeast, yeast extract, dietetic supplements, growth factors, fodder yeast
Chemicals or Drugs	Bioethanol, CO_2, glycerol, vitamins, aroma compounds
Proteins	Hormones, vaccines, antibodies, interferons, blood proteins, enzymes

Economic value of fermented by yeast beverages and food is huge. The three main products are the well-known beer, bread and wine, but yeasts also play a relevant role in other products. In cocoa fermentation, *Hanseniaspora guilliermondii* or *Hanseniaspora opuntiae* generally dominate the early part of fermentation after which *S. cerevisiae*, *Kluyveromyces marxianus*, *P. membranifaciens*, *P. kudriavzevii* and some *Candida* spp. are most often dominant (Ho et al., 2014). In addition, more than 23 different yeast species have been isolated from kefir grains and from fermented beverages of different origins. The predominant species are *Saccharomyces cerevisiae*, *S. unisporus*, *C. kefyr*,

and *K. marxianus* (Prado et al., 2015). Also, the composition of cheese microbiota is very complex and includes different yeast species depending on the kind of cheese (Mounier et al., 2008). Some of them form part of cheese starters contributing to its correct development, and other come from contaminant sources and may not be so desirable (Banjara et al., 2015).

Getting back to the main fermented food products by *S. cerevisiae*, beer occupies the first place in volume of produced product. According to the Barth-Haas Group, the estimated worldwide beer production was 1.96 billion hL in 2016, and China lead production by far with 460 million hL (Barth-Haas, 2017). In 2014, European Union beer market was valued at 51 billion €, and held 2.3 million direct or indirect jobs, confirming a positive growth tendency (The contribution made by beer to european economy). Regarding wine production, it kept stable in the last years, around 270 million hL, however, it dropped in 2017 due to weather conditions (**Figure 1**). In Spain, wine industry a key sector, it was the third leader producer country in 2016, with 33.5 million hL, a 15% of the worldwide wine production (Organisation Internationale de la Vigne et du Vin, 2017). At last, the global market for baker's yeast production reached nearly $7.100 million in 2016, and it is expected to grow up to $10.7 billion by 2022 (Yeasts, Yeast Extracts, Autolysates and Related Products: The Global Market: CHM053C | BCC Research).

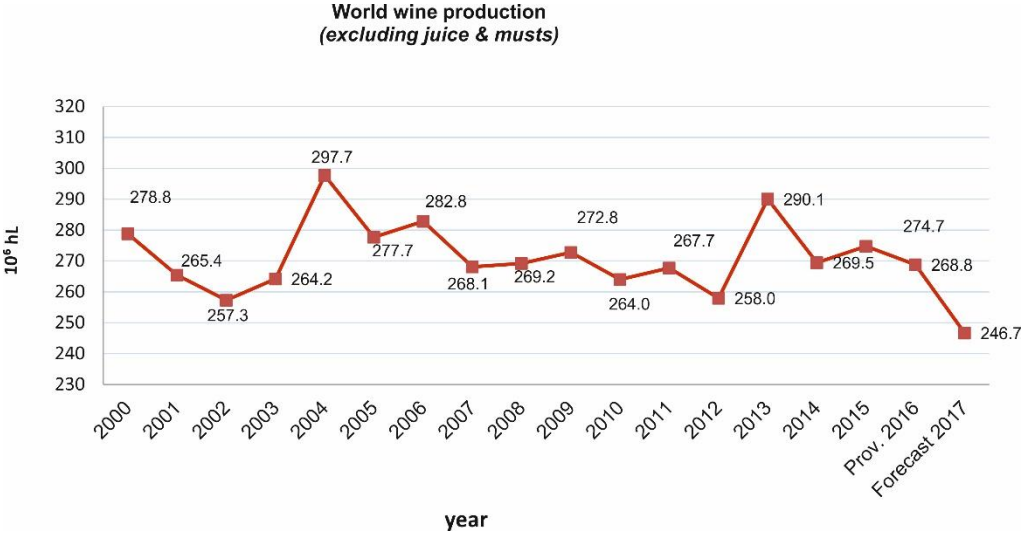


Figure 1| Modified from (Organisation Internationale de la Vigne et du Vin, 2017)

Due to their interesting physiological properties, stablished genetic manipulation methods, and the qualification as GRAS (Generally Recognized as Safe) organisms, *S. cerevisiae* will continue to be the main organism for its use in industry. Precisely this could benefit the use of its closest relatives of the genus *Saccharomyces*.

3. Winemaking process

Vinification or winemaking is the production of wine. It is influenced by numerous factors such as viticulture practices, soil quality, the cultivar of grape, the temperature of fermentation or the yeast selected (or not) to carry out the fermentation (Vivier and Pretorius, 2002). Despite being an ancient process, winemaking has maintained its basic steps almost unaltered along history. It starts with grapes being harvested and crushed. Afterwards, the wine is fermented in barrels made of wood, stainless steel or concrete vats. Red wine (**Figure 1A**) and white wine (**Figure 1B**) production processes present relevant differences.

Alcoholic fermentation occurs together with maceration in the case of red wines, and in the presence of grape lees that contain anthocyanins pigments, which are responsible in great extent for the difference in the composition and taste of red and white wines. After alcoholic fermentation, a second type of fermentation called the malolactic fermentation (MLF) takes place. MLF transform malic acid into lactic acid, lowering acidity of wine and thus altering organoleptic characteristics.

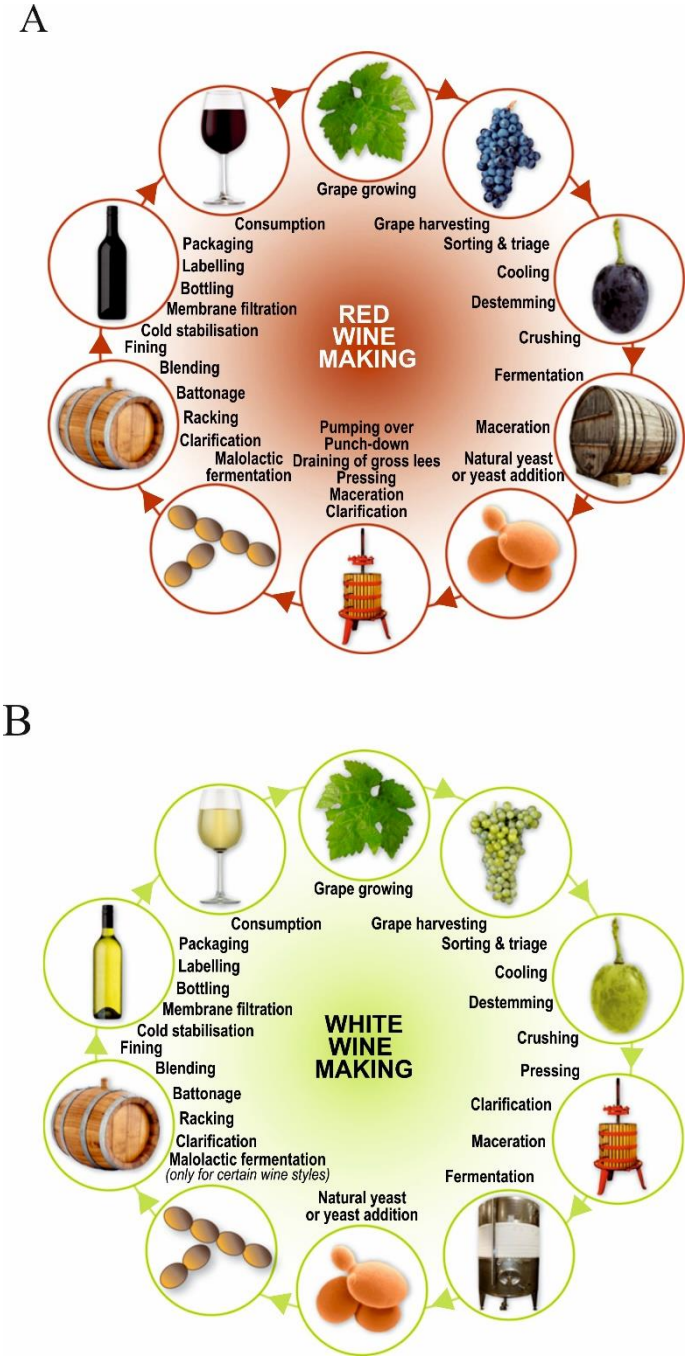


Figure 2| Main steps of red (A) and white (B) wine (modified from (Jolly et al., 2014))

3.1. Microbiota associated with wine fermentation

The microflora naturally present in wine must is highly complex, including yeast, bacteria and molds. Moreover, the composition of microbiota varies according to climatic conditions, viticultural practices, stage of grape ripening, physical damage of grapes, and the presence of fungicides applied to vineyards (Pretorius et al., 1999). Alcoholic fermentation is initiated by a high proportion of oxidative and weakly fermentative yeast (Ghosh et al., 2015; Jolly et al., 2003b; Wang et al., 2015a). These species are rapidly outgrown by strongly fermentative yeast, normally *S. cerevisiae*, which dominates the middle and end of fermentation (Bagheri et al., 2016; Ghosh et al., 2015; Jolly et al., 2003a; Morgan, 2016; Portillo and Mas, 2016; Pretorius et al., 1999; Setati et al., 2015; Tristezza et al., 2016; Wang et al., 2015a; Zott et al., 2008). The growth and metabolic activity of these yeast species are influenced by physicochemical conditions of fermentation process (Mendoza et al., 2009; Sainz et al., 2003). However, beyond such environmental factors, ecological interactions between yeast species will determine the wine fermentation dynamics and final product characteristics (Bagheri et al., 2017; Morales et al., 2015; Nissen et al., 2003; Pina et al., 2004; Renault et al., 2013; Sadoudi et al., 2012; Shekhawat et al., 2016; Wang et al., 2015b).

Bacterial community can be divided into two main groups: lactic acid bacteria (LAB) and acetic acid bacteria (AAB). The most frequently found LAB belong to the genera *Lactobacillus* and *Oenococcus*. They are responsible for the malolactic fermentation that takes place in red wines and normally in white wines with high volatile acidity after alcoholic fermentation. They consume malolactic from the wine and produce lactic acid and other metabolites. This step adds

complexity to the aroma profile and softens acidity (Bartowsky, 2005; Cappello et al., 2017). In the case of AAB, they are obligate aerobic organisms that belong to the family of *Acetobacteraceae*. They are considered one of the main wine spoilage agents because they consume ethanol and produce acetic acid, characteristic that allow the transformation of wine into vinegar. AAB usually do not develop during winemaking due to the harsh conditions of the environment (semi-anaerobiosis, high ethanol and SO_2 levels), but aeration that sometimes is applied to the process can foster their growth (Bartowsky and Henschke, 2008).

Among yeasts, more than 20 different genera have been found *Aureobasidium, Auriculibuller, Brettanomyces, Bulleromyces, Candida, Cryptococcus, Debaryomyces, Hanseniaspora, Issatchenkia, Kazachstania, Kluyveromyces, Lachancea, Lipomyces, Metschnikowia, Phaemoniella, Pichia, Rhodosporidium, Rhodotorula, Saccharomyces, Sporidiobolus, Sporobolomyces, Starmerella, Torulaspora, Wickerhamomyces, Yarrowia, Zygoascus, and Zygosaccharomyces* (Bagheri et al., 2016; Barnett, 2001; Grangeteau, 2016). Some of this yeast, in a suitable proportion, can improve organoleptic quality and complexity of wines (Clemente-Jimenez et al., 2005; Fleet, 2008; Jolly et al., 2014; Zohre and Erten, 2002). On the other hand, some of them can spoil wine (Ciani and Picciotti, 1995). This is the main reason why *S. cerevisiae* is inoculated in most industrial fermentations, to inhibit the presence of undesirable organism. Despite not being dominant on grape berries surface or in the initial stages of fermentation when other genera such as *Hanseniospora* accounts for most of the yeast population, *Saccharomyces*, and in almost all cases *S. cerevisiae*, plays a primary role in the winemaking process (Pretorius, 2000, 2003).

Traditionally, wine fermentation has been carried out in a spontaneous way by indigenous yeasts present on the grapes when harvested or introduced from the equipment and cellar during the vinification process. At present, most wine-producing companies carry out the fermentation process by adding a pure *Saccharomyces* yeast strain to the must, thus improving reproducibility in fermentations and in final product quality.

3.2. Alcoholic fermentation

Alcoholic fermentation is the most important biotransformation during winemaking. It is conducted by yeast species that belong to the natural microbiota of the grape musts or are added to it. During alcoholic fermentation, yeast metabolize the sugars present in the grape juice to obtain energy, producing ethanol and carbon dioxide (Barnett, 2000) according to the next stoichiometry:

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2$$

90% to 95% of sugars are converted into ethanol and carbon dioxide. Only 1-2% of carbon source is used for cell growth and maintenance, and the rest 4-9% end up in secondary metabolites such as glycerol, acetic acid, high alcohols and esters (Boulton et al., 1999).

The ability to conduct alcoholic fermentation under high sugar concentration conditions and even in the presence of oxygen was called Crabtree effect (De Deken, 1966). Following the "Make-Accumulate-Consume" life strategy, *S. cerevisiae* has been described as an organism able to efficiently consume sugars and produce high amounts of ethanol that kills its competitors due to toxicity. When sugar is depleted from the medium, its metabolism switches to aerobic consumption of ethanol. The outstanding capability of *S. cerevisiae* to follow this

behavior guaranteed its implementation success in grape juice (Hagman et al., 2013).

3.3. Winemaking main challenges

As we mentioned above, wine production is a highly conserved process, however, industry is facing demanding tasks at different levels. First, the adaptation to new market demands. Wine consumption is decreasing in the last years, so the search for products that better suit to new opening markets is a must. For instance, sweeter wines and fruity aromas fit better with young people preference. Besides these new trends, there is also an increasing consciousness about effects of alcohol uptake on health as well as road safety.

One of the most important challenges for winemaking industry is climate change effect on composition and properties of grape (Borneman et al., 2013). Enological maturity of grape must depends of three factors: Sugar content and acidity, which determine final ethanol concentration of wines, phenols content which color and astringency depend on, and primary aromas, which also affects wine taste and bouquet. Owing to global warming effect, this oenological maturity is becoming more and more difficult to achieve in real vineyards because higher temperatures accelerate sugar maturity of grapes and disrupt phenolic maturity, thus, provoking an unbalance between these two factors (Jones et al., 2005). If the winery waits until the phenolic maturity is achieved before harvesting, the higher amount of sugars results in overproduction of ethanol. Moreover, temperatures also negatively affects wine acidity (Mozell and Thach, 2014) and anthocyanins involved in color characteristics of wines (Spayd et al., 2002). Thus, this situation is leading to wines low in acidity, decreased

color intensity and stability, as well as too alcoholic, increasing the perception of heat and altering the perception of aroma complexity.

Although current technology allows for great ability to monitor and control the process, stuck and sluggish fermentations remain major challenges for the international wine industry. Bisson (1999) defined incomplete or "stuck" fermentations as those having a higher than desired residual sugar content at the end of alcoholic fermentation (>2-4 g L^{-1}), while slow or "sluggish" fermentations are characterized by a low rate of sugar consumption by the yeast. As well as the obvious problem of presenting longer fermentation times, sluggish fermentations also provides AAB and LAB with an opportunity for proliferation, and are more vulnerable to oxidation, with the subsequent increased probability of spoiling the product (Pizarro et al., 2007). Most common factors responsible for stuck and sluggish fermentations include nutrient deficiencies or imbalances (vitamins, minerals and nitrogen), high ethanol levels, high acidity, high sugar concentrations (> 300 g L^{-1}), fructose accumulation, presence of inhibitory wild yeast and the accumulation of toxic by-products (fatty acids, peptides, acetic acid or sulfites). Furthermore, extremes in fermentation temperature act in combination with must-associated factors, especially nitrogen deficiency (Alexandre and Charpentier, 1998; Charoenchai et al., 1998; Coleman et al., 2007). Restarting stuck fermentations is an extremely difficult task, and usually highly time and economic demanding (Quirós et al., 2014; Santos et al., 2008).

Aromatic compounds synthesized by yeasts and lactic acid bacteria such as higher alcohols, acetate esters, and ethyl esters constitute an essential factor for the aromatic profile of wines (Saerens et al., 2010). Low temperature fermentations improve not only the retention of these highly volatile compounds,

but also their production (Killian and Ough, 1979). This leads to the current tendency of conducting fermentations at low temperatures (10 °C-15 °C) for white and rosé, so that the resulting wines present richer and more complex aroma profiles acquired during the process. However, length of fermentation and risk of stuck fermentation increases because the optimal growth temperature of the wine yeast *S. cerevisiae* is far higher than these (Bisson, 1999; Salvadó et al., 2011b). Besides, the ecology of wine fermentation can be modified, with the risk of undesired alcohol resistant *non-Saccharomyces* yeast prevalence (Fleet, 2003). In addition, low temperature modifies the gene expression and physiology of yeast in general, altering the synthesis of secondary metabolites, and rearranging lipid membranes composition, whose fluidity decreases with temperature, affecting the transport of metabolites (Tronchoni et al., 2009, 2012).

3.4. Alternative yeast in winemaking

Originally, wine yeasts were commercialized in the form of compressed moist cakes and in the form of liquid cultures. In 1964, the successful drying of compressed yeast resulted in the marketing of active dry wine yeasts. This form of commercial yeasts is most suitable for use in wineries and their addition has become widely accepted. In the modern wine production, selected yeasts are used to inoculate grape must to control the fermentation, to reduce the risk of contamination, to increase the reproducibility, and to generate specific characteristics in the wine (Querol et al., 2018). A big number of selected dry yeast strains are available from different companies. Most of the commercialized strains are *S. cerevisiae*; however, there is an increasing interest in other species. The use of alternative yeast or tailored microbial starters is one of the main approaches for overcoming the above discussed issues. As we already mentioned,

many non-*Saccharomyces* yeasts are found in grape, vineyard, or even early stages of wine fermentation. Due to their highly diverse potentially interesting properties, some of them have been explored for improving wine production or characteristics. Numerous research projects applied some of these yeasts for reducing ethanol content using due to their lower yield of ethanol production on sugars, although most of the studies have not concluded with a considerable reduction (Gonzalez et al., 2013; Jolly et al., 2014; Varela et al., 2017). Also, their variate nature is an opportunity to explore their potential for aroma diversity generation (Fleet, 2003; Jolly et al., 2014; Lleixà et al., 2016), with even good results at the sensory level (Cordero Otero et al., 2003; Garcia et al., 2002; González et al., 2018; Nissen and Arneborg, 2003; Sadoudi et al., 2012). Even though, the main problem with these yeast species is that they are rapidly outcompeted by *S. cerevisiae* (Albergaria et al., 2010; Arneborg et al., 2005; Ciani et al., 2016a; Granchi et al., 1998; Nissen et al., 2003; Pérez-Nevado et al., 2006; Torija et al., 2001; Vendramini et al., 2017), thus, they present a limited efficacy that requires from strategies such as oxygenation, sequential inoculation or co-inoculation (Andorrà et al., 2012; Bely et al., 2008; Canonico et al., 2016, 2017; Ciani et al., 2010; Garcia et al., 2002; Gobbi et al., 2013; Gonzalez et al., 2013; Izquierdo Cañas et al., 2014; Loira et al., 2014; Shekhawat et al., 2016; Taillandier et al., 2014; Tristezza et al., 2016; Zara et al., 2014). In fact, fermentations inoculated with the non-*Saccharomyces* yeasts *T. delbrueckii*, *K. thermotolerans*, *Lachacea thermotolerans*, *C. pulcherrima*, *P. kluiveri* and *Metschnikowia pulcherrima* are used in actual commercial productions (Hansen, 2004a, 2004b, 2013; Laffort, 2013; Lallemand, 2012).

Nevertheless, there is also a growing interest in alternative yeast belonging to the *Saccharomyces* genus, such as *S. uvarum* or *S. kudriavzevii*, or interspecific hybrids from these species (Querol et al., 2018).

4. *Saccharomyces* Genus

During the XX Century, *Saccharomyces* genus has been revised several times. Due to the lack of molecular technology that we enjoy these days, researchers added and erased many taxa based on morphological or physiological properties.

The genus *Saccharomyces* belongs to the kingdom Fungi, the phylum *Ascomycota* (as the sexual reproduction is based on the formation of ascospores), the subphylum *Saccharomycotina*, the class *Saccharomycetes*, the order *Saccharomycetales* and the family *Saccharomycetaceae*. The taxonomy of the *Saccharomyces* genus currently involves eight species (**Figure 3**): *S. cerevisiae*, *S. kudriavzevii, S. uvarum, S. paradoxus, S. jurei, S. mikatae, S. arboricolus,* and *S. eubayanus* (Borneman and Pretorius, 2015; Boynton and Greig, 2014; Hittinger, 2013; Naseeb et al., 2017). In addition, recent studies have reported possibility of the existence of other species within the genus that remain to be isolated from their environment (Legras et al., 2018; Peter et al., 2018). Moreover, the pure species has formed different hybrids through multiple hybridization events, normally associated with human biotechnological processes. This is the case of *S. pastorianus*, the lager beer fermenter which is probably the most relevant industrial hybrid, whose parents are *S. cerevisiae* and the recently isolated in Argentina, North America, New Zealand and China *S. eubayanus* (Bing et al., 2014; Gayevskiy and Goddard, 2016; Libkind et al., 2011; Peris et al., 2014). *S. bayanus*, a hybrid isolated from sider and wine fermentations, was considered the parent of *S. pastorianus* until molecular

analysis from Nguyen *et al.* (2011) and Pérez-Través *et al.* (2014b) revealed that it was a "mosaic" yeast from *S. cerevisiae*, *S. uvarum* and what they provisionally named *S. lagerae*, which resulted to be the true lager yeast parent *S. eubayanus*.

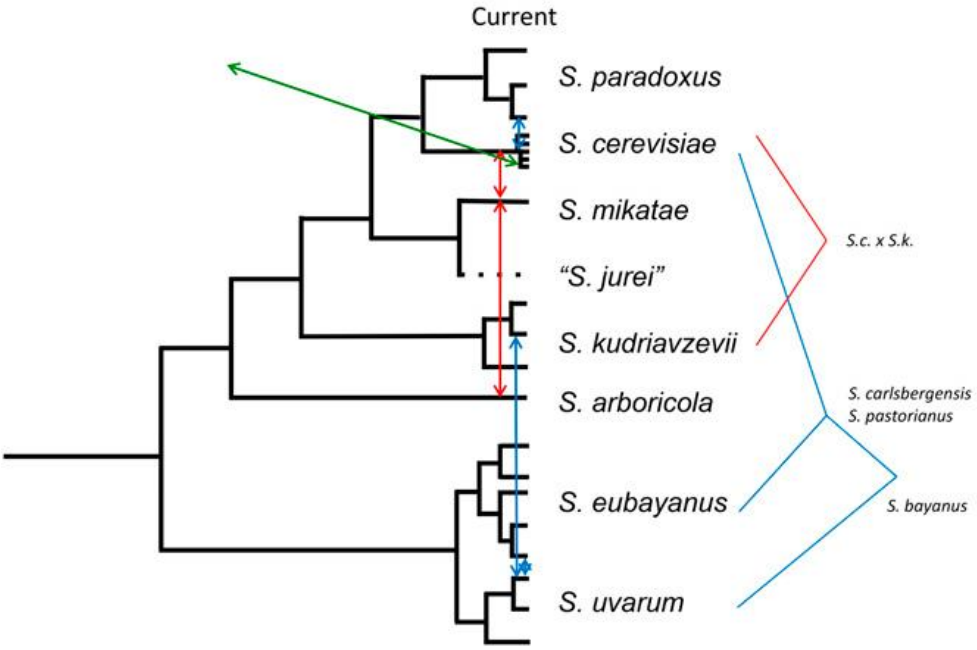


Figure 3| Philogeny of the *Saccharomyces* genus (adapted from Dujon and Louis, 2017).

The ecology of *Saccharomyces* species is diverse. Several species of this genus have been only found in natural environments. This is the case of *S. mikatae, S. kudriavzevii, S. arboricola, S. eubayanus* and *S. jurei.* They are normally found in oak barks or leafs, or in soils surrounding those (Gómez-Alonso et al., 2007; Libkind et al., 2011; Lopes et al., 2010; Naumov et al., 2000a; Peris et al., 2016; Sampaio and Gonçalves, 2008; Wang and Bai, 2008). Other species are found both in human related environments and in nature such as *S. uvarum* and *S. paradoxus* (Demuyter et al., 2004; González Flores et al., 2017; Naumov et al.,

2000b, 2002; Redžepović et al., 2002; Rodríguez et al., 2014; Torriani et al., 1999). Although *S. cerevisiae* is dominant in bread, ale beer, most wine and cider fermentations, it is normally in very low proportion with respect to other *Saccharomyces* species when found in natural environments (Serjeant et al., 2008).

Moreover, a number of *Saccharomyces* interspecific hybrids have been found associated with industrial production apart from the already mentioned. Commonly, hybrids *S. cerevisiae x S. kudriavzevii* have been isolated from wine, beer and cider. Also hybrids *S. cerevisiae x S. eubayanus* have been found in wine and cider. In the case of *S. cerevisiae x S. uvarum* hybrids, they are found only in wines (González et al., 2006; Pérez-Torrado et al., 2018; Peris et al., 2018; Querol et al., 2018). Even hybrids of three different species have been isolated from industrial fermentations (Boynton and Greig, 2014). In general, these hybrids are characterized by incorporating the wine-related more suitable characteristics of the parents. For instance, *S. cerevisiae x S. kudriavzevii* hybrids normally present fermentative capability similar to *S. cerevisiae* wine strains and also *S. kudriavzevii*'s cryotolerance (Erny et al., 2012; Ortiz-Tovar et al., 2018). Thus, generation of interspecific hybrids via rare mating under laboratory controlled conditions, constitute a strategy for obtaining new fermenter yeasts meeting the market demands (Pérez-Través et al., 2015).

From a genomics point of view, species of the *Saccharomyces* genus have sixteen chromosomes and sinteny is generally conserved among them, except for some translocation events (Borneman and Pretorius, 2015). Also, non-Mendelian generics elements may be present e.g. Ty retrotransposons and 2µm plasmid in the nucleus; mitochondrial DNA in the mitochondria; and killer viral-like

particles containing orthologue genes exist among the species in the genus (Scannell et al., 2011), including a core genome, which could be called the *Saccharomyces* pangenome, that includes the main functions like genetic and signaling regulation, metabolic pathways, cell cycle, etc. That explains why most of these yeasts are able to adjust to a typical fermentative medium and conduct fermentation up to some extent. However, despite this apparent similarity, the closest species to *S. cerevisiae* is *S. paradoxus* and the identity between their orthologue genes is ~98%. That is approximately the same as between human and mouse. Whereas the further species is *S. uvarum*, with an 80% identity, similar as between human and chicken (Dujon, 2006). Under this divergence that ultimately manifests in many phenotypic differences there is a huge potential for winemaking innovation as we will introduce next. For instance, *S. paradoxus* is currently used for winemaking in Croatia (Redžepović et al., 2002). Here, we are going to focus in *S. cerevisiae* and two alternative species of interest, *S. uvarum* and *S. kudriavzevii*.

4.1. *Saccharomyces cerevisiae*

The origin of *S. cerevisiae* remains a relevant question to be answered. Diverse populations are found in numerous different environments, often inhabiting liquid or moist areas. Common habitats include soil, plant exudates, animal tissues, and vineyards. They include diverse geographical environments, from tropical regions, to cold Northern climates (Fay and Benavides, 2005; Landry et al., 2006). Last studies point out to a single “out-of-China” origin (Peter et al., 2018; Wang et al., 2012). Anyways, its natural niche remains unclear. Goddard and Greig (Goddard and Greig, 2015) recently proposed that *S. cerevisiae* is a natural yeast with no niche, changing its location as resources are depleted,

maybe using insects as vectors, which are attracted by ethanol and aroma synthetized by this yeast (Buser et al., 2014). Moreover, some independent domestication events took place giving rise to the different domesticated clades that conform the industrial *S. cerevisiae* subgroups (Liti et al., 2009).

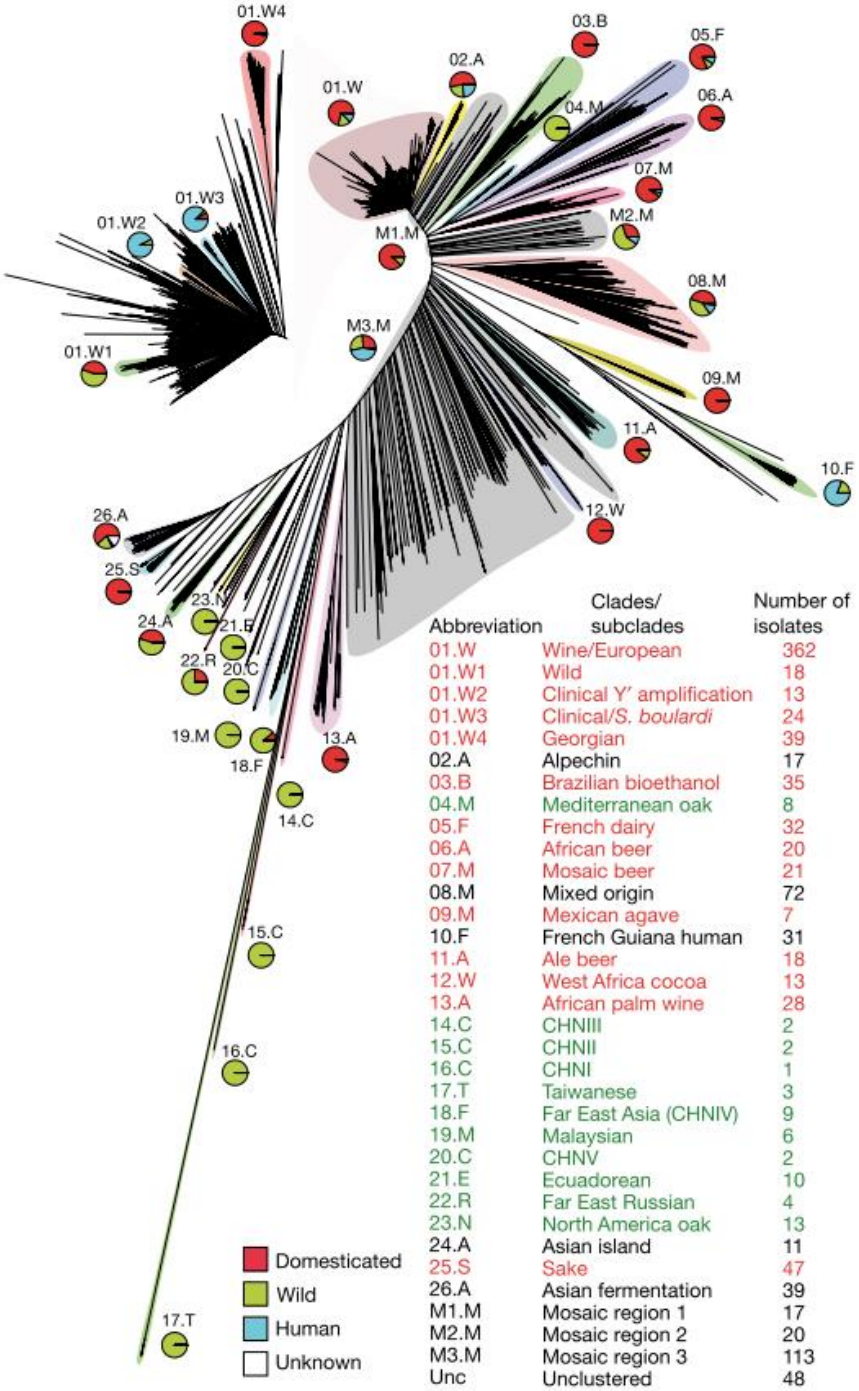
Industrial *S. cerevisiae* strains are highly specialized organisms, which have evolved to grow in the different environments or ecological niches that have been provided by human activity. This specialization has been associated with some genome characteristics, such as diploid genome with the presence of aneuploidies or polyploidies, high level of chromosome length polymorphism, homotallism, genome renewal and allopolyploid/hybrid genomes (Mortimer et al., 1994; Querol et al., 2003). For example, ploidy can increase the copy number and subsequent dosage and expression of beneficial genes, whilst decreasing the effects of deleterious mutations. These extra gene copies can diverge and adopt new functions, increasing heterozygosity and allowing for greater scope for adaptation to an ever-changing external environment.

The development of NGS technology allowed the sequencing of multiple *S. cereviae* strains from different origins and classify them according to genomic differences, which revealed the existence of numerous clades, as the wine clade, within the species (Peter et al., 2018) (**Figure 4**). Subsequently the suitability of the lab strain S288c as a "reference" strain is becoming less clear as it does not share many elements found in other species, especially an important number of ORFs present in other strains depending on their niche. In addition, *S. cerevisiae* yeasts from different origins have revealed the presence of horizontal gene transfers that could be involved in adaptation to industrial environment (Legras

et al., 2018; Marsit and Dequin, 2015; Morales and Dujon, 2012; Novo et al., 2009; Peter et al., 2018).

S. cerevisiae wine strains have received increasing attention in the last years. Recently, the gain of three large genomic regions (A, B, C) from distant yeast species have been discovered (Borneman et al., 2011; Galeote et al., 2010; Novo et al., 2009). Region C was transferred from *T. microellipsoides* and contains two duplicated genes encoding oligopeptides transporters which confer yeast with improved capability of nitrogen source uptake in wine fermentation (Marsit et al., 2016). In addition outcrossing rate within human related environment strains, especially wine strains, is higher than within natural strains, which is considered to be an important driving force for domestication (Magwene et al., 2011). Copy number variation is also relevant as we mentioned before, in the case of wine strains genes, *YKL222, MCH2,* ADH7, genes involved in heavy metals resistance *CUP1* or *PUT1* related to in nitrogen metabolism, are present in multicopy (Ibáñez et al., 2014; Legras et al., 2018).

Considering the great amount of currently available data, other genetic elements such SNPs fixed in wine population will be associated with phenotypic features of the different clades, constituting a field of remarkably accessibility to be explored in the near future. However, similar analysis of the rest of the *Saccharomyces* species of interest remains to be addressed.



Abbreviation	Clades/ subclades	Number of isolates
01.W	Wine/European	362
01.W1	Wild	18
01.W2	Clinical Y′ amplification	13
01.W3	Clinical/*S. boulardi*	24
01.W4	Georgian	39
02.A	Alpechin	17
03.B	Brazilian bioethanol	35
04.M	Mediterranean oak	8
05.F	French dairy	32
06.A	African beer	20
07.M	Mosaic beer	21
08.M	Mixed origin	72
09.M	Mexican agave	7
10.F	French Guiana human	31
11.A	Ale beer	18
12.W	West Africa cocoa	13
13.A	African palm wine	28
14.C	CHNIII	2
15.C	CHNII	2
16.C	CHNI	1
17.T	Taiwanese	3
18.F	Far East Asia (CHNIV)	9
19.M	Malaysian	6
20.C	CHNV	2
21.E	Ecuadorean	10
22.R	Far East Russian	4
23.N	North America oak	13
24.A	Asian island	11
25.S	Sake	47
26.A	Asian fermentation	39
M1.M	Mosaic region 1	17
M2.M	Mosaic region 2	20
M3.M	Mosaic region 3	113
Unc	Unclustered	48

Figure 4| Neighbor-joining tree of 1,011 sequenced genomes, built using the biallelic SNPs (Withdrawn from Peter et al., 2018).

4.2. *Saccharomyces uvarum*

S. uvarum is one of the cryotolerant yeasts of the genus that have been investigated for its application in winemaking industry. Although several strains have been sporadically isolated from insects, tree fluxes or mushroom (Naumov et al., 2003), it has been found mainly in industrial environments, such as wine and cider fermentation processes performed at low temperatures (Demuyter et al., 2004; Naumov et al., 2000b, 2001; Rodríguez et al., 2014). From the oenological point of view, as compared to *S. cerevisiae*, *S. uvarum* has shown higher production of glycerol and lower production of ethanol and acetic acid (Giudici et al., 1995; González Flores et al., 2017; Pérez-Torrado et al., 2016; Rodríguez et al., 2014). Additionally, *S. uvarum* has been characterized by its higher capability to release desirable flavor components, such as 2-phenylethanol and 2-phenylethyl acetate (Gamero et al., 2013; Masneuf-Pomarède et al., 2010). Recently, further assessment of the behavior of this yeast with natural must and limited aeration have been carried away (Contreras et al., 2015; Varela et al., 2016, 2017). All these factors make it a good candidate for its use to solve the current challenges of winemaking industry.

4.3. *Saccharomyces kudriavzevii*

S. kudriavzevii was first isolated from decaying leaves in Japan (Naumov et al., 2000a). Given the existence of hybrids of this species in traditional wine and brewing in Europe (Gonzalez et al., 2007), researchers made queries how this species could form them. The answers came recently with the isolation of *S. kudriavzevii* strains from oak trees in Portugal and Spain (Lopes et al., 2010; Sampaio and Gonçalves, 2008). Despite, the geographical distance, there is low divergence between the two main clades in this species, the Japanese and the European clades (Hittinger et al., 2010) .

Different aspects of *S. kudriavzevii* biology have been investigated due to its interest for oenology, which was first assessed in regard to its ability to grow at very low temperatures in comparison to *S. cerevisiae* (Salvadó et al., 2011b). That makes it potentially appropriate for avoiding stuck fermentations in low temperature processes. In fact, *S. kudriavzevii* is able to conduct fermentation when inoculated in sterile wine must (Gonzalez et al., 2007; Peris et al., 2016). Moreover, differences in the physiology of *S. kudriavzevii* and *S. cerevisiae* lead to interesting oenological properties.

In comparison to *S. cerevisiae*, *S. kudriavzevii* fermented musts contain more glycerol and less ethanol (Gonzalez et al., 2007; Pérez-Torrado et al., 2017a; Peris et al., 2016). Glycerol is a metabolite involved in resistance to osmotic and cold stress (Remize et al., 2001; Tulha et al., 2010). It is considered to contribute to wine quality by providing slight sweet- ness, smoothness, and fullness, reducing wine astringency (Goold et al., 2017). In *S. kudriavzevii,* the differentiated import/efflux capacity under hyperosmotic stress results was proposed to be the reason of higher glycerol content in wines (Pérez-Torrado et al., 2016). Regarding ethanol, its content reduction in wine is desirable for many wineries, as mentioned above.

Other interesting feature of this species is the aroma profile of wine fermented by it. Despite they have been isolated only from natural environments far from wineries, *S. kudriavzevii* strains produce larger amounts of higher alcohols and 2-phenylethanol (rose aroma) at low temperatures (Stribny et al., 2015).

Thus, the use of *S. kudriavzevii* could constitute an opportunity for wineries which seek targeting new industry challenges by the use of yeast similar to the

traditionally used that can provide with improvements in compositions of final product.

4.4. Yeast life cycle and growth kinetics in fermentation

Saccharomyces yeasts can multiply either asexually by vegetative multiplication or sexually by sporulation and crossing. Under optimal nutritional and cultural conditions, these yeasts remain diploid and they adopt the most common mode of reproduction of Ascomycetes; vegetative growth by budding. The cell division cycle for vegetative growth is divided into four main stages: G1 (period before DNA replication), S (DNA replication), G2 (period between S and mitosis) and M (Mistosis and cytokinesis). Once mitosis is concluded, the nascent nucleus and organelles migrate into the bud, cytokinesis starts and septum is formed in the isthmus between mother and daughter cells, completing cell division.

Saccharomyces yeast present two possible alleles for the sexual locus *MAT* (MATa and MATα). Diploid cells have a copy of each of the MAT alleles. When nutrient sources in the media fall short, yeasts entry into sexual phase of life cycle. It involves the induction of meiosis, with the subsequent formation of four haploid spores inside an ascus that functions as an isolator and protector from the environment. Cells can be under this state for longs periods until the detect accessibility to nutrients, when they restart growth. Haploid cells can grow by vegetative division, however, if a MATa cell and a MATα cell meet, they tend to mate with high probability to gain the ideal diploid state. Moreover, most *Saccharomyces* strains are homothallic, meaning that haploid cells can switch sexual type increasing the chances of mating.

Population growth of yeast is the result of cell division and cell cycle progression under the appropriate growth conditions, growth kinetics responds to a typical microbial growth curve, which comprises four stages of phases: latency phase (or lag phase), exponential growth phase (or log phase), diauxic phase, and stationary phase (**Figure 5**). Lag phase (λ) reflects the time required for the yeasts to adjust to their new environment, normally by synthesizing the necessary ribosomes and enzymes to generate the highest growth rate. Yeast characteristics, Initial population size and medium conditions are the main factors determining the duration of this stage. Then, cells begin active metabolism, DNA replication and cell division. In the second phase, cells are already adjusted to the environment and achieve their specific maximal growth rate (μ_{max}). Again, μ_{max} is highly influenced by the growth medium, temperature, and strain; although for a *Saccharomyces* yeast under favorable conditions generation time ranges from 90 to 120 minutes. Third growth stage is diauxic phase, a slow growth period characterized by a change from fermentative to respiratory metabolism due to lack of fermentable carbon sources in the medium, which makes the oxygenic consumption of ethanol obligatory. Finally, due to the depletion of limiting nutrients or the presence of growth inhibitors, cell metabolism and cell division decay, and the number of cells in the population is maintained. Cells can survive during long periods of time thanks to modifications in their cell wall, storage of carbon reserve metabolites (trehalose and glycogen) and to a dramatic slowdown of transcription and translation. After that, cells start autolysis and die.

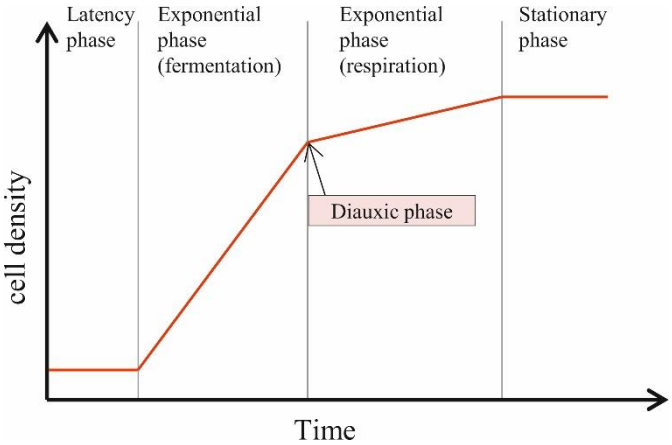


Figure 5| Typical *Saccharomyces* growth curve

5. Yeast population dynamics during wine fermentation

As we briefly explained above, spontaneous fermentation is a complex biochemical process characterized by the sequential growth of different yeast. The environment conditions and the population size of each species will determine which yeast dominates the process according to their fitness at the different stages. At the beginning, non-*Saccharomyces* yeasts are more numerous. They are characterized by low ethanol tolerance, low fermentative capacity and a limited growth phase. Initially, yeast from genera such as *Hanseniospora* or *Candida* can present high density level (10^4-10^5 cells/mL), and they can reach populations of 10^7-10^8 cells/mL until the ethanol level is too high (30-40 g/L). Then, *Saccharomyces* takes over thanks to its strong fermentative capability and high ethanol tolerance (**Figure 6**) (Bagheri et al., 2016; Pretorius, 2000).

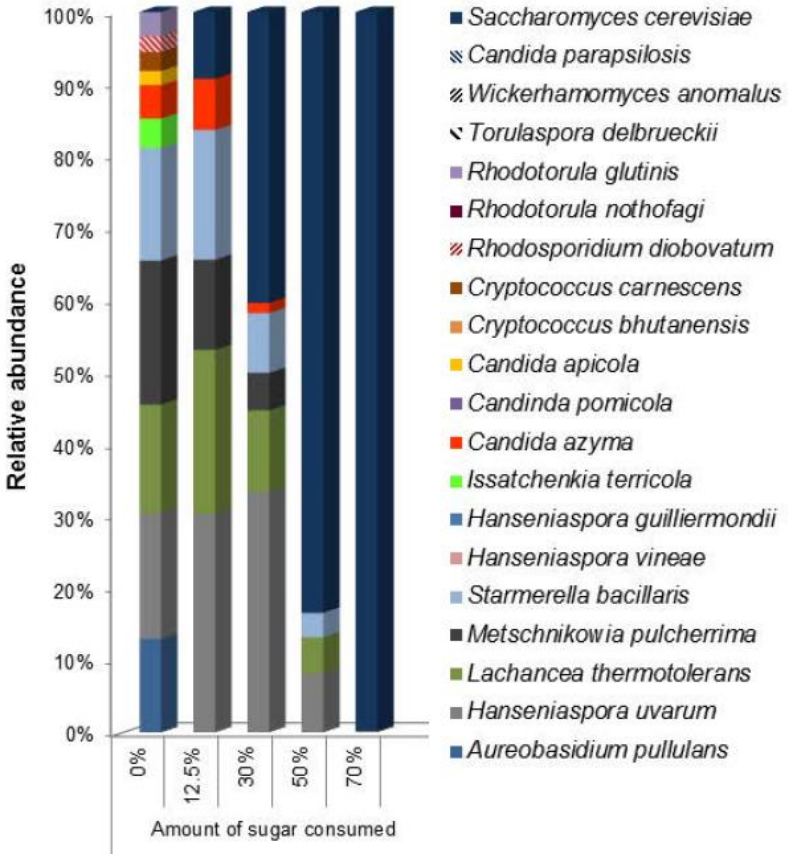


Figure 6| The occurrence and evolution of yeast species at different stages of a spontaneous fermentation (withdrawn from Bagheri et al., 2016).

The ability of yeasts to effectively ferment grape sugars depends on their growth fitness under the harsh conditions of the environments. This capacity is determined by different regulatory mechanisms that constitute the physiological response to stresses.

High Osmolarity

Grape must is a high-density substrate medium that contains a considerable concentration of osmotically active substances, specifically fructose and glucose. This high osmolality is one of the main stresses yeasts have to deal with,

consisting in the potential loose of intracellular water which can cause cell death. Yeast response may involve alterations of cell wall composition and cytoskeleton. Also, the synthesis of the inert osmolite compound glycerol to reestablish the osmotic equilibrium (Hohmann, 2002).

Low pH

In addition, yeasts have to face very low pH of grape must (~3) when their optimal pH generally rages from 4.0 to 6.0. In fact, pH can affect yeasts fitness and physiology, and subsequently wine composition (Arroyo-López et al., 2009; Serra et al., 2005; Zemančíková et al., 2018). Under low pH conditions in the medium, weak acids are mostly in their undissociated form, being able to trespass the plasma membrane. Once in the cytoplasm, these acids dissociate due to the higher intracellular pH, liberating protons which promotes cell acidification (Orij et al., 2011). Cell acidification leads to inhibition of essential metabolic functions such as glycolysis (Bracey et al., 1998). To counter fight this phenomenon, the studied mechanism of *S. cerevisiae* consists on pumping the excess of protons out of the cell by the H^+-ATPase Pma1p. The counterpart of this homeostasis mechanism is a considerable leak of energy resources (Bracey et al., 1998). Several studies have focused on how several *Saccharomyces* species and interspecific hybrids fitness respond to must pH (Arroyo-López et al., 2009; Charoenchai et al., 1998; Serra et al., 2005). In general, this genus is well adapted to must pH in comparison to other variables such as temperature. Moreover, production of weak acids by species such as *S. uvarum* has been used as a mechanism to inhibit growth of non-desirable organisms in fermentation due to cell acidification (Caridi and Corte, 1997).

Ethanol toxicity

Ethanol generated during wine fermentation is highly toxic for most of the yeast. This compounds inhibits metabolism and growth (Ingram and Buttke, 1985; Viana et al., 2012). The main target of ethanol is plasma membrane due to the solubility of its hydroxyl group in aqueous and lipidic matrices, increasing plasma membrane fluidity and permeability. Ethanol also damages mitochondria, reducing the rate of respiration and ATP levels, provoking formation of ROS and acetaldehyde, and generating DNA damages, lipid peroxidation and oxidative stress (Alexandre et al., 2001; Costa and Moradas-Ferreira, 2001; Du and Takagi, 2007). Furthermore, it inhibits the activity of glycolytic enzymes, increases heat shock proteins expression, petit mutations and causes vacuole acidification caused by the entrance of protons through the damaged cell wall (Hu et al., 2006; Meaden et al., 1999; Rosa and Sá-Correia, 1991, 1996). Another of the important effects is the transmembrane transport non-competitive exponential inhibition, affecting sugars, ammonia and amino acids uptake (Leão and van Uden, 1984). Ethanol tolerance is mostly based on the ability to keep the plasma membrane fluidity (Alexandre et al., 1994; Ding et al., 2009; Huffer et al., 2011). Sterols are indispensable to keep this fluidity; indeed, ethanol tolerant yeast strains exhibit elevated ergosterol levels in their membrane. They also present higher ratios of phosphatidylinositol-to-phosphatidylcholine and larger amounts of longer unsaturated fatty acids (Aguilera et al., 2006; Arneborg et al., 1995). Moreover, the implication of intracellular pH homeostasis, trehalose biosynthesis genes, and heat shock proteins expression have been proven in ethanol resistance (Alexandre et al., 2001). More recently, unfolded protein response (UPR) have been demonstrated to increase tolerance to ethanol in *S. cerevisiae* (Navarro-Tapia et al., 2016).

Ethanol is especially toxic for most non-*Saccharomyces* yeasts in comparison to *Saccharomyces* (Arroyo-López et al., 2010b), although there are some exceptions such as *Hanseniospora* species or *T. delbruekii* (Cocolin et al., 2000; Fleet, 2003; Jolly et al., 2003b; Pérez-Nevado et al., 2006; Pina et al., 2004). Within the *Saccharomyces* genus, *S. cerevisiae* is the most resistant species, especially strains related to industrial process in which ethanol is presence in elevated concentrations, such as bioethanol production or winemaking. *S. paradoxus*, *S. uvarum* and *S. kudriavzevii* follow *S. cerevisiae* in this order, always presenting tolerance above 7-8% (v/v) of ethanol (Arroyo-López et al., 2010b).

Nitrogen limitation

A numerous variety of nitrogen sources are available for yeast in wine must, including ammonium, amino acids, and peptides, that are necessary for protein synthesis and cell growth and maintenance. Although, nitrogen composition of musts is something quite variable depending on biotic and abiotic factors, in general, ammonium is the preferred nitrogen source and is rapidly depleted from the media (Crépin et al., 2012), since it is the main intermediate between catabolic and anabolic pathways (ter Schure et al., 2000). Glutamine, glutamate, and asparagine, were also proven to be good nitrogen sources for yeasts growth. On the contrary proline, allantoin, or urea are described as non-preferred nitrogen sources (Crépin et al., 2012).

Nitrogen regulates the formation of biomass as long as it is the limiting nutrient in grape must. When assimilable nitrogen is depleted from the fermentation media, yeasts enter into stationary phase. Thus, ammonium and amino acid uptake ability determines competitive success in some extent. In fact, cases have been described where the initial microflora (non-*Saccharomyces*) rapidly

assimilated most of the available nitrogen sources, impeding *S. cerevisiae* appropriate growth, which lead to stuck fermentations (Bisson, 1999; Constantí et al., 1998; Fleet, 2003; Medina et al., 2012). Among the species of the genera, and even depending on the strain origin, there are differences in nitrogen uptake preference and efficiency (Su et al., 2018).

Cold stress

As we already mentioned, low temperature fermentation (12 °C - 15 °C) is becoming a common practice in winemaking. However, *S. cerevisiae*'s optimal temperature is around 30 °C (Salvadó et al., 2011b). That means a restrictive temperature for this species that increases growth lag phase and reduces the growth rate due to the necessary acclimation to this stress. Interestingly, low temperature favors cell viability (Torija, 2003).

Cell physiology is greatly affected by low temperature and the response comprises many elements in order to maintain metabolic functions. One of the main affected cell structures is plasma membrane, which loses its fluidity reducing intra and extracellular the transport and diffusion rates of compounds and ions (Inouye and Phadtare, 2004). The cell counteract this effect by producing altered unsaturated fatty acids that increases fluidity in membranes (López-malo et al., 2014). Similarly, low temperature stabilizes secondary structures of proteins and nucleic acids. The mRNA 5' untranslated region forms saccessible for the ribosome during translation (Al-Fageeh and Smales, 2006). To balance the problems related to protein misfolding, cell synthetizes heat shock proteins (HSPs), which acts as chaperones and help restoring enzyme activity at low temperatures (Murata et al., 2006).

Furthermore, yeast cells produce protectant compounds under cold stress, such as trehalose, glycogen or glycerol (Sahara et al., 2002). Trehalose is a disaccharide that acts as a chemical chaperone for membrane and protein stabilization. Glycerol protects cells by its osmotic homeostasis function, coping with the effect of osmotic shrinkage during freeze and thaw cycles (Panadero et al., 2006). In fact, differentiation in glycerol export and uptake from the media among the *Saccharomyces* genera, explains part of the cold cryotolerant characteristics of *S. uvarum* and *S. kudriavzevii* (Oliveira et al., 2014; Pérez-Torrado et al., 2016). At last, antioxidant compounds and enzymes, including glutathione, catalase and superoxide dismutase, are all induced under low temperature conditions, detoxifying reactive oxygen species (ROS) for the maintenance of viability (García-Ríos et al., 2016; Murata et al., 2006; Paget et al., 2014; Zhang et al., 2003).

Strains' extent of adaptation to low temperatures, will determine their utility as starters in low temperature fermentations.

General stress response

Initial studies on the yeast response to environmental fluctuations were performed in laboratory media, very different from wine must in composition, and termed "environmental stress response" (ESR). Expression analysis have determined that approximately 10-15% of the yeast genes are differentially expressed as part of ESR as a common signature (Causton et al., 2001; Gasch, 2003; Gasch et al., 2000; Mitchell et al., 2009). ESR appears to control cell cycle progression, reestablish energy supply balance to account for these environmental fluctuations, as well as providing a protective role against stress (Giaever et al., 2002; Lai et al., 2005).

Fermentation stress response (FSR) during winemaking has also been assessed (Marks et al., 2008). In this case, 40% of genes showed differential expression, and 28% of them have unknown function. Part of the genes involved in FSR (around 20%) overlaps with ESR. Those are related to chaperone proteins and heat shock proteins (HSPs), involved in repairing the damage caused by environmental stress and prevent any further damage from taking place. Chaperones and HSPs are also the most highly expressed genes at the late-stationary phase and reflects a priority for protein synthesis (Rossignol et al., 2009; Zuzuarregui et al., 2006). FSR also includes hyperosmotic response genes, such as glycerol synthesis genes, and genes expressed specifically under high sugar concentration. Also, genes involved in ethanol toxicity, nitrogen depletion and oxidative stress were found.

Although stress responses are usually assessed in model organisms, there are important differences at the species and also at the strain level (Tronchoni et al., 2017; Zuzuarregui et al., 2005). This is mostly owed to adaptive evolution to the environments these organisms come from (Fay et al., 2004). For instance, the cryotolerant *S. kudriavzevii* better resists cold shocks because responsive genes to this stress are constitutively expressed at a higher rate than in *S. cerevisiae* (Tronchoni et al., 2014).

2.1. Yeast-yeast interactions

In recent decades, many studies have contributed to the understanding of wine microbiota ecology and population dynamics (Albergaria et al., 2010; Bagheri et al., 2016; Kemsawasd et al., 2015; Nissen et al., 2003; Pérez-Nevado et al., 2006; Renault et al., 2013; Taillandier et al., 2014). As mentioned above, *S. cerevisiae*

is the dominant species during the fermentation, outcompeting the rest of the yeast from the must after the first days. The reason underlying *S. cerevisiae* dominance in wine fermentation rely on its competitive advantage in the changing environment (Bauer and Pretorius, 2000). The classical factors responsible for such imposition such as acidic pH or ethanol presence have been already discussed; however, microbial interactions also can deeply affect the growth and metabolic activity of yeasts (Bisson, 1999; Fleet, 2003).

From an ecological point of view, microbial interactions can be classified into positive (+), negative (-) and neutral (0); and are mainly four: mutualism (+/+), commensalism (+/0), amensalism (-/0) and competition (-/-). All of them can be found in wine fermentation (Liu et al., 2015). Amensalism takes place in cases where the growth of one microorganism is repressed by the secretion of a toxic metabolite by another microorganism, not affecting to the fitness of the latter. Competition for nutrients is another of the most important interactions modulating microbial populations during this process. There are also reported cases of commensalism between one *Saccharomyces* and one non-*Saccharomyces* species, e.g., the high extracellular proteolytic activity of some non-*Saccharomyces* yeast causes the release of amino acids from proteins present in the must that can be used by *S. cerevisiae* as nitrogen source (Fleet, 2003).

Among the competition mechanisms, there is a possible classification regarding whether contact between the cells of the involved strains is necessary or not. This way, competition by altering media composition such as nutrient depletion, ethanol synthesis, or release of toxic compounds does not require direct cell-to-cell contact. In 2005, for the first time, Arneborg *et al.* (2005) proved that competition mechanism of *S. cerevisiae* against the non-*Saccharomyces* yeast *H.*

uvarum, was based on the physical surrounding of the competitor, involving cell-to-cell contact, or at least, close proximity (**Figure 7**). This is compatible with the hypothesis that *S. cerevisiae* intense metabolism provokes peaks of high temperature that would have a devastating effect on non-*Saccharomyces* yeasts (Goddard, 2008). More recently, *S. cerevisiae* was shown to secrete GADPH derived peptides that remain attached to the cell wall, which and resulted toxic to several non-*Saccharomyces* yeasts (Branco et al., 2014, 2018; Kemsawasd et al., 2015), which would also imply cell-to-cell contact. Moreover, a diversity of other studies conducted in the last years revealed a great importance of direct contact in growth arrest or early death of *S. cerevisiae*'s competitors (Nissen and Arneborg, 2003; Pérez-Nevado et al., 2006; Rivero et al., 2015; Taillandier et al., 2014; Wang et al., 2015b).

6.

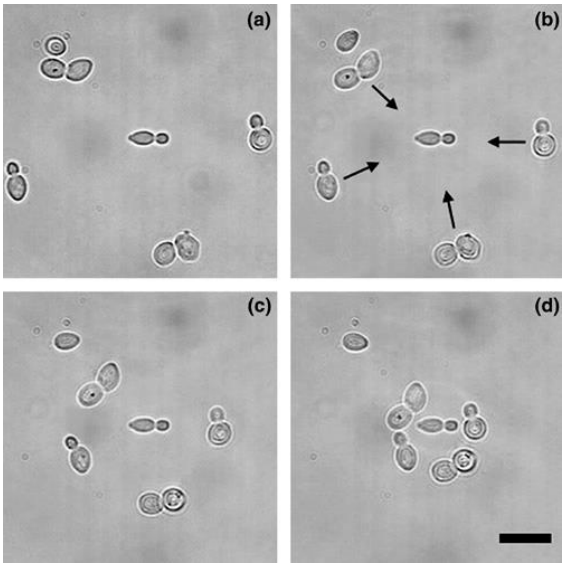


Figure 7| Dynamic microscopy imaging of a mixed culture of *S. cerevisiae* and *H. uvarum*. Arrows show the direction of movement. Images from one representative experiment were

recorded at (a) 0 s; (b) 30 s; (c) 60 s; and (d) 90 s. Scale bar represents 10 μm. Withdrawn from (Arneborg et al., 2005).

Most of these mechanisms are considered or even proved to be constitutive in *S. cerevisiae*. However, there is evidence that *S. cerevisiae* can undertake a modulation of its genetic expression to face the biotic stress caused by competition (Tronchoni et al., 2017), and this may be related to faster nutrient uptake and thus, depletion from the media (Curiel et al., 2017), which we had classified as a non-contact dependent competition mechanism. This reveals the high complexity underlying the phenomenon of competition.

Nevertheless, practically the totality of published works related to *S. cerevisiae*'s competitive traits in wine fermentation involved a non-*Saccharomcyes* organism. Despite the increasing interest on alternative *Saccharomyces* yeasts, their behavior in co-cultures has not received much attention except for few cases. In 2015, Williams *et al.* (2015) showed that *S. cerevisiae* is able to displace other *Saccharomyces* species in high sugar environments like wine must, and that this trait evolved much more recently than Whole Genome Duplication event. Pérez-Torrado et al., (2017b) proposed a mechanistic model for the competition between two *S. cerevisiae* strains based on cell-to-cell contact and the relation between sulphite secretion and resistance, which also points to a very recent appearance of some competitive traits owing to the fact that two different strains of the same species present such phenotypic differences.

More directly related to my thesis is the previous work carried out by our group in which a *S. cerevisiae* wine strain and a *S. kudriavzevii* strain were co-inoculated in fermentations set up at different temperatures (Arroyo-López et al., 2011). They showed how *S. kudriavzevii* was totally depleted from the medium

at the higher assayed temperature (31 °C) in the mixed culture, despite been completely viable when grown alone. More strikingly is that growth arrest occurred always before for *S. kudriavzevii* in mixed cultures, even at cold temperatures expected to be more suitable for its fitness (Salvadó et al., 2011b) (**Figure 8**). This suggests the existence of elements other than the classically considered ethanol toxicity and temperature tolerance for the interaction between two different *Saccharomyces* species.

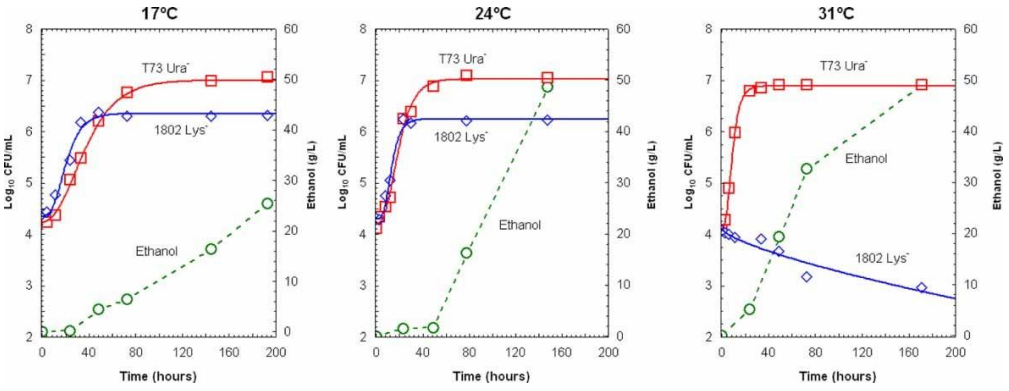


Figure 8| Population evolution of the strains *S. cerevisiae* T73 and *S. kudriavzevii* IFO 1802T in mixed fermentations performed at 17 °C, 24 °C and 31 °C.

OBJECTIVES

Nowadays, improvement of fermented foods through the modulation or replacement of the natural microbiota is key to industry nowadays. Due to the new demands of the wine market and the challenges given by climate change, there is a growing interest in the use of specialized yeasts that provide the final product with suitable properties that overcome these issues. Different species of the *Saccharomyces* genus have been proposed as good candidates for wine improvement. However, implantation of these alternative yeasts is one of the main difficulties in industry due to exclusion by the high competitive fitness of *S. cerevisiae* naturally present in musts. Thus, the study of their behavior in fermentation when sharing the environment with a wine yeast representative, is important to characterize their real utility in fermentation, so that they could be used as a component of a starter culture, under certain conditions.

Taking the previous issues in consideration the present thesis aimed to achieve the following objectives:

1. The analysis of competitions between *S. cerevisiae* and other *Saccharomyces* species during wine fermentation, by the assessment of the effect of:
    1.1. Competition on the fitness of both *S. cerevisiae* and the other species of interest
    1.2. Competition on the final wine composition
    1.3. Competition on the fermentation kinetics
    1.4. Low temperature on competitive fitness
    1.5. The origin of the strains in their behaviors during competition.

2. The improvement of the competitive fitness of *S. kudriavzevii* under regular winemaking temperature conditions by the evaluation of the effect of:
   2.1. The use of controlled oxygenation.
   2.2. The inoculation of high proportions of *S kudriavzevii*.
   2.3. The sequential inoculation of *S. kudriavzevii* before *S. cerevisiae*.
   2.4. The different strategies on the fermentation kinetics and the final product composition
3. The analysis of the interaction, at the molecular level, between *S. cerevisiae* and *S. kudriavzevii* during the competition under fermentative conditions, using different approaches:
   3.1. Analysis of the transcriptomic response of *S. cerevisiae* and *S. kudriavzevii* to competition at different time points of the fermentation, to identify possible specific.
   3.2. To experimentally validate these mechanisms
   3.3. Analysis of the differences in the competition response depending on the origin of the competing strains.
   3.4. Evaluation of the effect of the fermentation temperature on the interactions during competition
   3.5. Comparison with a case study of co-fermentations of *S. cerevisiae* with *S. uvarum*

This doctoral thesis has been organized into three chapters:

I. Effect of temperature on the prevalence of *Saccharomyces* non *cerevisiae* species against a *S. cerevisiae* wine strain in wine

fermentation: competition, physiological fitness, and influence in final wine composition, addresses **objective 1**.

II. The use of mixed populations of *Saccharomyces cerevisiae* and *S. kudriavzevii* to reduce ethanol content in wine: limited aeration, inoculum proportions and sequential inoculation, corresponds to **objective 2.**

III. Dominance of wine *Saccharomyces cerevisiae* strains over *S. kudriavzevii* in industrial fermentation competitions is related to an acceleration of nutrient uptake and utilization, deals with **objective 3**.

CHAPTER I

Effect of Temperature on the Prevalence of *Saccharomyces* Non *cerevisiae* Species against a *S. cerevisiae* Wine Strain in Wine Fermentation: Competition, Physiological Fitness, and Influence in Final Wine Composition

1. Introduction

Wine is the product of complex interactions among yeast, bacteria and other fungi that begin in vineyards and continue with the fermentation process. Different yeast species are predominant on the surface of grape skins and in the winery environment (Albergaria and Arneborg, 2016; Sabate et al., 2002), and *S. cerevisiae* is recognized as being the main microorganism responsible for this process (Pretorius, 2000). However, other *Saccharomyces* species (*Saccharomyces* non-*cerevisiae* yeasts, SNC) may play an important role in wine fermentation under certain conditions. In this way *S. uvarum* is less frequent than *S. cerevisiae* in wines, but appears to be predominant in European wine regions with an oceanic climate where wine fermentations are performed at lower temperatures; e.g., the Basque Country, Spain (Rementeria, 2003), Alsace, France (Demuyter et al., 2004), Val de Loire, Sauternes, and Jurançon in France (Naumov et al., 2000b), Valpolicella, Italy (Torriani et al., 1999), Tokaj in Hungary and Slovakia (Antunovics et al., 2005; Naumov et al., 2002; Sipiczki et al., 2001), and Yalta, the Ukraine (Naumov and Nikonenko, 1987). *S. paradoxus* is a natural species worldwide distributed with a fortuitous presence in vineyards and fermentation processes (Valero et al., 2007). However, some strains of this species have been described as predominant in Croatian vineyards (Redžepović et al., 2002), and exhibit interesting enological properties.

The fermentations conducted by natural interspecific *Saccharomyces* hybrids, such as *S.cerevisiae* × *S.kudriavzevii* and *S.cerevisiae* × *S.uvarum*, have also been described in European wine regions with oceanic and continental climates (northern Spain, Alsace, Germany, Switzerland, Austria, Croatia, Hungary and

Moldavia), close to the northern limit of grapevine distribution (Erny et al., 2012; González et al., 2006; Masneuf et al., 1998; Peris et al., 2012).

Despite these exceptions, presence of SNC in the final stages of the fermentation process is quite rare. This is because *S. cerevisiae* can competitively displace other yeast species from wine fermentations, both SNC (Arroyo-López et al., 2011; Williams et al., 2015) and non-*Saccharomyces* yeasts (Holm Hansen et al., 2001; Pérez-Nevado et al., 2006). Different mechanisms have been proposed to explain the higher competing capability of *S. cerevisiae* compared to non-*Saccharomyces* yeasts which, in most cases, are not mutually exclusive, but complementary.

The vigorous fermentative capacity of *S. cerevisiae* yeasts in both the presence (Crabtree effect) and absence of oxygen has been recognized as the main strategy to outcompete other microbial species present in must. *S. cerevisiae* consumes sugar resources faster, and the ethanol and CO_2 produced during fermentation can be harmful or less tolerated by their competitors. Once competitors are overcome, *S. cerevisiae* can then use accumulated ethanol as a substrate for aerobic respiration. This ecological strategy is called make-accumulate-consume (Piškur et al., 2006; Thomson et al., 2005), and provides a selective advantage to *S. cerevisiae* to outcompete other microorganisms. Different non *Saccharomyces* yeast, as well as bacteria, have also been proven to be very sensitive to the killer peptides or toxic compounds produced by *S. cerevisiae* (Albergaria et al., 2010; Branco et al., 2014; Pérez-Nevado et al., 2006; Wang et al., 2015b, 2016), which may play a key role during competition. Finally, the higher *S. cerevisiae* cell density has also been postulated as being an important factor that contribute to the exclusion of non-

Saccharomyces yeasts (Arneborg et al., 2005; Holm Hansen et al., 2001; Nissen et al., 2003, 2004; Nissen and Arneborg, 2003).

Other *Saccharomyces* species share very similar physiological properties with *S. cerevisiae* and, hence, similar ecological strategies. However, wine *S. cerevisiae* yeasts show better adaptation to survive under the stressful environmental conditions occurring during alcohol fermentation; e.g., high concentrations of sugar or ethanol, low pH and nutritional depletion, which provides them with a competitive advantage (Albergaria and Arneborg, 2016).

Another important advantage of *S. cerevisiae* on SNC species is its efficient growth at a wide range of temperatures, especially at higher temperatures (32 °C). This has also been considered an important trait that explains its dominance during wine fermentation (Salvadó et al., 2011a). Goddard (2008) also observed that *S. cerevisiae* is even able to significantly increase the environmental temperature during vigorous fermentation. Arroyo-López et al. (2011) also demonstrated that *S. cerevisiae* was able to outcompete *S. kudriavzevii* even at temperatures that are more suitable to the latter (Salvadó et al., 2011b). However, *S. paradoxus* has been shown to be present during grape fermentation processes when competing with *S. cerevisiae* at both 22 °C and 30 °C (Williams et al., 2015). Therefore, very little is known about the behavior of other SNC in competition with *S. cerevisiae* in winemaking environments at low temperatures.

In the 21st century, the wine industry must respond to the challenges posed by both new consumers' demands and changes in grape composition and properties due to climate change. Consumers demand products with lower

alcohol content and fruitier aromas, which lead winemakers to lower fermentation temperatures, as far as 10-12 °C, to preserve aroma compounds in wines. Climate change influences grape must characteristics (acidity, content in sugars or tannins, etc.), which has an impact on final product quality. Also due to climatic change there is a gap between the maturity according to sugar content and the maturity of the phenolic compounds of the grape. Therefore, sugar concentration in musts reaches higher levels, which leads to wines with higher ethanol content.

These facts strongly challenge the quality and acceptance of the final product which leads to the necessity of improvements in oenological practices, among which the development of new yeast starters adapted to the new imposed conditions are of chief importance. Previous studies have shown that unconventional SNC yeast species, such as *S. kudriavzevii* and *S. uvarum*, could be good candidates to achieve those goals. This is because they exhibit good fermentative capabilities at low temperatures (Salvadó et al., 2011b), produce wines with lower alcohol and higher glycerol amounts (Arroyo-López et al., 2010a; Oliveira et al., 2014; Pérez-Torrado et al., 2016), and contribute with good aromatic profiles (Díaz-Montaño et al., 2008; Gamero et al., 2011, 2013, 2014; Gonzalez et al., 2007; Lopandic et al., 2007; Stribny et al., 2015). As well as *S. uvarum* and *S. kudriavzevii*, we also included *S. paradoxus*, the closest species to *S. cerevisiae* among those of the *Saccharomyces* genera, which has been already tested for its fermentative capacity as we mentioned above; and *S. eubayanus*, the cryotolerant and recently discovered parental of lager yeast, found in natural fermented beverages from indigenous South American communities (Libkind et al., 2011; Rodríguez et al., 2014), which

makes it a good candidate for screening new properties that might increase the diversity of current commercial wines. Yet despite their potential, these species may have difficulties in competing at the industry level with *S. cerevisiae*, which in most of the cases exhibits better ethanol resistance and the ability to ferment at higher temperatures.

In the present chapter, we analyzed the survival capacity of SNC in competing with *S. cerevisiae* during fermentation at different temperatures to identify those traits that influence their competitive capabilities and to evaluate their industrial potential. Whereas genetic markers are the standard to differentiate *Saccharomyces* strains in a complex culture, a quantitative PCR (QPCR)-based approach was designed to avoid them and their possible effect on gene expression or relative fitness. This approach consists on a relative quantification of the proportion of cells based on the QPCR amplification of a gene with species-specific primers using total DNA isolated from a mix of two strains.

2. Results

2.1. Specificity and sensitivity of the QPCR assay

Six pairs of primers were designed, one for each strain, except for the two *S. uvarum* strains, which share primers. To check for specificity, primers were tested by conventional PCR amplification. Bands of the desired size were observed in all cases. Absence of bands from the PCR reactions of the total DNA isolated from the competitor strain confirmed strain specificity.

To assess the technique's sensitivity for the relative quantification of different yeast strains in co-culture, mixes of cells in known proportions were prepared

for each assayed competition; i.e., our reference *S. cerevisiae* winery strain T73 against *S. kudriavzevii* strain CR85, *S. uvarum* strain BMV58, *S. uvarum* strain CECT12600, *S. paradoxus* strain 54 or *S. eubayanus* strain NPCC1292, and the wild *S. cerevisiae* strain YPS128 against *S. kudriavzevii* strain CR85. The obtained QPCR results about the theoretical proportions can be seen in **Figure I-1**. Data were fitted to linear regression models and coefficients that came very close to the normal for all cases were obtained (**Table I-1**). These results were statistically significant according to the Fisher test (**Table I-1**). Thus the method is suitable for the quantification of the different *Saccharomyces* strains mixed in a culture.

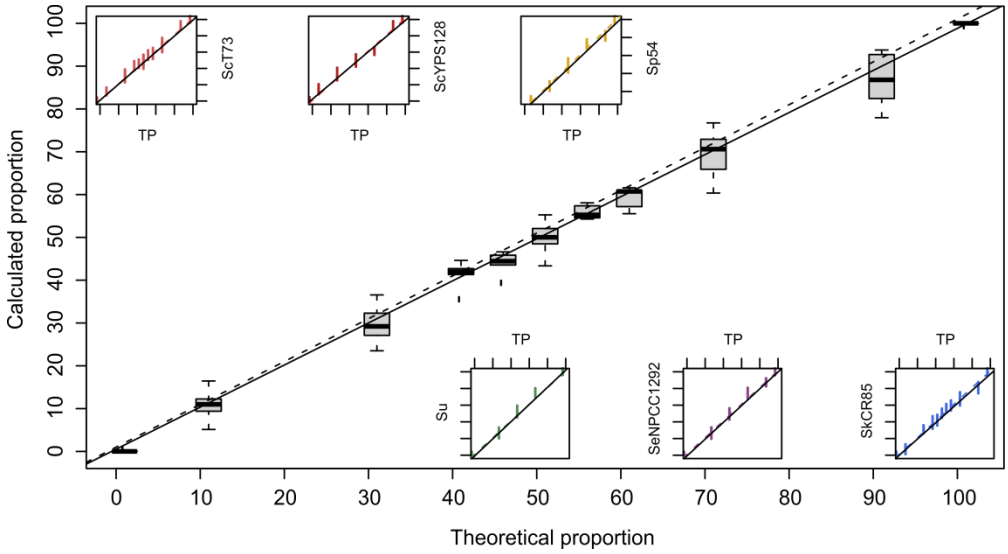


Figure I-1| Calculated relative quantification by QPCR against theoretical values. Boxplot shows the summary of all the data, while small graphics show the dispersion for each specific pair of primers. Data sets were adjusted to a linear model. Dotted lines represent normal distribution and full lines denote adjustments.

Table I-1| Linear model adjustment results for the calculated relative QPCR quantification (*y*) against the theoretical values (*X*).

Pair of primers	A	B	R2	p-value
***S. cerevisiae* T73**	1.0089	-0.5715	0.9924	$< 2.2\times10^{-16}$
***S. paradoxus* 54**	1.0074	1.2536	0.9829	1.473×10^{-15}
***S. eubayanus* NPCC1292**	0.9834	1.4564	0.9905	$< 2.2\times10^{-16}$
***S. uvarum* BMV58/CECT12600**	0.9640	1.2897	0.9924	3.655×10^{-15}
***S. cerevisiae*YPS128**	1.0214	-1.6341	0.9867	$< 2.2\times10^{-16}$
***S. kudriavzevii* CR85**	1.0182	0.5339	0.9905	$< 2.2\times10^{-16}$
All	10060	0.043	0.9889	$< 2.2\times10^{-16}$

A is the regression coefficient and **B** is the error term. p-values were obtained applying a *Fisher* test.

2.2. Yeast competitions

To assess the effect of competition at low temperature on the intrinsic growth rate (*r*) of the SNC species, we performed a series of fermentations conducted by yeast strains *S. paradoxus* 54, *S. uvarum* BMV58, *S. uvarum* CECT12600, *S. kudriavzevii* CR85 and *S. eubayanus* NPCC1292 in competition with wine *S. cerevisiae* strain T73. We also tested the behavior of wild *S. cerevisiae* strain YPS128 in competition with *S. kudriavzevii* CR85. These competition experiments were performed in batch fermentations of synthetic must (SM) at 8 °C, 12 °C and 20 °C. Fermentations at a moderate temperature condition (25 °C) were also performed as a control of *S. cerevisiae's* imposition on cryotolerant yeasts. Monoculture fermentations, inoculated with the same strains, were performed as controls under the same conditions.

Figure I-2 shows the percentages of the strains under competition when fermentation reached the stationary growth phase. These results offer an

overview of the output of competitions during fermentation at different temperatures. We can see that wine *S. cerevisiae* T73 was able to exclude all the other *Saccharomyces* strains during fermentation at 25 °C. At 20 ºC, T73 also outcompeted all the strains, except for wine *S. uvarum* BMV58, which was present in similar percentages. However, at low temperature, 12 ºC, T73 co-existed with *S. eubayanus*, *S. kudriavzevii* and *S. paradoxus*, but was displaced by both the *S. uvarum* strains.

In the competitions between wild strains *S. cerevisiae* YPS128 and *S. kudriavzevii* CR85, YPS128 clearly outcompeted CR85 at 25 ºC, they co-existed at 20 ºC, but CR85 certainly dominated at low temperatures.

The most suitable indicator of these effects is the relative intrinsic growth rates ($R\Delta r$) based on the difference of growth rates when the strain is grown in a mixed culture and in a single culture (**Figure I-3**). It is important to note that there was no significant positive effect on the growth of any strain as a result of the presence of a competitor. For *S. cerevisiae*, these effects were negative at low temperatures, but null or insignificant at high temperatures (**Figure I-3**). For *S. uvarum* and *S. kudriavzevii*, a trend in the opposite direction was noted as the effect was less negative (or insignificant) at low temperatures, and the negative effect increased with temperature (**Figure I-3 C-F**). Finally for *S. paradoxus and S. eubayanus*, the strongest negative effect occurred at medium temperature (20 ºC) (**Figure I-3 A** and **B**).

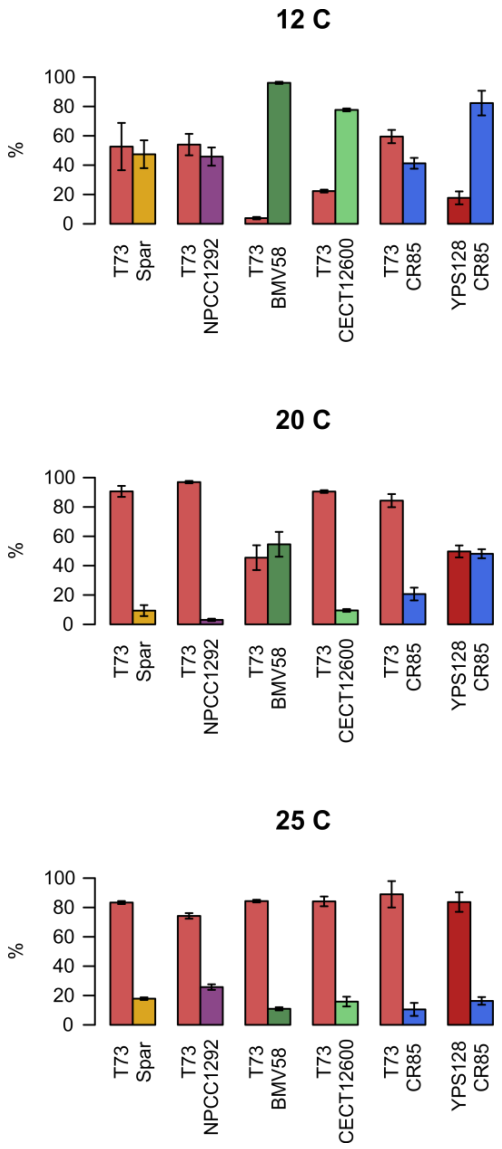


Figure I-2| Presence of both strains from each competition when their highest cell densities were reached. Values are the mean of three replicates. Error bars represent SD.

Despite these results being quite explicative about domination during competition, it is interesting to obtain a quantitative measurement of the effect that presence of a particular yeast can have on its competitor's growth.

The comparison made between the performances of strains of the same species, but with different origins, showed that *S. cerevisiae* T73 and *S. uvarum* BMV58 wine strains were considerably less affected by their competitors than the strains with other origins, such as *S. cerevisiae* YPS128 and *S. uvarum* CECT12600 (**Figure I-3 C-F**).

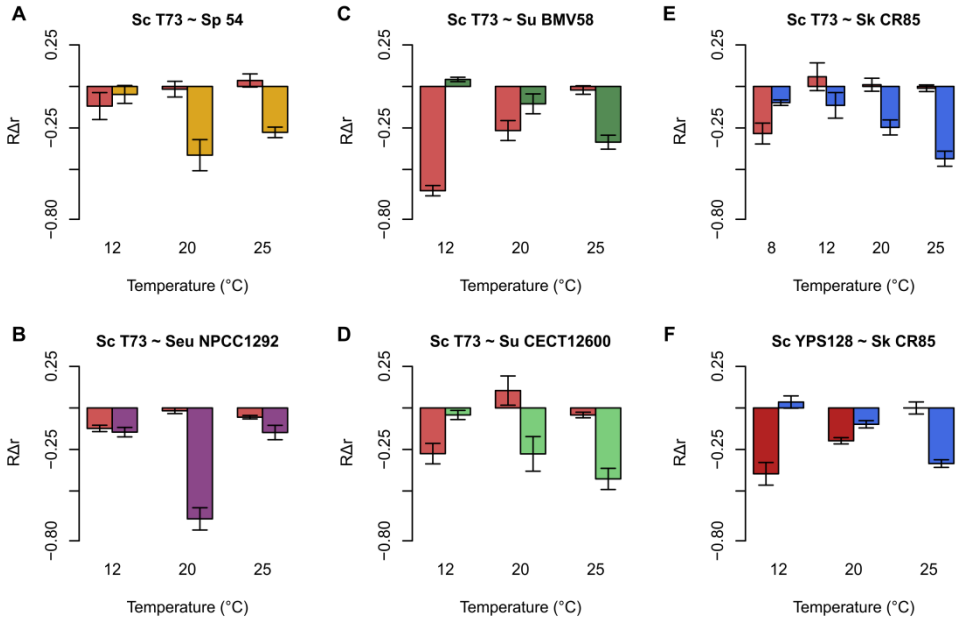


Figure I-3| Relative intrinsic growth rate ($R\Delta r = (r_{competition} - r_{single})/r_{single}$) caused by the effect of competition at different temperatures for each involved strain. Values are the means from triplicate experiments. Error bars represent SD.

Prevalence during fermentation seemed to be clearly related to temperature adaptation. The correlations of growth parameters maximum growth rate and lag phase duration (**Table I-2**) with the relative increment in the intrinsic growth rate were calculated. Positive correlations with $R^2 \sim 0.4$ were obtained for both parameters.

Competitions between *S. cerevisiae* T73 and *S. paradoxus* 54

When competing with *S. paradoxus* strain 54, T73 achieved slightly lower intrinsic growth rate at 12 °C compared to a single fermentation. However, at 20 °C and 25 °C, its growth fitness is maintained (**Figure I-3A**). Strain 54 performed normally at low temperature, but was clearly affected at 20 °C and 25 °C (**Figure I-3A**), and was almost totally excluded from fermentation (**Figure I-2**). Although both species were phylogenetically closely related, the wine *S. cerevisiae* strain seemed superior in this competition. Furthermore, it is interesting to note that at all tested temperatures, the dominant strain T73 had a shorter lag phase (λ, **Table I-2**).

Competitions between *S. cerevisiae* T73 and *S. eubayanus* NPCC1292

In competition both strains maintained their capability to grow in co-cultures at 12 °C and 25 °C, according to the slight drop in their intrinsic growth rate parameter compared to single cultures. Strikingly at intermediate temperatures, NPCC1292 was clearly outcompeted by T73 (**Figure I-3B**), when its lag phase became noticeably longer (**Table I-2**). Although the intrinsic growth rate of NPCC1292 was only slightly affected at 25 °C, this strain was present at a low percentage during fermentation (**Figure I-2**). This can be explained by a low cell density during not only competition, but also during single culture fermentation (data not shown).

Competitions between *S. cerevisiae* T73 and *S. uvarum* strains

Here we assessed the competitive adaptation capacity of a wine and a non-fermentative *S. uvarum*. Wine *S. uvarum* strain BMV58 competed better at low temperatures (12 and 20 ºC), and severely affected T73 growth. This effect reverted as temperature rose. We can see that T73 shows a clear advantage at 25 °C (**Figure I-3C**).

To test whether the same trend could be observed with a non-wine strain, we performed the same experiment using strain *S. uvarum* CECT12600. The behavior of the differential intrinsic growth rates was similar, but in this case *S. uvarum* CECT12600 obtained lower values and had a less intense effect on T73 (**Figure I-3D**) than BMV58, which showed better competitive fitness in fermentative environments.

Finally, it is important to remark that *S. cerevisiae* T73 had a shorter lag phase (λ) than *S. uvarum* BMV58 during the competitions at 20 °C and 25 °C (**Table I-2**), but at similar maximum growth rates (μ_{max}), and BMV58 was able to co-exist with T73 during the competition at 20 °C, but not at 25 ºC (**Figure I-3**). At 12 °C, both *S. uvarum* strains had a shorter lag phase and higher maximum growth rates than *S. cerevisiae* (**Table I-2**), and were dominant during fermentation (**Figure I-2**).

Competitions between *S. kudriavzevii* CR85 and *S. cerevisiae* strains

Wine strain *S. cerevisiae* T73 is not affected by most temperature conditions when competing with *S. kudriavzevii*. However, at 8º C, a clear negative effect on the relative intrinsic growth rate (*r*) on T73 can be observed. *S. kudriavzevii* CR85 was always affected by presence of T73, although its impact was softer at 8 ºC and *S. kudriavzevii* became more competitive (**Figure I-3E**).

To test if T73 resistance during competition, even at a very low temperature, was to some extent dependent on its better adaptation to fermentation environments, the wild *S. cerevisiae* strain YPS128, isolated from an oak bark, was used in the competitions assays with *S. kudriavzevii*. **Figure I-3F** shows noticeable differences in the competition at 12 °C, where CR85 clearly

outcompetes YPS128, and it exhibited an intrinsic growth rate that was markedly affected. In the competitions at 20 °C, in which *S. kudriavzevii* predominated (**Figure I-2**), YPS128 underwent a greater negative effect (**Figure I-3**). Contrarily at 25 °C, CR85 was clear at a disadvantage (**Figure I-3F**). Therefore, the fermentative origin of the *S. cerevisiae* yeasts seems to correlate with better performance in fermentation.

The growth parameters from **Table I-2** could explain most of these results. At 8 °C, when *S. Kudriavzevii* outcompeted *S. cerevisiae* T73, the winner (**Figure I-2**) had a higher maximum growth rate and a shorter lag phase. At 12 °C, *S. kudriavzevii* presented a shorter lag phase than both the *S. cerevisiae* strains, and a higher maximum growth rate than the wild *S. cerevisiae* strain, which was clearly affected under these conditions (**Figure I-3F**). *S. cerevisiae* wine strain T73 had a clearly higher μ at 12 ºC, which could be the reason why T73 became dominant as fermentation continued (**Figure I-2**). At 20 °C, both the *S. cerevisiae* strains already exhibited better growth capabilities in synthetic must (**Table I-2**), but there were clear differences in their performance during the competition against *S. kudriavzevii* as the wine strain was a much better competitor than the wild strain (**Figure I-2 and Figure I-3E-F**).

Correlation between growth parameters and competitive advantage

We assessed whether there was any correlation between the fact of having better growth parameters in single culture and the imposition during our competition experiments (**Table I-2**). Linear correlations between $R\Delta r$ and $R\Delta\mu$ or $R\Delta\lambda$ were obtained, with R^2 values of 0.40 and 0.42, respectively. Significance was tested by the Fisher's test, and the resulting p-values were 0.005158 and 0.003681, respectively.

A graphical summary of the three parameters used in the analysis for each competition is depicted in **Figure I-4**. In most cases, low $R\Delta r$ values corresponded to low $R\Delta\mu$ and $R\Delta\lambda$ values. This indicates that a more affected strain during co-fermentations exhibits worse growth parameters in single culture than its competitor (**Figure I-3**, **Table I-2**).

There are some exceptions however, as already mentioned above, such as the competition between T73 and BMV58 at 20 °C, at which *S. uvarum* had a similar $R\Delta\mu$ and a notably worse $R\Delta\lambda$ value, but competition had a remarkably negative effect on *S. cerevisiae*. Interestingly, **Figure I-4** shows comparatively slight $R\Delta\mu$ or $R\Delta\lambda$ differences together with extreme $R\Delta r$ values (T73-NPCC1292, 20 °C), and vice versa (T73-NPCC1292, 25 °C). This indicates that co-culture fermentations may be influenced by other competitive growth strategies.

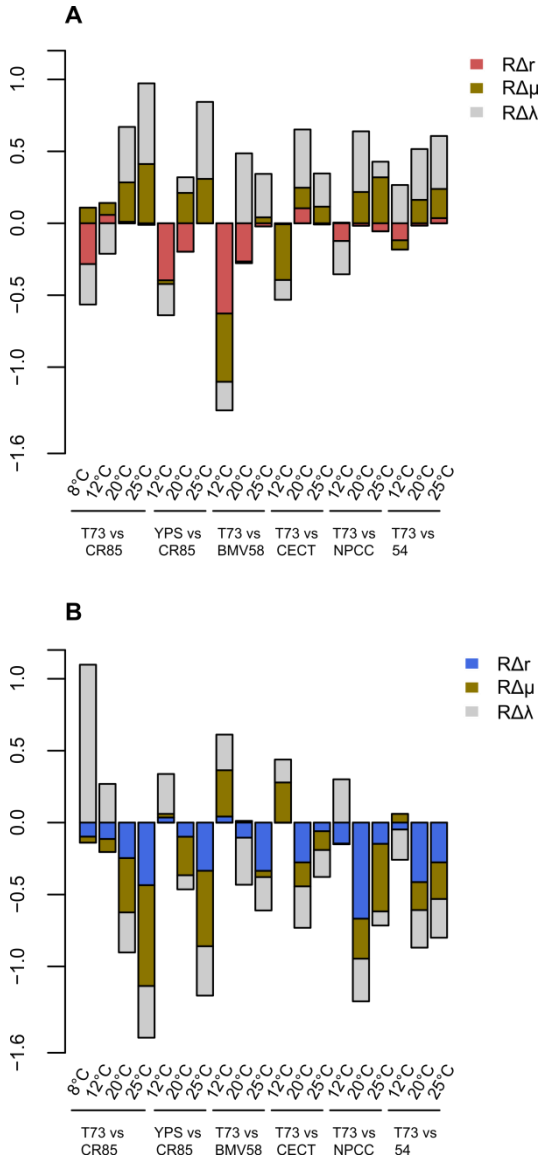


Figure I-5| Comparative of performance in competition and growth kinetics parameters in single culture for *S. cerevisiae* (A) and competitor strains (B).

Table I-2| Growth parameters obtained by the Gompertz equation proposed by Zwietering *et al.* (1990) for the different strains in SM at different temperatures

	8 °C		12 °C		20 °C		25 °C	
Strain	**μ_{max} (h^{-1})**	**λ (h)**	**μ_{max} (h^{-1})**	**λ (h)**	**μ_{max} (h^{-1})**	**λ (h)**	**μ_{max} (h^{-1})**	**λ (h)**
***S. cerevisiae* T73**	0.0083±0.0002 ^{c}	247.77±9.1^{e}	0.0217±0.0005^{b}	82.5652±0.9^{c}	0.0602±0.002^{a}	18.6771±0.4^{a}	0.1740±0.006^{a}	9.3204±0.6^{a}
***S. paradoxus* 54**	0.0080 ±0.0003$^{b.c}$	260.69±9.1^{f}	0.0231±0.001^{c}	104.5469±2.4^{a}	0.0504±0.003^{c}	25.2771±1.0^{c}	0.1387±0.002^{c}	12.7527±1.5^{d}
***S. eubayanus* NPCC1292**	0.0098±0.0012^{d}	143.58±2.5^{a}	0.0216±0.0006^{b}	63.4613±2.1^{e}	0.0471±0.002^{d}	26.5521±0.4^{d}	0.1184±0.002^{d}	10.3316±0.2$^{a.b}$
***S. uvarum* BMV58**	0.0181±0.0009^{f}	147.09±4.1^{a}	0.0320 ±0.0008^{e}	66.1784 ±1.1^{d}	0.0609±0.002^{a}	27.7608±0.4^{e}	0.1668±0.021$^{a.b}$	12.1358±1.0$^{c.d}$
***S. uvarum* CECT12600**	0.0153±0.0005^{e}	160.02±1.8^{b}	0.0301 ±0.0006^{d}	71.2131 ±2.0^{b}	0.0516±0.0009^{c}	26.2352±0.5^{d}	0.1539±0.005^{b}	11.4711±0.3$^{b.c}$
***S. cerevisiae* YPS128**	0.0067±0.0003^{a}	220.15±7.4^{d}	0.0194±0.0003^{a}	83.1130±2.2^{c}	0.0554±0.001^{b}	23.3442±0.5^{b}	0.1560±0.004^{b}	9.5580±0.7^{a}
***S. kudriavzevii* CR85**	0.0074±0.0002$^{a. b}$	178.10±4.7^{c}	0.0199 ± 0.0005^{a}	65.0655 ±2.1$^{d.e}$	0.0437±0.002^{e}	25.8793±1.0$^{c.d}$	0.1023±0.007^{e}	14.5462±0.6^{e}

μ_{max} is maximum growth rate and λ is lag phase duration. Values are given as mean±standard deviation. The values followed by different superindexes in the same column are significantly different according to the Tukey HSD test ($\alpha = 0.05$, n = 10).

2.3. Influence of competition on fermentation parameters

Yeast characterization as wine fermenters must include aspects like the ability to consume all the sugars present in must at a suitable pace, or the capability to produce a wine with high quality standards according to consumer demands. **Table I-3** includes different fermentation kinetic parameters: maximum sugar consumption rate (*m*) and fermentation lag phase (*l*), inferred from mass loss during fermentation, as well as the time taken to consume 90% of the initial sugar content (t_{90}). Final product composition is also a key factor. Thus, we measured glucose, fructose, glycerol and ethanol concentrations at the end of fermentation, that is, when no mass loss was observed (**Table I-4**). This data set is useful to determine the mixed yeast cultures that could potentially improve some wine characteristics.

Reference wine strain *S. cerevisiae* T73 is characterized by the production of relatively low glycerol values (5-6 g L^{-1}) and high ethanol content (> 12 %), as observed in **Table I-4**. It also accomplishes quite a high sugar consumption rate at 20 °C (**Table I-3**) and 25 °C (**Table I-3**), but a low one at 12 °C (**Table I-3**), which is consistent with the temperature adaptation of *S. cerevisiae* to grow at higher temperatures than cryotolerant species *S. uvarum* and *S. kudriavzevii*. In most cases, and according to the ANOVA analysis, its *l* and t_{90} belong to the group of the shortest times (**Table I-3**).

Interestingly, some co-cultures improved these fermentation parameters; e.g., T73 with either *S. kudriavzevii* CR85 or *S. uvarum* BMV58 at 12 °C increased the *m*, and reduced t_{90} (**Table I-3**). At 25 °C, the combinations of T73 with *S. kudriavzevii* and *S. uvarum* once again seemed to improve the fermentation

kinetics. A reduction of t_{90} for the three co-cultures (T73-CR85, T73-BMV58 and T73-CECT12600) was also observed at 25 °C (**Table I-3**). These fermentation parameters improved compared to their respective single culture fermentations (**Table I-3**), which is indicative of synergic interactions.

Unlike the 12 °C and 25 °C conditions, practically no fermentation parameters or compounds improved at 20 °C (**Tables 3 and 4**). The competitions against *S. paradoxus* seemed disadvantageous at 20 °C and 25 °C, which also occurred when competing with CR85 at 20 °C and with NPCC1292 at 25 °C (**Table I-3**).

Despite their diverse origins, all the strains were able to complete their fermentations at 25 ºC except *S. eubayanus* NPCC1292 (**Table I-4**). By the end of the fermentations conducted by this strain, the final product contained large amounts of glucose, and especially fructose. At low and medium temperatures (12 °C and 20 °C) some strains also left a considerable amount of sugars, such as *S. cerevisiae* YPS128 or competences NPCC1292-T73, CECT12600-T73, 54-T73 and CR85-YPS128 (**Table I-4**). Interestingly, most of them were able to ferment all the sugars when cultured alone (**Table I-4**), so this could result in an antagonist effect for these pairs of strains. Moreover, some other parameters also reflected worse performances during co-fermentations, specifically t_{90} of T73-NPCC1292 at 12 °C, or T73-54 and T73-CECT12600 at 20 °C (**Table I-3**).

As previously mentioned, a more profitable interaction is observed for CR85-T73 at low temperatures. At 12 °C both strains co-existed during fermentation in similar proportions (**Figure I-2**), which led to a final product with a lower

ethanol concentration and a higher glycerol content than those obtained for the fermentations conducted by T73 alone (**Table I-4**). Ethanol concentrations also lowered during co-inoculated fermentations at 20 °C and at 25 °C, but the conservative ANOVA test did not support the significance of these differences (**Table I-4**). With the co-cultures of T73 with *S. uvarum*, no significant improvements in the final product composition were observed, although mean glycerol values and ethanol concentrations showed a positive tendency compared to the single *S. cerevisiae* fermentations at 12 °C and 20 °C (**Table I-4**).

Table I-3: Kinetics parameters of fermentations

	12 °C			20 °C			25 °C		
Fermentation	**m (g L^{-1} h^{-1})**	**l (h)**	**t_{90} (h)**	**m (g L^{-1} h^{-1})**	**l (h)**	**t_{90} (h)**	**m (g L^{-1} h^{-1})**	**l (h)**	**t_{90} (h)**
T73	**0.31±0.01^{a,b}**	**91.71±1.10^{a,b,c}**	**472.92±5.36^{a,b,c}**	**0.90±0.06^{d,e}**	**26.70±1.24^{a,b}**	**171.24±10.73^{a,b}**	**1.14±0.17^{b,c,d}**	**6.42±1.69^{b,c}**	**149.22±17.43^{b,c}**
54	0.28±0.01^{a,b}	131.64±2.41^{e}	589.40±7.35^{c,d}	0.82±0.05^{b,c,d,e}	33.96±3.05^{c,d}	181.52±8.54^{a,b}	1.51±0.33^{d,e}	9.37±2.47^{b,c,d}	122.13±12.43^{a,b}
T73-54	0.31±0.00^{a}	97.06±3.10^{b,c,d}	583.58±8.43^{c,d}	0.86±0.16$^{b.c.d,e}$	31.38±4.25$^{b.c}$	247.05±28.78^{c}	0.87±0.01$^{a.b}$	12.71±0.51^{d,e,f}	171.69±10.93^{c}
NPCC1292	0.21±0.00^{a}	76.27±6.64$^{a.b}$	Na	0.59±0.01$^{a.b}$	30.14±0.89$^{a.b.c}$	Na	0.65±0.08^{a}	23.46±1.59^{h}	Na
T73-NPCC1292	0.27±0.01^{a}	79.47±5.87$^{a.b}$	656.23±103.49^{d}	1.01±0.08^{e}	27.87±0.99^{a,b,c}	198.77±6.62^{b}	0.91±0.10$^{a.b}$	17.23±1.67^{g}	162.83±14.64^{c}
BMV58	0.30±0.01$^{a.b}$	85.12±1.58$^{a.b.c}$	505.21±8.14^{b,c}	0.71±0.04$^{a.b.c,d}$	85.72±1.50^{e}	260.97±9.17^{c}	0.89±0.02$^{a.b,c}$	2.09±0.72^{a}	179.49±4.74^{c}
T73-BMV58	0.49±0.01$^{c.d}$	71.20±2.32^{a}	320.76±6.27^{a}	0.63±0.03$^{a.b.c}$	84.49±1.58^{e}	283.37±17.12^{c,d}	1.47±0.14^{d,e}	15.86±0.46^{f,g}	104.81±7.54^{a}
CECT12600	0.50±0.01^{d}	92.59±3.77$^{a.b.c.d}$	383.64±26.29^{a,b}	1.00±0.15^{e}	31.43±2.40$^{b.c}$	172.47±23.94^{a,b}	1.68±0.03^{e}	21.79±0.16^{h}	100.45±2.40^{a}
T73-CECT12600	0.38±0.01$^{b.c}$	80.79±6.18$^{a.b}$	421.02±18.23^{a,b}	1.00±0.06^{e}	23.18±2.15^{a}	146.04±7.17^{a}	1.72±0.23^{e}	15.71±0.63^{e,f,g}	99.89±17.82^{a}
YPS128	0.27±0.01$^{a.b}$	97.84±9.08$^{b.c.d}$	727.23±5.36^{d}	0.88±0.03$^{c.d,e}$	31.39±0.62$^{b.c}$	172.66±4.49^{a,b}	1.37±0.03$^{c.d,e}$	11.98±0.40^{d,e}	116.24±2.03^{a,b}
YPS128-CR85	0.31±0.01$^{a.b}$	96.23±4.05$^{b.c.d}$	489.54±6.61^{b,c}	0.88±0.04$^{c.d,e}$	27.71±1.88$^{a.b.c}$	321.89±0.50^{d}	1.43±0.07^{d,e}	10.37±0.77^{c,d}	111.70±8.21^{a,b}
CR85	0.32±0.01$^{a.b}$	116.35±2.49$^{d.e}$	502.88±7.59^{b,c}	1.02±0.00^{e}	38.50±1.16^{d}	156.49±0.87^{a,b}	0.87±0.07^{a,b}	15.68±0.62$^{e.f,g}$	184.99±13.71^{b}
T73-CR85	0.52±0.14^{d}	103.32±19.91$^{c.d}$	382.17±26.05^{a,b}	0.54±0.07^{a}	24.15±1.86^{a}	259.85±19.33^{c}	1.65±0.11^{e}	6.17±0.78^{b}	98.44±9.57^{a}

m is the maximum sugar consumption rate, **l** is the fermentation lag phase duration, and **t_{90}** is the time employed to consume 90% of the sugars present in the initial must. Parameters are obtained through an adjustment to Gompertz (Zwietering et al., 1990). Values given as mean±standard deviation of three biological replicates. An ANOVA analysis was carried out. The values followed by different superindexes in the same column are significantly different according to the Tukey HSD test ($\alpha = 0.05$).

Table I-4: Chemical composition of the fermented SM obtained through HPLC

	12 °C				20 °C				25 °C			
Fermentation	**glucose**	**fructose**	**glycerol**	**ethanol**	**glucose**	**fructose**	**glycerol**	**ethanol**	**glucose**	**fructose**	**glycerol**	**ethanol**
T73	**0.10±0.04^{a,b}**	**6.64±1.04^{a,b,c,d}**	**5.09±0.03^{a}**	**12.49±0.28^{c,d}**	**0.02±0.01^{a}**	**4.16±0.60^{a,b,c}**	**5.53±0.24^{a,b,c}**	**12.47±0.48^{d,e}**	**0±0^{a,b}**	**0±0^{a}**	**5.78±0.08^{a,b}**	**12.67±0.10^{c,d,e}**
54	0±0^{a,b}	2.90±0.33^{a,b}	5.03±0.04^{a}	11.83±0.10^{b,c,d}	0.02±0.02^{a}	4.06±2.11^{a,b,c}	6.08±0,29^{b,c,d}	11.21±0.34^{b,c,d}	0.21±0.02^{a,b}	8.91±0.31^{c}	7.57±0.25^{e}	10.76±0.04^{a,b}
T73-54	0.51±0.10^{a,b}	14.88±0.70^{e}	5.06±0.12^{a}	12.09±0.28^{b,c,d}	0±0^{a}	4.30±0.41^{a,b,c}	5.95±0.37^{b,c,d}	13.19±0.44^{e,f}	0.28±0.06^{a,b}	8.5±1.94^{b,c}	5.92±0.12^{a,b,c}	12.25±0.40^{b,c,d,e}
NPCC1292	6.66±0.80^{c}	41.05±0.50^{f}	7.68±0.40^{d}	9.69±0.54^{a}	2.25±0.24^{b}	24,54±2,45^{e}	6.66±0.08^{d,e}	9.89±0.01^{a,b}	2.73±1.20^{c}	31.47±5.03^{e}	7.46±0.16^{e}	10.14±0.23^{a}
T73-NPCC1292	0.69±0.58^{b}	13.45±5.03^{d,e}	5.95±0.64^{a,b}	11.40±0.70^{b,c,d}	0.35±0.34^{a}	9.31±3.09^{c,d}	6.76±0.27^{d,e}	13.97±0.58^{f}	0±0^{a}	0.56±0.80^{a}	6.14±0.09^{a,b,c}	12.70±0.18^{e}
BMV58	0±0^{a,b}	0.70±0.62^{a}	5.15±0.17^{a}	11.71±0.46^{b,c,d}	0±0^{a}	2.93±2.55^{a,b}	4.72±0.14^{a}	9.51±0.16^{a}	0±0^{a,b}	4.15±1.36^{a,b,c}	7.13±0.20^{d,e}	12.27±0.08^{b,c,d,e}
T73-BMV58	0±0^{a,b}	4.91±1.34^{a,b}	5.85±0.05^{a,b}	11.83±0.16^{b,c,d}	0±0^{a}	0±0^{a}	5.50±0.01^{a,b}	10.97±0.74^{a,b,c,d}	0±0^{a}	3.59±1.45^{a}	5.95±0.14^{a,b}	12.64±0.09^{c,e}
CECT12600	0.12±0.12^{a,b}	9.80±2.94^{b,c,d,e}	6.05±0.08^{a,b,c}	11.26±0.38^{a,b,c,d}	0±0^{a}	0±0^{a}	5.85±0.08^{b,c,d}	12.27±0.07^{d,e}	0±0^{a,b}	3.11±0.53^{a}	6.31±0.31^{b,c,d}	11.49±0.45^{a,b,c,d,e}
T73-CECT12600	0±0^{a}	3.92±1.46^{a,b}	6.00±0.42^{a,b}	12.44±0.70^{c}	0.59±0.23^{a}	12.76±2.45^{d}	5.31±0.01^{a,b}	11.51±0.57^{c,d}	0±0^{a,b}	1.13±1.13^{a}	5.46±0.36^{a,b}	11.11±0.24^{a,b,d}
YPS128	0.26±0.09^{a,b}	8.35±1.08^{a,b,c,d,e}	6.88±0.57^{b,c,d}	10.87±0.31^{b,c,d}	0.26±0.07^{a}	8.35±1.08^{b,c,d}	6.88±0.57^{d,e}	12.03±0.10^{c,d,e}	0.01±0.01^{a,b}	3.52±0.36^{a}	6.06±0.15^{a,b,c}	10.78±0.94^{a,b}
YPS128-CR85	0±0^{a,b}	3.13±0.28^{a,b}	7.07±0.07^{b,c,d}	11.76±0.74^{b,c,d}	0.56±0.36^{a}	11.31±4.53^{d}	6.63±0.29^{c,d,e}	11.78±0.24^{c,d,e}	0.14±0.11^{ab}	4.31±1.87^{a,b,c}	6.17±0.90^{a,b,c}	10.35±0.77^{a}
CR85	0.0±0^{a,b}	4.89±2.81^{c,d,e}	7.34±0.23^{c,d}	10.87±0.46^{a,b,d}	0.01±0.01^{a}	4.13±0.31^{a,b,c}	7.61±0.94^{e}	10.54±1.25^{a,b,c}	0.85±0.14^{b}	16.35±0.90^{d}	6.93±0.51^{c,d,e}	10.46±0.46^{a}
T73-CR85	0.12±0.17^{a,b}	6.42±5.37^{a,b,c}	7.24±1.32^{c,d}	10.36±1.41^{a,b}	0.05±0.75^{a}	2.73±3.11^{a,b}	5.22±0.51^{a,b}	11.44±0.26^{c,d}	0.03±0.04^{a,b}	3.95±1.28^{a,b}	5.35±0.24^{a}	11.17±0.72^{a,b,c,d}

Glucose, fructose and glycerol are given in g L^{-1}, and ethanol in %. Values are given as mean±standard deviation of three biological replicates and two HPLC detection runs. An ANOVA analysis was carried out. The values followed by different superindexes in the same column are significantly different according to the Tukey HSD test ($\alpha = 0.05$).

3. Discussion

Accurate quantification of different *Saccharomyces* yeasts in co-cultures

Natural auxotrophic or drug-resistant mutants and strains genetically modified with reporter genes have been used to monitor yeast competences in co-cultures or during fermentation, which involves demanding tasks such as mutant selection or construction, CFU enumeration in selective media, or flow cytometry (Arroyo-López et al., 2011; García-Ríos et al., 2014). Our results indicate that a more straightforward QPCR-based method, which does not require previous cell type separation, is suitable for the relative quantification of yeasts. In fact quantification by QPCR of different organisms in wine, including *Saccharomyces* yeasts, has already been applied (Andorrà et al., 2010; Neeley et al., 2005; Vendrame et al., 2014). However, to our knowledge, this technique has never been used to date to differentiate *Saccharomyces* yeasts during competition in the same environment. This novel approach can be applicable to broaden our knowledge about the ecology of the *Saccharomyces* yeast when competing for the same niche.

Temperature adaptation affects domination in a fermentative environment

Cryotolerant species *S. kudriavzevii* and *S. uvarum* have been used in this work given the trend to perform fermentation at lower temperatures in the wine industry to preserve the aroma fraction (Beltran et al., 2002; Gamero et al., 2013; Şener and Yildirim, 2013; Torija et al., 2001). Our results clearly show a longer prevalence of these species in fermentations at low temperatures. However at 25º C, *S. cerevisiae* outgrow them. It can also be

drawn from our data that this effect is modulated by the adaptation of strains to different habitats, where wine strains are always more competitive no matter what the temperature. This was observed not only for *S. cerevisiae*, but also for *S. uvarum*. Therefore, adaptation to high sugar environments could be another trait that influences fermentation domination as indicated by Barrajón et. al (2011).

Salvadó et. al (2011b) analyzed the thermotolerance of different *Saccharomyces* species using their growth kinetics parameters as measurable indicators. Growth ability under settled conditions could be considered as a suitable predictor for the imposition of one strain on another in competition. However, previous works (García-Ríos et al., 2014), as well as ours, have revealed that domination of environments is a more complex trait in *Saccharomyces* yeasts. We showed that in most cases a higher μ_{max} and a lower λ correlate to greater invulnerability in co-fermentation than the competitor strain. Nevertheless, we observed that the intensity of the effect is widely variable, and in some cases we found that the contrary happens; i.e., *S. eubayanus* NPCC1292 in the competition against *S. cerevisiae* T73 at 12 °C, whose growth was affected despite having a shorter lag phase. *S. cerevisiae* YPS128, a strain isolated from oak trees, performed worse than expected against *S. kudriavzevii* CR85 at 20 °C. Something similar occurred with the competition between *S. cerevisiae* T73 and *S. uvarum* BMV58 for both wine strains at 20 °C: T73 had a noticeably shorter λ and a similar μ_{max}, but was clearly weakened by BMV58. Thus it is conceivable that an interaction among yeasts or their side products takes place as part of the

competition mechanism. The nature of this mechanism is something that we loosely look into in **Chapter III** of the present thesis.

Coinoculation of *Saccharomyces* yeasts can be potentially beneficial for fermentations and final product composition

One of the main goals when studying alternative organisms for their use in food fermentation is achieving new characteristics of interest, that these organisms give rise to. With wine, numerous studies have been carried out with non-*Saccharomyces* yeasts, and most have focused on improving or enriching of aroma profiles, whereas others have focused more on controlling the final product concentration of specific compounds, such as ethanol or acetic acid (Andorrà et al., 2012; Canonico et al., 2015, 2016; Contreras et al., 2014; Curiel et al., 2017; Izquierdo Cañas et al., 2014; Medina et al., 2013; Morales et al., 2015; Rantsiou et al., 2012; Rodrigues et al., 2016; Varela et al., 2017; Zara et al., 2014). However, fewer studies have been published about fermentation characterization by combining *Saccharomyces* strains or using uncommon *Saccharomyces* species (Arroyo-López et al., 2011; Barrajón et al., 2011; Cheraiti et al., 2005; Gustafsson et al., 2016; Howell et al., 2006; King et al., 2010; Saberi et al., 2012; Varela et al., 2016; Williams et al., 2015). Just as some of these investigations have suggested, our results showed that the final product composition of co-fermented musts cannot always be predicted from those of mono-fermentations. We observed a range of different scenarios: synergic or antagonist effects, as well as simply additive, depending on the strains and the assayed conditions. Nevertheless, we found some promising combinations of a wine *S. cerevisiae* strain with a SNC one; e.g., the remarkable case of the co-inoculation of *S. cerevisiae* T73

and *S. kudriavzevii* CR85 at low temperatures, which improved the efficiency of the process as regards single inoculations by increasing the maximum sugar consumption rate, and which also yielded a final product that contained less ethanol and more glycerol.

From the kinetics point of view, the co-fermentations of our wine *S. cerevisiae* strain with *S. uvarum* also revealed a positive effect, which was more visible at 12 °C and 25 °C. This kind of synergic effect has been observed in previous works, where the addition of fructophilic yeast *S. bombicola* (similarly to *S. uvarum*) led to faster fructose and glucose consumption (Milanovic et al., 2012). In our case it was also noteworthy that the viability of T73 during competition against wine strain BMV58 was negatively influenced. However against the non-winery strain CECT12600, it did not diminish, which means that winery strains could be more capable of sensing other yeasts in fermentative media and over-activate sugar consumption to take advantage of them. This hypothesis would also be supported by our results about the maximum sugar consumption rate (*m*) of the co-fermentations carried out by our reference wine strains *S. cerevisiae* T73 with *S. uvarum* or *S. kudriavzevii*. At any rate it is remarkable that reduced t_{90} values occurred during the fermentations run at 25 ºC for the cases of T73-CR85, T73-BMV58 and T73-CECT12600, when SNC strains were present in small proportions. This could be indicating that *S. cerevisiae* T73 responded to interactions during competences by increasing its metabolism.

To summarize, we have confirmed the great capacity of *S. cerevisiae* to dominate fermentative environments at traditional process temperatures (Arroyo-López et al., 2011; Holm Hansen et al., 2001; Pérez-Nevado et al.,

2006; Williams et al., 2015). However, some cryotolerant *Saccharomyces* yeasts, particularly *S. uvarum*, can seriously compromise *S. cerevisiae* fitness during competences at lower temperatures, which explains why *S. uvarum* can replace *S. cerevisiae* during wine fermentations in European regions with oceanic and continental climates (Demuyter et al., 2004; Naumov et al., 2000b, 2002; Redžepović et al., 2002; Rementeria, 2003; Sipiczki et al., 2001). From a biotechnological point of view, the application of cryotolerant *Saccharomyces* species as starters for wine fermentation at low temperature could avoid its colonization by undesirable microorganisms that has been reported by other authors (Ciani and Comitini, 2006).

Our results also suggest that adaptation to winemaking establishes noticeable differences in the performance of *Saccharomyces* yeasts when competing during wine fermentation. Thus profounder research on *Saccharomyces* yeasts' physical and biochemical interactions is necessary to optimize the composition of such starter cultures, which would make them even more interesting for industrial purposes. As a hint, at low temperatures we obtained improvements in the final composition of important compounds, such as higher glycerol contents and a lower ethanol yield, as well as the better fermentation performance of some yeast combinations, especially those of the *S. cerevisiae* with cryotolerant SNC species.

CHAPTER II

The Use of Mixed Populations of *Saccharomyces cerevisiae* and *S. kudriavzevii* to Reduce Ethanol Content in Wine: Limited Aeration, Inoculum Proportions and Sequential Inoculation

1. Introduction

Wine composition is the product of complex interactions among yeast and bacteria that take place in vineyards and wineries, although one yeast species, *Saccharomyces cerevisiae*, is generally the main microorganism responsible for winemaking process (Pretorius, 2000). Its vigorous fermentative capacity, even in the presence of oxygen (Crabtree effect), makes *S. cerevisiae* a very efficient ethanol producer, strategy that allows its imposition over the rest of the microbiota during fermentation due to the toxicity of this compound (Piškur et al., 2006; Thomson et al., 2005).

However, this high ethanol production capability may be disadvantageous taking into account the challenges currently faced by the wine industry. In the first place, global warming provokes a gap during grape ripening between phenolic maturity and sugar content. If grapes are harvested when the sugar content is appropriate but the phenolic maturity has not been reached, wines can show altered aroma, flavor, mouth feel, and astringency. On the contrary, if grapes are harvested when their phenolic maturity is the appropriate, their sugar contents are higher, giving rise to wines with increasing ethanol concentrations (Jones et al., 2005). This higher ethanol content is undesirable according to consumers' new demands, because affects flavor complexity sensing (Goldner et al., 2009), and its excessive consumption is harmful for health and road safety.

A variety of measures are taken at the different winemaking stages to overcome the problem of the higher ethanol levels in wines. These include new agronomical methods for grape cultivation (Intrigliolo and Castel, 2009),

the use of mixed musts from grapes at different ripening stages (Kontoudakis et al., 2011), the use of engineered yeasts producing lower ethanol yields (Varela et al., 2012), or the partial dealcoholisation of wines by chemical or physical procedures (Belisario-Sánchez et al., 2012; Diban et al., 2008; Gómez-Plaza et al., 1999; Hernández et al., 2010; Offeman et al., 2010; Pilipovik and Riverol, 2005). However, some of these approaches have little impact on ethanol contents, negatively affect the quality of wine, are highly expensive industrial processes, or contravene the current regulations about the use of GMO.

In addition, a wide range of different biological strategies have been proposed to reduce alcohol contents in wines (Kutyna et al., 2010). The use of non-conventional yeast strains in winemaking stands out for its potential. Several non-*Saccharomyces* yeasts, usually in combination with *S. cerevisiae*, have been tested to reduce ethanol yields during wine fermentation (Ciani et al., 2016b; Comitini et al., 2011; Contreras et al., 2014, 2015; Quirós et al., 2014; Sadoudi et al., 2012). Different strategies have been carried out to improve the fermentation performance of these non-*Saccharomyces* yeasts, such as sequential inoculation or co-inoculation at increased proportions with *S. cerevisiae*, to provide new characteristics to the final wines (Andorrà et al., 2012; Canonico et al., 2016; Gobbi et al., 2013; Izquierdo Cañas et al., 2014; Jolly et al., 2014; Loira et al., 2014). Another approach to reduce alcohol content in wines is the supply of oxygen to the fermenters, under a controlled flowrate, to promote the respiratory consumption of sugars by these non-*Saccharomyces* yeasts (Gonzalez et al., 2013; Rodrigues et al., 2016). However, temperature under industrial winemaking conditions is generally

close to 25 °C, which does not allow for any of these alternative yeasts to survive the first hours of the process (Nissen and Arneborg, 2003; Pérez-Nevado et al., 2006; Torija, 2003; Williams et al., 2015)

Alternative *Saccharomyces* yeasts, such as *S. kudriavzevii* or *S. uvarum*, can help to solve some of the new challenges of the wine industry. These species exhibit physiological properties that are especially relevant during the winemaking process, such as their good fermentative capabilities at low temperatures, resulting in wines with lower alcohol and higher glycerol amounts (Pérez-Torrado et al., 2017a; Varela et al., 2016). In the case of *S. kudriavzevii*, this species displays a different metabolic regulation concerning ethanol and glycerol syntheses (Arroyo-López et al., 2010a; Oliveira et al., 2014; Pérez-Torrado et al., 2016). Regarding its implantation in fermentation, temperature appears as an important factor to determine the preponderance of *S. cerevisiae* during the process as described previously by several authors (Arroyo-López et al., 2011; Nissen and Arneborg, 2003; Pérez-Nevado et al., 2006; Salvadó et al., 2011a; Torija, 2003; Williams et al., 2015).

However, none of the techniques used to favor the growth of non-*Saccharomyces* yeasts, such as co-inoculation, sequential inoculation or microoxigenation, have been applied to the species *Saccharomyces kudriavzevii* to favor their presence during wine fermentation. In this chapter, we first analyzed the presence of *S. kudriavzevii* during co-fermentation with a *S. cerevisiae* wine strain under different aeration conditions to select the most suitable one. Next, we studied the effect of *S. kudriavzevii* enrichment in the inoculum with and without external oxygen supply, and finally the effect of sequential inoculation of the strains.

2. Results

2.1. Determining the Air Flow Conditions Favoring *S. kudriavzevii* Presence in Mixed Fermentations with *S. cerevisiae*

A controlled aeration system feeding a set of fermentations co-inoculated with *S. cerevisiae* and *S. kudriavzevii* in a ratio 1:1 with 4 different air flow rates: 1, 5, 10, and 20 VVH (10 gas volumes/culture volume/hour) was installed. **Figure II-1** shows a clear disadvantage of *S. kudriavzevii* even in the presence of an external oxygen input. However, air flow rate seems to have an influence on the time that *S. kudriavzevii* can remain in the culture in substantial proportions, and thus, can have a more relevant role during fermentation. The percentage of *S. kudriavzevii* was higher than 30% during the first 48 h in fermentations performed with air flows of 10 and 20 VVH. , However, after 48h of fermentation a faster decline of the *S. kudriavzevii* population is observed, which suggests that aeration only favors *S. kudriavzevii* growth at the beginning of the fermentations

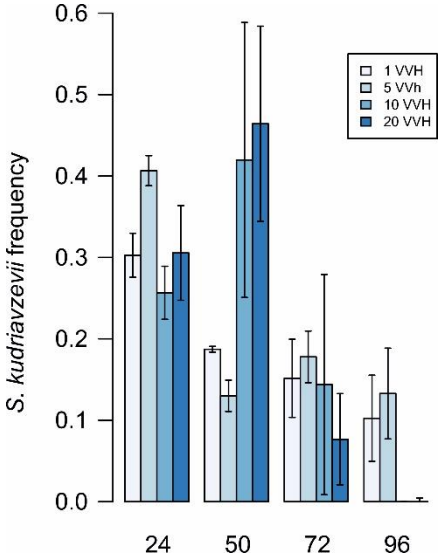


Figure II-1| *S. kudriavzevii* frequency under different aeration conditions. Values are mean of 3 replicates. Error lines represent standard deviations.

2.2. Assaying Different *S. cerevisiae*/*S. kudriavzevii* Inoculation Proportions in Fermentations with and without Air Supply

According to these previous data, aeration was applied only for short periods (48 h) for subsequent fermentations because longer aeration time does not favor growth of *S. kudriavzevii*, and also could increase the final acetic acid concentrations in wines, due to respiration (Salmon, 2006). To test whether a higher inoculation from the beginning of the fermentation, in combination with aeration, could improve *S. kudriavzevii*'s competitive performance, starters composed by *S. cerevisiae*/*S. kudriavzevii* proportions of 1:3 and 1:9 in were inoculated into fermentations supplied with an air flow rate of 20 VVH during the first 48 hours. Fermentations in the same conditions without aeration were also included to analyze the effect of the yeast species proportions alone.

There were significant differences between aerated and non-aerated fermentations. First, there is a considerable reduction of the fermentation time at which all sugars were totally consumed. Whereas unaerated fermentations took 10 days to finish, aerated fermentations took only 7 days. Second, a clear effect on the maximum cell density was observed, thus, single cultures of *S. cerevisiae* and *S. kudriavzevii* with air supply reached OD_{600} values around 25, however OD_{600} values for single cultures without aeration were around 20 and 15, respectively.

Regarding yeast proportion changes during fermentations, the initial inoculum proportion of 1:3 shows a slight increase of the frequency of *S. kudriavzevii* at the final fermentation stage due to limited air supply (**Figure II-2A and B**). However, this inoculation ratio does not provide, with respect to the 1:1 proportion a clear competition advantage for *S. kudriavzevii*. However, when the inoculation proportion was 1:9 and without aeration (**Figure II-2C**), *S. kudriavzevii* is able to remain at frequencies higher than 40% for 4 days, although at the end, is outcompeted by *S. cerevisiae*. Strikingly, the addition of the oxygen supply to inoculation proportions of 1:9 seems to provide a favorable environment for *S. kudriavzevii* imposition (**Figure II-2D**).

Sequential inoculation is one of the most common strategies proposed for the preservation of non-dominant microorganisms during food fermentations (Contreras et al., 2014; Gobbi et al., 2013; Loira et al., 2014). In the present study, this strategy was also applied by inoculating a set of bottles only with *S. kudriavzevii* at the beginning, and adding *S. cerevisiae* after 24 hours in a

proportion of 1%. In this case, *S. cerevisiae* was able to increase its frequency to 40% at the end of the fermentations (**Figure II-2E**).

As a summary of these results, the use of aeration has a slight impact on the relative competitive fitness of *S. kudriavzevii* when inoculated at equal proportions with *S. cerevisiae*. However, highly biased proportions of *S. kudriavzevii*, as well as sequential inoculations, can extend the presence of this less competitive species of interest to promote its impact in the fermentation process. Nevertheless, the combination of aeration and biased inoculation synergistically improves *S. kudriavzevii* presence during fermentation.

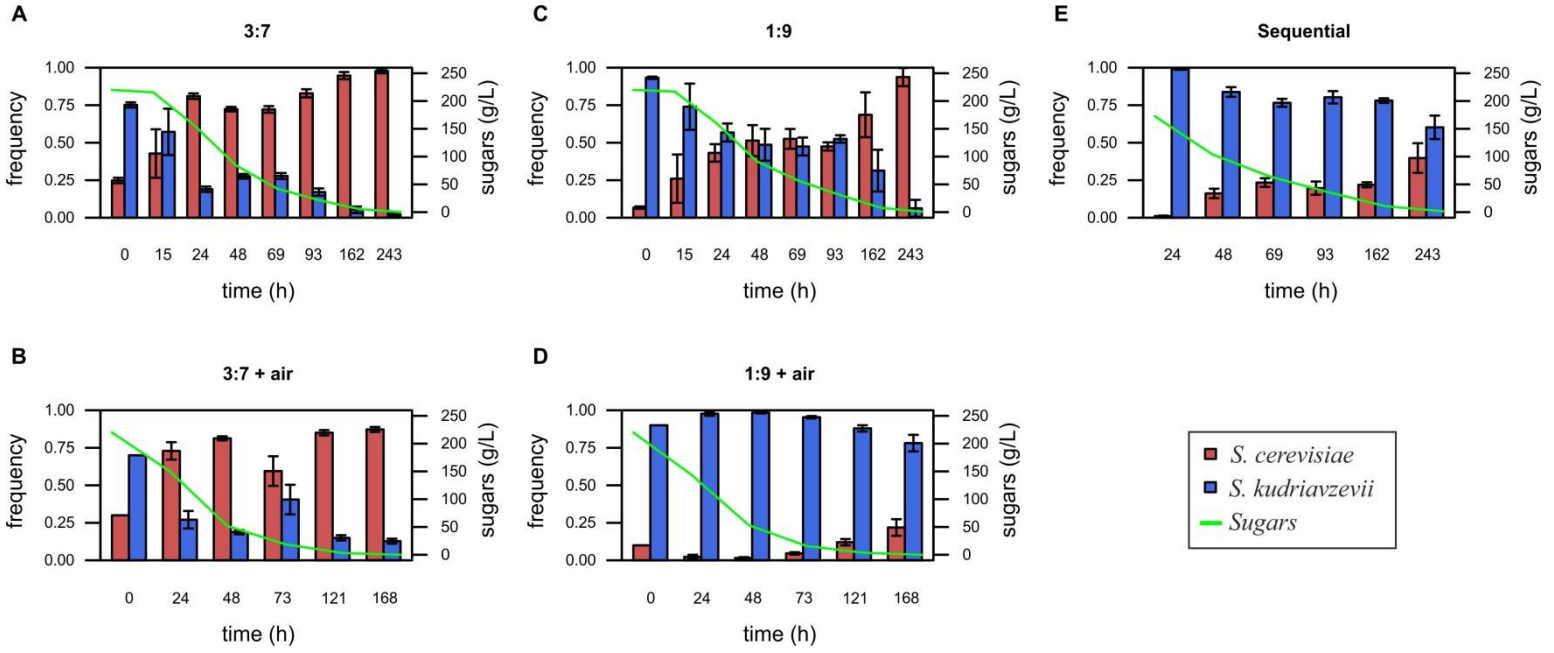


Figure II-2| *Saccharomyces cerevisiae* and *S. kudriavzevii* frequency during fermentation under different conditions: inoculum proportion 3:7 without air (A), inoculum proportion 3:7 with aeration during the first 48 hours (B), inoculum proportion 1:9 without air (C), inoculum proportion 1:9 with aeration during the first 48 hours (D), and sequential inoculation (E). Values are mean for 3 replicates. Error bars represent standard deviations. The sum of glucose and fructose concentrations in the must at every time point was also shown.

2.3.Effect of the Different Inoculation-Aeration Strategies on the Final Fermentation Product

To determine if these strategies really improve wine fermentations, the final wine composition was evaluated by HPLC analysis. First, it is important to remark that in all assayed conditions fermentations were finished with the consumption of all sugars present in the original must, except for fermentations performed only with single cultures of *S. kudriavzevii* (**Table II-1**), and under aeration, fructose was totally consumed.

Glycerol concentrations were clearly higher in all conditions in which *S. kudriavzevii* is present, compared to fermentations performed only with the reference *S. cerevisiae* wine strain, except for the 1:1 proportion with aeration. This glycerol production increase was especially relevant in fermentations with sequential inoculation (**Table II-1**).

Ethanol reduction was accomplished in fermentations with microaeration (up to 1.9% v/v less) and with sequential inoculation (**Table II-1**). However, the ethanol reduction achieved by increasing respiration rate had the counterpart of an acetic acid content increase between 0.5 and 0.7 g/L in bottles under limited aeration, which was not observed in non-aerated fermentations.

Table II-1| Chemical composition of the fermented SM obtained through HPLC

***Sc* : *Sk* proportion**	**Aeration (VVH)**	**Glucose (g/L)**	**Fructose (g/L)**	**Glycerol (g/L)**	**Ethanol (%)**	**Acetic acid (g/L)**
1:0	0	0.00 ± 0.00^{a}	0.12 ± 0.03^{a}	$5.86\pm0.11^{a,b}$	$13.13\pm0.09^{a,c}$	1.05 ± 0.01^{a}
0:1	0	0.02 ± 0.03^{a}	4.11 ± 2.34^{b}	7.73 ± 0.46^{d}	$12.50\pm0.26^{a,b}$	1.27 ± 0.03^{a}
1:1	0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	6.24 ± 0.29^{a}	13.27 ± 0.50^{c}	1.16 ± 0.13^{a}
3:7	0	0.00 ± 0.00^{a}	0.15 ± 0.10^{a}	$6.13\pm0.09^{a,b}$	$13.04\pm0.05^{a,c}$	1.15 ± 0.03^{a}
1:9	0	0.00 ± 0.00^{a}	0.78 ± 0.75^{a}	6.53 ± 0.12^{a}	$13.00\pm0.16^{a,c}$	1.22 ± 0.01^{a}
Sequential	0	0.00 ± 0.00^{a}	1.63 ± 0.18^{a}	$7.47\pm0.21^{c,d}$	$12.46\pm0.08^{a,b}$	1.13 ± 0.05^{a}
1:1	20	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	5.36 ± 0.40^{b}	12.12 ± 0.33^{b}	1.57 ± 0.10^{b}
3:7	20	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	6.24 ± 0.55^{a}	12.09 ± 0.18^{b}	1.61 ± 0.23^{b}
1:9	20	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	$6.61\pm0.07^{a,c}$	11.26 ± 0.19^{d}	1.79 ± 0.02^{b}

Values are given as mean±standard deviation of three biological replicates and two HPLC detection runs. An ANOVA analysis was carried out. The values followed by different superindexes in the same column are significantly different according to the *Tukey HSD* test ($\alpha = 0.05$).

3. Discussion

In the last century, alcohol abuse became considered as one of the most important health problems in the world, and promoted new behavioral strategies against alcohol consumption. In addition, because of global warming, in wine-growing regions with a Mediterranean climate there is excessive ripening of the grape, which produces musts with a higher concentration of sugars (Jones et al., 2005), and hence, higher alcohol yields, implying a higher tax burden, which makes wines less competitive, and a rejection by the consumer for health reasons, road safety, etc.

Therefore, wine industry must respond to these challenges posed both by new consumer demands and by changes in the composition and properties of the grape must due to climate change. These demands have a significant impact on the quality and acceptance of the final wines and require improvements in the enological practices, among which the development of new yeast starters exhibiting lower ethanol yields during wine fermentation is of chief importance.

Different approaches in the use of yeast starters have been proposed to reduce alcohol contents in wines (Schmidtke et al., 2012; Varela et al., 2015). They include controlled aeration, starter strain proportion adjustment, or inoculation of dominant yeast species after a non-*Saccharomyces* yeast of interest (Ciani et al., 2016; Comitini et al., 2011; Contreras et al., 2014, 2015; Quirós et al., 2014; Sadoudi et al., 2012). In the present study, we adapted these strategies to foster a *Saccharomyces* non-*cerevisiae* strain (*S. kudriavzevii* CR85) presence in synthetic must fermentation. This yeast had

been proved to foster decreased ethanol content, and also to increase fermentation kinetics and glycerol concentration in a 1:1 inoculum proportion with *S. cerevisiae* under low temperatures conditions. In contrast, this effect was not found under regular red winemaking temperatures as we reported in **Chapter I**, probably due to some of the already proposed competition mechanisms, such as antimicrobial GAPDH-derived peptides produced by *S. cerevisiae* (Branco et al., 2016), lower sulphite tolerance and efflux capacity (Pérez-Torrado et al., 2017b), or early nutrient depletion by *S. cerevisiae* (Fleet, 2003). However, the results reported in the present work show that *S. kudriavzevii* presence during an important period of the fermentation was achieved at regular industrial temperatures.

Although *S. kudriavzevii* and *S. cerevisiae* show long-term Crabtree effect, the carbon flux ratio between respiration and fermentation under aerobic conditions seem to be slightly higher in *S. kudriavzevii* CR85 compared to *S. cerevisiae* T73 (our unpublished data). Thus, an external oxygen supply to a fermentation co-inoculated with these two yeast species may benefit *S. kudriavzevii* growth. Nevertheless, high oxygen levels can deteriorate important compounds of must, originating undesired metabolites correlated to respiration such as acetic acid (Salmon, 2006). Therefore, a fine tuning of the amount of oxygen introduced into the system seems to be critical for the final wine quality. A wide range of airflow rates, from 2.4 to 60 VVH have been used at laboratory scale (Shekhawat et al., 2016; Vilanova et al., 2007). Nevertheless, an air flow rate of 20 VVH has been showed to be on the top limit for acetic acid production when applied to *S. cerevisiae*

microvinification (Morales et al., 2015), therefore the screening for the most suitable condition was performed always below this value.

S. kudriavzevii performance under air supply conditions was observed to improve its competitive fitness against *S. cerevisiae*. Our results suggest, though, that despite maintaining an air supply during the whole fermentation, after 48 hours, *S. kudriavzevii* was outcompeted by *S. cerevisiae*. This, together with the fact that an aerobic environment produces a higher acetic acid accumulation up to 70%, led us to reduce aeration just for the first 48 hours of fermentation for the successive experiments. Nevertheless, it is noteworthy that, as observed by Moruno et al. (1993) and later confirmed by Beltrán et al. (2008), synthetic and natural musts have different impact on the final product composition, acetic acid levels are much higher for synthetic must, as can also be observed for our aerated conditions. Thus, due to laboratory experimental conditions, acetic acid values obtained in the present work are high even for non-aerated synthetic must fermentations performed with the *S. cerevisiae* wine strain, compared to natural must fermentation under industrial conditions (0.35 g/L). Therefore, acetic acid levels produced during fermentations with air supply could still be under the limits of regulation (~1 g/L) and consumers' acceptance when tested at industrial scale.

Despite the acetic acid increase, ethanol reduction is notable for the aerated fermentations, in concordance with previous studies (Morales et al., 2015; Shekhawat et al., 2016), and similar to ethanol reductions obtained in other works in which similar co-inoculation strategies with non-*Saccharomyces* yeasts have been followed (Ciani et al., 2016; Contreras et al., 2015; Englezos et al., 2016). However, this is the first study in which *Saccharomyces*

kudriavzevii was used to reduce ethanol yields, which, together with a recent study on the sequential inoculation of *S. uvarum* and *S. cerevisiae* (Varela et al., 2016), opens new approaches to the use of other *Saccharomyces* species. These species, in addition to their ethanol metabolic characteristics, also provide richer aroma profiles to wine (Stribny et al., 2015).

The analysis of the non-aerated fermentations also showed a slight ethanol yield reduction clearly correlated with the *S. kudriavzevii* proportions during the fermentation process under the different assayed conditions. Moreover, there also is a clear direct correlation between *S. kudriavzevii* proportions and glycerol production, another desirable enological characteristic of importance for wine quality because it contributes to wine body and astringency masking (Jolly et al., 2014). Glycerol and ethanol metabolism has been proven to differ in *S. kudriavzevii* with respect to *S. cerevisiae* (Arroyo-López et al., 2010; Pérez-Torrado et al., 2016). In fact, the cryotolerant *Saccharomyces* species have been proven to produce wines and ciders with higher glycerol contents than *S. cerevisiae* (Bertolini et al., 1996; González Flores et al., 2017; Masneuf-Pomarède et al., 2010; Peris et al., 2016), so their use could be of great interest for wine industry.

Among the strategies followed to favor *S. kudriavzevii* growth against *S. cerevisiae*, the co-inoculation with a proportion of *S. cerevisiae* lower than 10% and the sequential inoculation showed the more promising results. Air supply showed a synergistic effect in proportion *S. cerevisiae* / *S. kudriavzevii* 1:9, whereas it did not have a significant impact on the rest of the assayed inoculum proportions. These results agree with the fact that *S. cerevisiae* is better adapted to anaerobic conditions such as wine fermentation, and air

supply produces an imbalance in this environment, which promotes *S. kudriavzevii* survival. According to our results, it also seems feasible that a certain threshold in *S. cerevisiae* cell density is necessary to trigger *S. kudriavzevii* lack of viability. This also agrees with the previous observations indicating that the viability of a competitor strain is affected by its interaction with *S. cerevisiae* due to cell-to-cell contacts (Arneborg et al., 2005; Branco et al., 2016; Nissen et al., 2003; Pérez-Torrado et al., 2017b), or by microenvironment modifications produced by *S. cerevisiae* (Goddard, 2008). A rise in temperature due to the higher fermentative rate of *S. cerevisiae* (Goddard, 2008) can affect *S. kudriavzevii* viability (Arroyo-López et al., 2011)

In summary, the most promising results were obtained from the combination of different strategies for promoting *S. kudriavzevii* prevalence during wine fermentation, such as co-inoculation with a low proportion of *S. cerevisiae* (<10%) or sequential inoculation together with limited aeration, resulting in an ethanol yield reduction as well as a higher glycerol production. Aeration requires costly additional technology, but it is already implemented in the wine industry (Vidal and Aagaard, 2008; Vivas and Glories, 1996) to improve wine quality by accelerating the transformations of phenols reducing the astringency.

Finally, these results have to be confirmed in real grape must to evaluate not only the effect of aeration on yeast physiology but also a potential effect on sensory profile. In addition, lower aeration rates can also be tested at industrial scale, particularly for *S. cerevisiae* / *S kudriavzevii* proportions lower than 1:9. Moreover, deeper understanding of the interactions among

Saccharomyces yeasts are also needed in order to finely tune the optimal use of these tools to reduce ethanol contents in wine.

CHAPTER III

Dominance of wine *Saccharomyces cerevisiae* strains over *S. kudriavzevii* in industrial fermentation competitions is related to an acceleration of nutrient uptake and utilization

1. Introduction

In most natural environments, a vast diversity of microorganisms coexists and competes for space and resources. In many aspects, microbial habitats resemble ecological battlegrounds where microorganisms fight until domination or utter destruction of the opponent. Grape must is sugar-rich habitat for a complex microbiota of yeasts and bacteria that are usually replaced by just one or a few *Saccharomyces cerevisiae* strains after the first stages of wine fermentation (Fleet, 2003; Querol et al., 1994). In this paper, we understand the concept of dominance as the phenomenon that is observed in mixed microbial populations when one individual (strain) is outnumbered by another (Pérez-Torrado et al., 2017b).

Competitive interactions between *S. cerevisiae* and other naturally present microorganisms in wine must, mostly non-*Saccharomyces* yeast, have been the subject of diverse studies (Bagheri et al., 2016; Ciani et al., 2016a; Fleet, 2003). This interest has recently been propelled due to the fact that, in the last years, the use of alternative yeasts in winemaking has become a widespread trend to respond to the new demands of the wine industry (Jolly et al., 2014; Pérez-Torrado et al., 2017a). These demands come from, first, the effect of global warming on vines, which produces an uncoupling of sugar content and phenolic maturity in grapes resulting in higher ethanol yields; and two, an increasing market demanding wines with lower ethanol content and with diverse flavours and aroma.

S. cerevisiae yeasts are characterized by their high capability to ferment simple sugars into ethanol even in the presence of oxygen (Crabtree effect

(Crabtree, 1928)). Although alcohol fermentation is energetically much less efficient than aerobic respiration, it provides with a selective advantage to these yeasts to outcompete other microorganisms: sugar resources are consumed faster and the ethanol produced during fermentation (Goddard, 2008), as well as higher levels of heat and CO_2, can be harmful or less tolerated by their competitors (Conant and Wolfe, 2007; Hagman et al., 2013; Merico et al., 2007; Piškur et al., 2006; Piškur and Langkjaer, 2004; Williams et al., 2015). Also, nitrogen source consumption and biomass production are more efficient in *S. cerevisiae* (Andorrà et al., 2012; Monteiro and Bisson, 1991). Therefore, a more efficient nutrient uptake seems to be one of the most important factors for *S. cerevisiae* dominance.

Strikingly, some studies suggested a secondary or irrelevant role for ethanol as a selective factor, and pointed to other factors as determinant of the competition outcome. Some authors proposed a relevant role to the production and release of toxic peptides by *S. cerevisiae*, such as those derived from glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Albergaria et al., 2010; Branco et al., 2014; Pérez-Nevado et al., 2006). Temperature, as already mentioned, has also been proven to be highly influential on competitions (Arroyo-López et al., 2011; Goddard, 2008). In fact, fermentations under low temperature conditions can benefit the competition capability of cryophilic *Saccharomyces* yeasts, such as *S. eubayanus*, *S. kudriavzevii* and *S. uvarum*, which can coexist with *S. cerevisiae* until the end of mixed–culture fermentations at low temperatures as can be observed in **Chapter I**.

Strains belonging to these three cryophilic *Saccharomyces* species were already proposed as promising starters for wine fermentations (Arroyo-López

et al., 2010a; Henriques et al., 2018; Peris et al., 2016). They exhibit physiological properties that are especially relevant during the winemaking process, such as their good performance in fermentations at low temperatures, resulting in wines with lower alcohol and higher glycerol contents (Pérez-Torrado et al., 2017a; Varela et al., 2016), as well as the production of larger and diverse amounts of aromatic compounds (Gamero et al., 2013; Stribny et al., 2015). *S. kudriavzevii*, as a member of the *Saccharomyces* genus, exhibits a higher genetic and, physiological similarity with *S. cerevisiae*, as well as species-specific differences (Arroyo-López et al., 2010a; Gamero et al., 2013; Peris et al., 2016; Stribny et al., 2015, 2016), including a better cold adaptation (Tronchoni et al., 2014). Characterization of its implantation when *S. cerevisiae* is present has been object of the present thesis and in other authors' studies (Arroyo-López et al., 2011). In fact, the main problem of their use, as occurs with most alternative yeast, resides in their implantation and persistence during wine fermentations. In **Chapter I**, we showed that *S. cerevisiae* is not affected by most temperature conditions when competing with *S. kudriavzevii* during fermentation, except at very low temperatures, i.e. 8º C. It is interesting to note that low temperature fermentations, in which *S. kudriavzevii* coexist with *S. cerevisiae* in high proportions (close to 50%), produce wines containing less ethanol and higher amounts of glycerol than wine fermentations conducted only by *S. cerevisiae*, however, higher temperatures result in domination of the culture by *S. cerevisiae*, with very low proportion of *S. kudriavzevii*.

Factors of presumable relevance in the domination phenomenon are cell-to-cell contacts and interactions, as assessed in previous studies (Kemsawasd et

al., 2015; Nissen and Arneborg, 2003; Renault et al., 2013; Wang et al., 2015b). Moreover, quorum sensing mediated mechanisms have been proposed as taking place during competition (Rivero et al., 2015). In fact, competitor cells have to be in close proximity for an effective response to competition (Arneborg et al., 2005; Pérez-Torrado et al., 2017b). However, little information is available about the recognition mechanisms and specific responses of *Saccharomyces* yeasts to the presence of a competitor. This information could be of especial relevance to understand yeast interactions during wine fermentation because they potentially affect yeast metabolism and growth, and thus alter the final characteristics and quality of wine.

Transcriptomic analyses have the potential to unveil the cell response to competition at the molecular level. The gene expression program of *S. cerevisiae* during wine fermentation has been profiled in previous studies (Barbosa et al., 2015; Mendes et al., 2017; Novo et al., 2013; Rossignol et al., 2003; Zuzuarregui et al., 2006). Previous studies using this approach regarding competition focused in the interactions among *S. cerevisiae* and bacteria or far distant non-*Saccharomyces* yeasts. More recently, the use of RNAseq allowed to study differential expression not only in *S. cerevisiae*, but also in the co-inoculated competitor *Torulaspora delbrueckii* yeast, observing an activation of both growth and carbon metabolism, which seemed to occur earlier in *S. cerevisiae* (Tronchoni et al., 2017). These authors observed expression activation of genes related to sugar and nitrogen metabolism under aerobic conditions when *S. cerevisiae* was cultured with other non-*Saccharomyces* yeast (Curiel et al., 2017).

In the present work, we performed a transcriptomic analysis during wine

fermentations co-inoculated with a wine *S. cerevisiae* strain and a strain representative of the closely related species *S. kudriavzevii* to shed light into the molecular mechanisms involved in the interaction between these two species that could be responsible of the dominance of *S. cerevisiae* in fermentations. Also, we included a *S. cerevisiae* strain isolated from oak tree bark in North America in order to check for this trait to be linked to the origin of a given population. Finally, we compared our observations with the case of the competition against strains of *S. uvarum*.

2. Results

2.1. Analysis of differential gene expression during competition between *S. cerevisiae* and *S. kudriavzevii*

The aim of this work is to elucidate the molecular response behind the dominance of *S. cerevisiae* when competing against the yeast of potential industrial interest *S. kudriavzevii* during wine fermentations. A transcriptome analysis of the wine yeast *S. cerevisiae* T73 in fermentation at 12 °C and 20 °C, with and without the presence of a *Saccharomyces* wild yeast, *S. kudriavzevii* CR85, was performed. Samples were collected at three different fermentation stages: early exponential phase (EEP), late exponential phase (LEP) and stationary phase (SP). These three time points at the very beginning of fermentation were selected based on previous results that showed that after these stages cell populations remained stable at the same proportions (**Figure III-1**). In addition, the same experiment, but this time using a *S. cerevisiae* strain isolated from oak bark, *S. cerevisiae* strain YPS128 and *S. kudriavzevii* CR85, was performed with the goal of elucidating whether the competition effect is associated at species or strain levels.

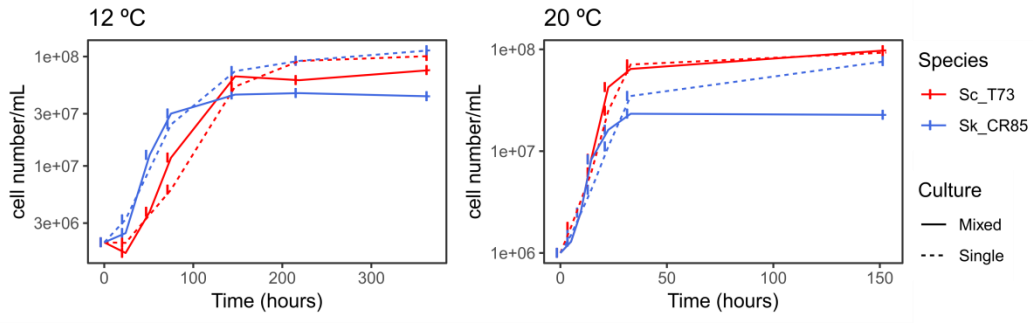


Figure III-1| Growth curves of *S. cerevisiae* T73 and *S. kudriavzevii* CR85 throughout all the fermentation at 12 °C and 20 °C. Cell number was measured by cell counting**.** Values are mean of three replicates**.**

The defined variables in the differential expression analysis were *time*, *temperature*, *culture* (single or co-inoculated) and *species*. PCA of our samples showed that 59% of variance corresponded to PCA component 1, which could be practically identified with the variable *species* itself (**Figure III-2**). Even when samples were clustered just according to genes exclusively affected by the variable *culture*, first branch unequivocally separated *S. cerevisiae* and *S. kudriavzevii* samples (**Figure III-3).** Although gene expression differences among *Saccharomyces* species are very interesting, the main objective of the present study is to determine the effect of the species competition on gene expression. For this reason, we decided to keep all of the available genes for each species. Thus, two *species*-specific datasets were used for the subsequent analyses.

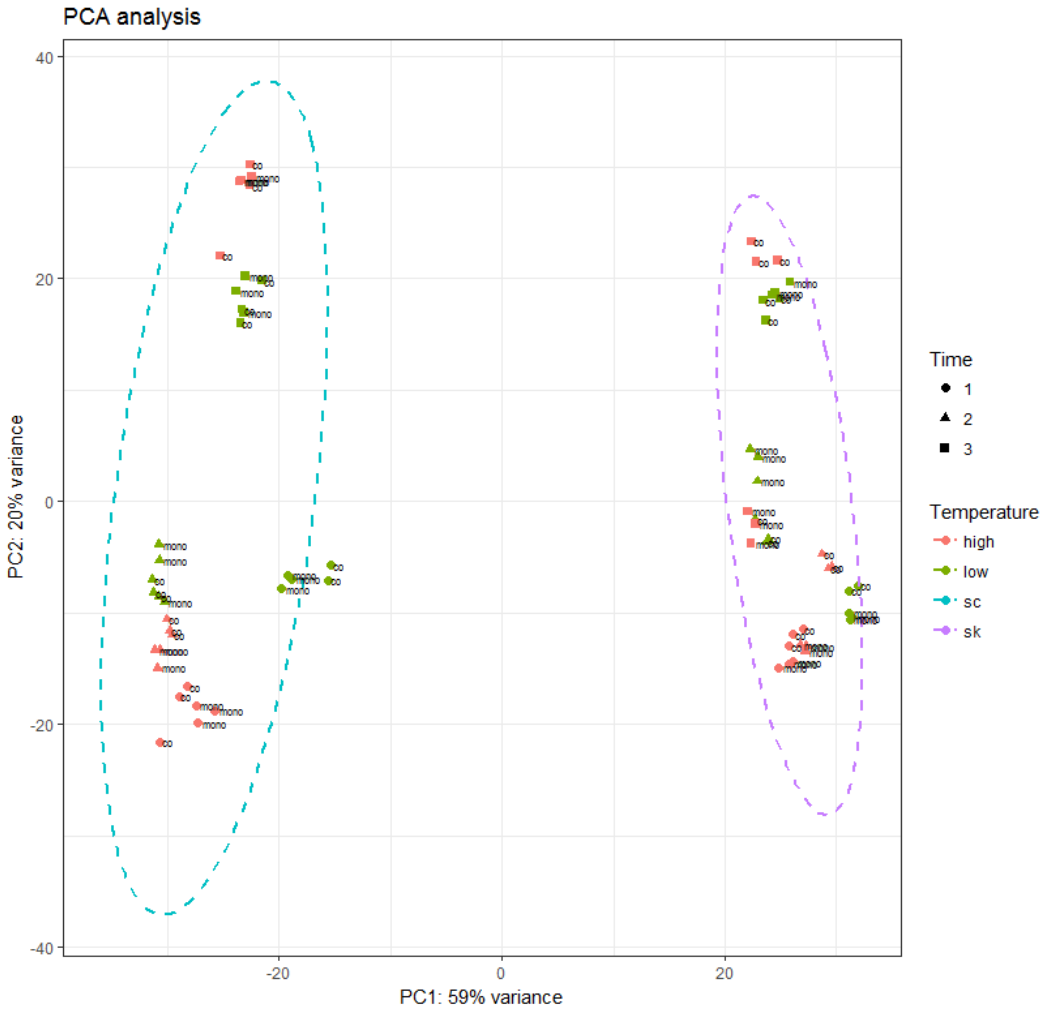


Figure III-2| Principal component analysis of differential gene expression for *S. cerevisiae* and *S. kudriavzevii*

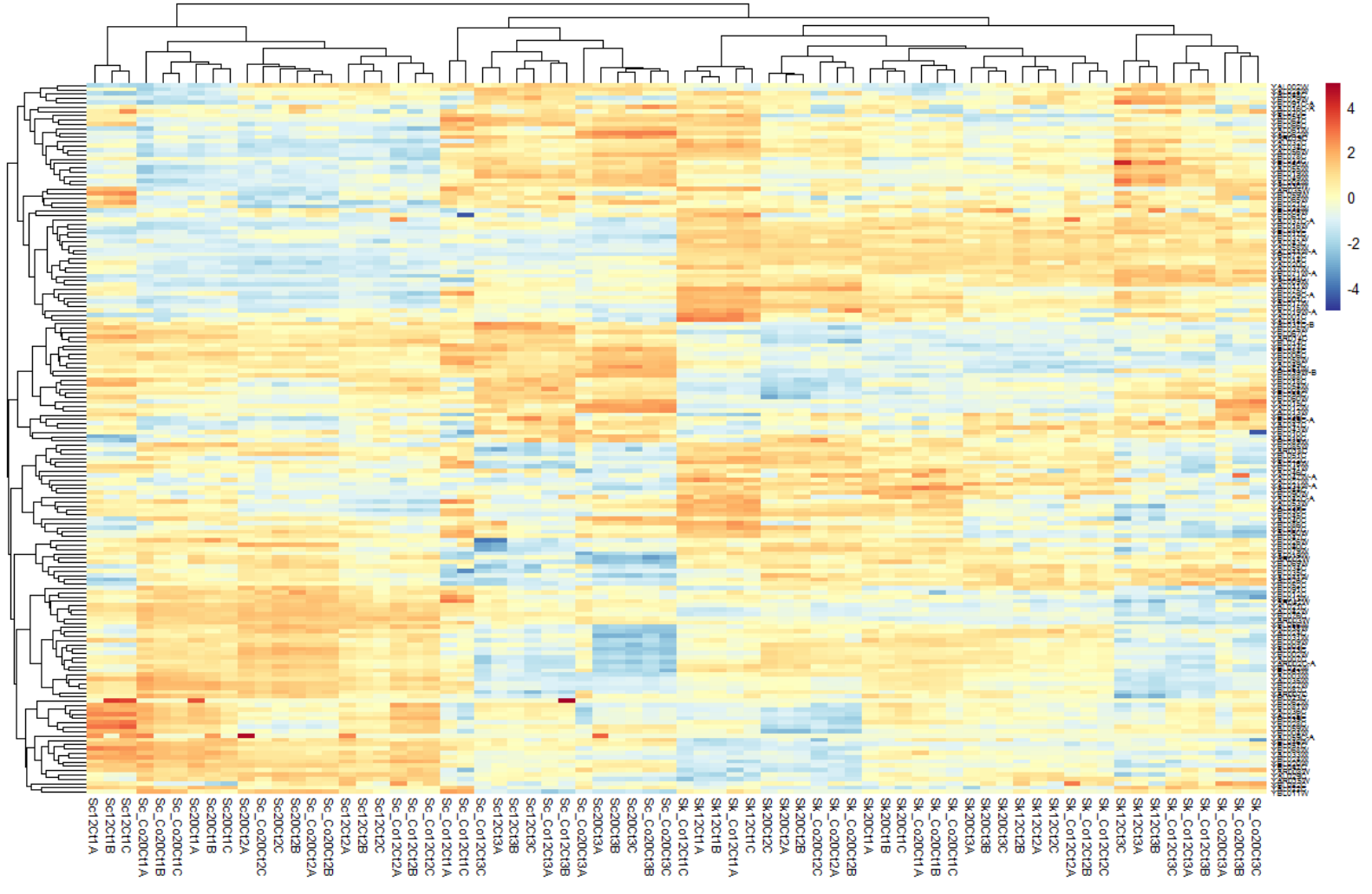


Figure III-3| Hierarchical clustering of genes affected by the variable *culture*. Euclidean distance matrix obtained from the expression matrix was used for clustering with *ward.D* method.

A PCA of the *S. cerevisiae* dataset showed that samples grouped mainly according to the variable *time*, meaning that the phase of fermentation was the main factor for sample variance. The variable *culture* accounted for less sample variance, that is, for lower levels of differential expression (**Figure III-1**). In the case of *S. kudriavzevii*, there was an overlap among the different fermentation stages; 20°C LEP and 12°C LEP samples cluster with 20°C EEP samples, and 20°C SP monoculture samples, respectively. In comparison to *S. kudriavzevii*, *S. cerevisiae's* variable *culture* seems to provide a higher sample variance. Differential gene expression analysis between mono and co-culture was carried out by contrast analysis for each species at each temperature and time point to avoid masking effect of these variables.

Interestingly, *S. cerevisiae* showed a stronger transcriptional response to competition during the EEP, higher at 12 °C than at 20 °C (**Figure III-5A**). This response decreases during the following stages at both temperatures, but faster at 20ºC. On the contrary, *S. kudriavzevii* presented during EEP a clearly higher response at 20ºC than at 12ºC, but, as the fermentation progresses, the number of differentially expressed genes increase at both temperatures, becoming very similar during LEP and identical at SP. These results are in agreement with the growth dynamics exhibited by both species in co-cultures compared to monocultures. *S. kudriavzevii* cell density was severely affected at SP, whilst *S. cerevisiae* remained practically unaltered at this stage (**Figure III-5B**).

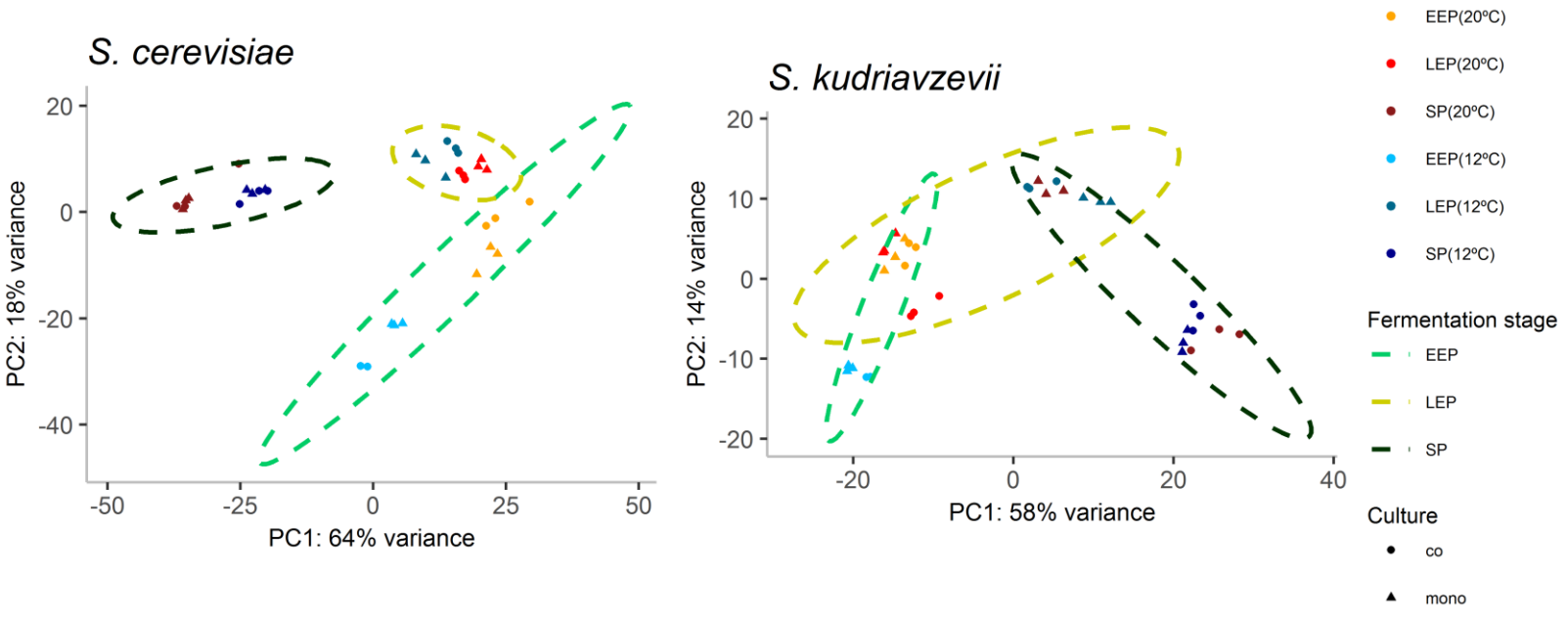


Figure III-4| Principal component analysis of differential gene expression for *S. cerevisiae* and *S. kudriavzevii*

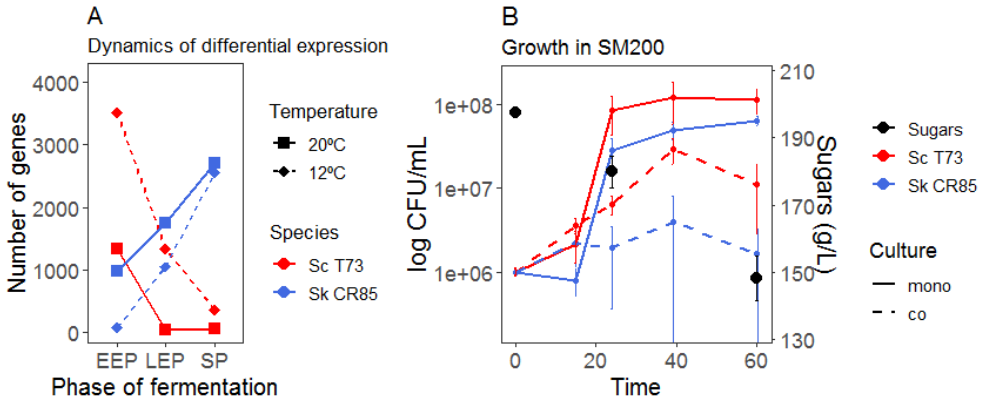


Figure III-5| (A) Number of differentially expressed genes in competition for each species at every temperature and phase of fermentation. **(B)** Growth curves of single (continuous curves) and mixed cultures (dashed curves) at 20 °C. Samples were taken at 15 h, 24 h, 39 h and 60 h. Values are mean of three to six replicates. Error bars represent standard deviation.

A first general overview of the differential expression analysis highlights a higher expression remodelling in *S. cerevisiae* T73 at EEP, which points to a detection of the competitor at the first stages of fermentation, and a response that might be more efficient in *S. cerevisiae* under these conditions. In the subsequent stages, the unsuccessful *S. kudriavzevii* showed a noticeable stress response due to its difficulties in competition culture, which is assessed below.

a) Differential expression in *S. cerevisiae* at EEP during competition

Gene expression modulation in response to competition was already noticeable at EEP. *S. cerevisiae* showed greater differential gene expression between monoculture and co-culture samples at 12°C than at 20°C (**Figure III-6A**). At 20°C, 680 genes were overexpressed in co-culture, and 658 genes were repressed (p-value < 0.05, **Table III-S2**). At 12°C, of the 3518 differentially expressed genes in co-culture, 1874 were overexpressed and 1644 repressed (**Table III-S2**). Assessing the function of those genes by

functional categories enrichment analysis, processes related to metabolism and cell growth were found at both temperatures (**Table III-S1**). With respect to repressed genes in co-culture, at 12°C we found many categories related to transcription, ribosome synthesis and translation (**Table III-S1**).

We found 198 upregulated and 79 downregulated genes in common at both temperatures, which were designated as culture-dependent (and temperature-independent) genes at this stage of fermentation. These genes were also classified into functional categories and clustered according to their expression level for *S. cerevisiae* at EEP (**Figure III-6A)**. One of the most relevant categories is gene expression regulation, with a high number of genes involved in histone modification and nucleosome regulation, and the basal transcription factor *CDC39*, which is indicative of a deep transcriptome reprogramming. In addition, genes involved in mRNA splicing are also present, as well as some genes related to cytoplasmic ribosomes and translation regulation. Among the repressed genes, those encoding mitochondrial ribosomal proteins are the most represented class. This agrees with the finding of upregulated genes related to respiration. This change in metabolism is coupled with an upregulation of mitosis and cell cycle progression, and the repression of telomere maintenance genes, which points to a faster cell proliferation. In addition, multiple stress response genes were also upregulated, especially those involved in oxidative stress and heavy metal detoxification. Also, glutathione seemed to be synthetized at EEP as well as a relevant set of ABC transporters involved in multidrug detoxification are also overexpressed.

Genes involved in mating pheromone response, as well as some meiosis activating genes, were also overexpressed (**Figure III-6A**). Genes involved in endocytosis, protein trafficking, protein degradation, and UPR response were among the upregulated genes. The overexpression of *GAT1* and *APG1*, genes encoding general amino acid transporters, amino acid biosynthesis and TOR signalling pathway genes, are considered as indicative of an acceleration of the nitrogen uptake and metabolism. In addition, iron and zinc uptake and homeostasis genes were also overexpressed. As for carbon metabolism, glycerol, ergosterol, long fatty acid, pentose phosphate pathway and acetate synthesis seemed to be favoured, with an important role of plasma membrane regulation.

To check which transcription factors were regulating gene expression during yeast competition, we used the *contrast* function of *DESeq2* package to generate an expression dataset similar to the previous one, but considering both temperatures. We manually assigned *p-value* 0 to the culture-dependent genes set and *p-value* 1 to the rest of genes. Then, this dataset was loaded into *PheNetic* (De Maeyer et al., 2015) web tool, which uses publicly available interactomics data to create networks from a given expression dataset, revealing possible master regulators and cellular processes relevant for the sample. In this case, the analysis determined Cin5p (*YOR028C*), Phd1p (*YKL043W*) and Spt23p (*YKL020C*) as the central transcription factors, which are involved in response to external stimulus and are known to recruit the general repressor Tup1p to certain promoters (Hanlon et al., 2011) (**Fig. 6B**). Other transcription factors known to be involved in external stimulus response were Yrm1p (*YOR172W*) and Cbf1p (*YJR060W*). In addition, Abf1p

(*YKL112W*), involved in vesicle trafficking; Sda1p (*YOR344C*), required for cytoskeleton organization and ribosome biogenesis; and Ihf1p (*YLR223C*), which regulates ribosomal genes transcription and is regulated by TOR signalling pathway, constituted the main nodes in the interaction network.

The typical gene expression along the fermentation for the differentially expressed genes at EEP is the dissipation of this response in the subsequent stages, as in *MIP1* (**Figure III-6C**). However, we could find only one gene, *HSP30,* which was kept downregulated in the co-culture with respect to the single culture, especially at 12 °C (**Figure III-6C**).

b) Differential expression in *S. cerevisiae* at LEP and SP during competition

At LEP, we found important differences with respect to temperature and at the species level. Regarding the number of differentially expressed genes, *S. cerevisiae* showed 29 at 20 °C and 1388 at 12 °C (**Figure III-5**). No significant enriched functional categories were obtained for the 20 °C condition. However, at 12°C, there was a clear response of membrane and cell wall remodelling. (**Table III-S1**). Also, several genes involved in iron homeostasis were overexpressed in co-culture (**Table III-S2**). 55 differentially expressed genes were found at SP, which are involved in meiotic phase entrance, translation repression, and response to DNA replication stress (**TableIII-S2**).

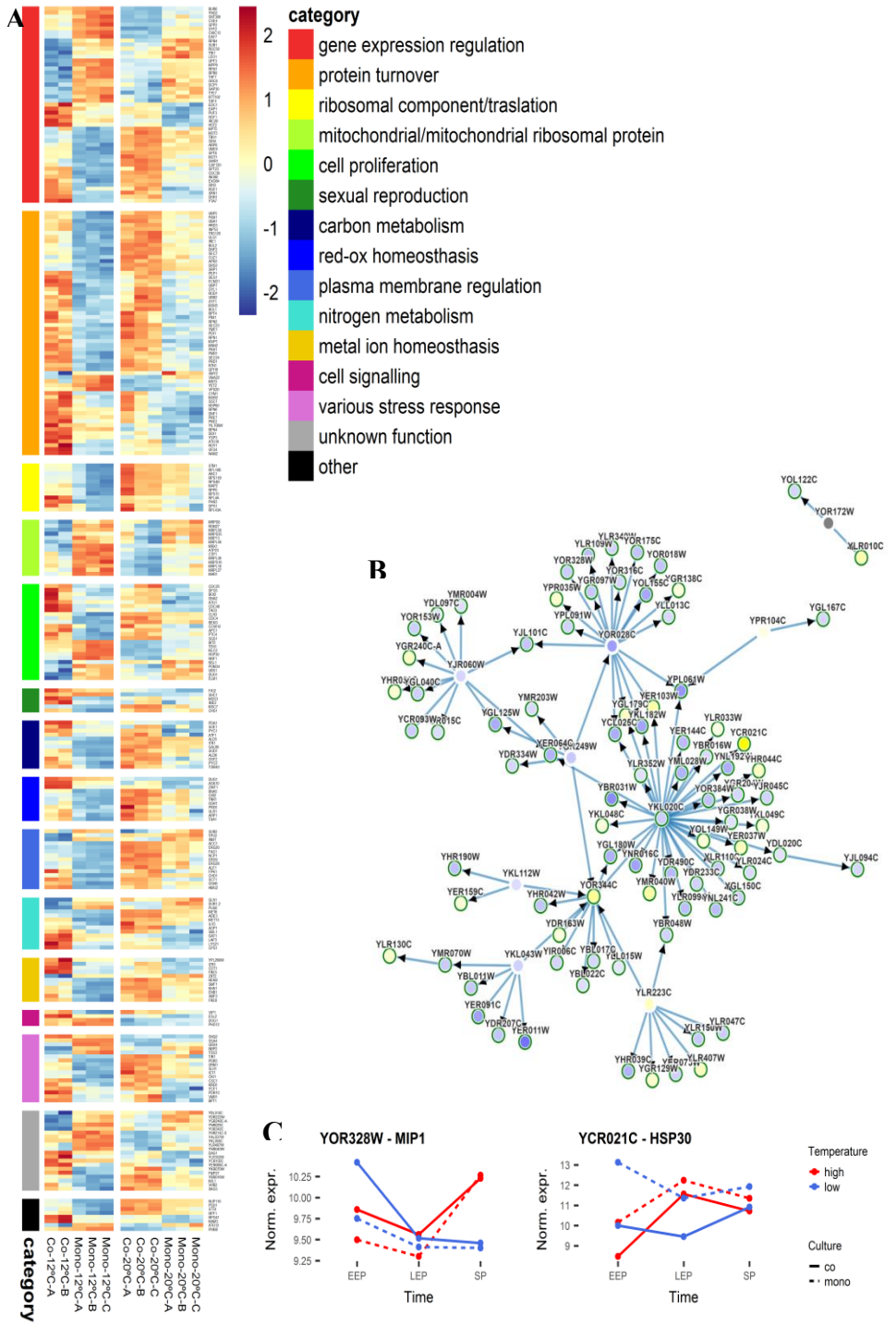


Figure III-6| **(A)** Hierarchical clustering of culture-dependent genes during EEP divided into functional categories. **(B)** Genetic interactions of culture-dependent genes given by Phenetics (network size = 100). **(C)** Expression profile (Norm. expr.) of genes *MIP1* and *HSP30*. Variance stabilizing transformation of the dispersion estimates dataset was used to reduce dependence of the variance on the mean.

Table III-1| Summary of the main GO terms and genes differentially expressed by *S. kudriavzevii* CR85 in competition with *S. cerevisiae* T73

		12 °C	**20 °C**
EEP	**UP**	sexual reproduction (*SPS18, FUS2, GAT3, CCH1, DON1, MSH5, 322YSW1*), stress response (*RSB1, FAA1, SLH1, FRT2*), nutrient homeostasis and transport (*IZH4, MLS1, HFA1, FAT3, TRK1, YIL166C, SUC2, PUS6, ZRT1, AGX1, ARG5, MPH2, HXT2*)	structural constituen of cell wall, plasma membrane, regulation of C-compound and carbohydrate, stress response, sugar transport, pseudohyphal growth and sporulation (*STE7, GPA2, DFG5, PTP2*), methionine transport and synthesis genes (*MUP3, MET6, SAM3*), iron ion uptake and homeostasis (*FRE3, FIT2, FIT3, TIR1, TIR3, FET4*), and aroma synthesis (*ATF1, ARO10*)
	DOWN	glucose catabolism,	translation, cell cycle
LEP	**UP**	Ergosterol biosynthetic process, aerobic respiration, integral to membrane response, membrane oxidative stress, sporulation or pseudohyphal growth (*IME1, RIM4, RIM11, SPO24, MSC7, YNL194C, SLZ1,ADY2, FMP45, DIG2, HYP2, RCK1, AQY1*), nutrient limitation (*GIS1, ADR1, SIP2, MRS4, ICY1, PUT1, HMX1, HXT2 ,HXT7 and GAL11*), response to drugs (*PMP3, PDR1, YOR1, FYV4, PDR5, CIN5*), aromatic compounds synthesis (ARO80), general stress response (*MSN4*)	
	DOWN	nucleotide binding	
SP	**UP**	ribosome biosynthesis, transcription, DNA-dependent, metal ion binding, ergosterol biosynthesis process, iron ion homeosthasis, sequence specific DNA binding	methionine biosynthesis process, transmembrane transport, oxidation-reduction process, sulfate assimilation, cysteine biosynthesis process, iron ion homeosthasis, zinc ion binding, fatty acid metabolic process, ergosterol biosynthetic process, sequence specific DNA binding
	DOWN	mitocondrial translation, proteolysis, oxidative stress respirtation, trehalose biosynthetic process and protein refolding	translation, mitosis, mitochondrial translation, proteasomal ubiquitin-independent, trehalose biosynthetic process and protein refolding

c) Differential Expression in *S. kudriavzevii* during competition

Comparatively to *S. cerevisiae*, *S. kudriavzevii* showed at EEP a lower response to the presence of *S. cerevisiae*, with 75 and 980 hits at 12°C and 20°C, respectively. Differential gene expression increased dramatically in the next stages as can be appreciated in **figure III-2**. At LEP, repressed genes at 20°C and 12°C arose to 1749 and 1043, respectively. Finally, a huge remodelling of expression in *S. kudriavzevii* took place at SP in the co-cultures with ~2,500 hits for both temperatures. All the genes and enriched GO terms can be explored in the supplementary material of this paper (**Tables S1 and S3**). However, we included a summary of the main enriched functional categories for *S. kudriavzevii* in **table III-1**. At EEP, there is already a slight response to the presence of *S. cerevisiae*. But, in concordance with the higher number of genes, the response becomes much higher in the next stages, with genes and related to stress response, nutrient homeostasis, and metabolism remodelling.

d) Identification of transcription factors responsible of the differential gene expression during competition

Datasets of differentially expressed genes for every time point, temperature and strain were analysed with *Phenetics* (**Table III-2**). At a first glimpse, we could observe the logical lack of central transcription factors for *S. kudriavzevii* during EEP at 12 °C and for *S. cerevisiae* during LEP and SP at 20 °C given the low number of genes in these datasets. Nonetheless, Cin5p, Phd1p and its paralog Sok2p, Mga1p, and Msn4p appeared as the most common factors for all the conditions.

Table III-2. Summary of nodal transcriptions factors in gene interaction networks obtained with *Phenetics*

	Temperature	EEP	LEP	SP
S. cerevisiae	12 °C	Cin5p, Mga1p, Msn4p, Phd1p, Swi5p, Tos8p	Cin5p, Hmo1p, Msn4p, Ste12p, Sip4p, Tos8p, Yox1p,	Met28p, Met32p, Tec1p, Thi2p, Tos8p, Sok2p
	20 °C	Aft1p, Hap1p, Mga2p, Msn2p, Msn4p, Phd1p, Spt23p, Tye7p, Ume5p		
S. kudriavzevii	12 °C		Adr1p, Cin5p, Hap1p, Met32p, , Mga1p, Phd1p, Sok2p, Yap5p	Cin5p, Hap4p, Met32p, Mga1p, Msn4p, Phd1p, Ste12p, Sok2p, Tos8p, Yap6p
	20 °C	Cin5p, Hap1p, Hap4p, Mga1p, Msn4p, Sok2p	Cin5p, Msn4p, Nrg1p, Sip4p, Sok2p, Swi5p	Cin5p, Mig1p, Phd1p, Sok2p, Yap5p, Yap6p

2.2. Nutrient consumption during competition

One of the most important results of the comparative transcriptome analysis is that competition favours the expression of genes related to nutrient uptake and cell division, which, in the case of the wine *S. cerevisiae* strain, occur from the first stages of the co-inoculated fermentation. To determine whether nutrient uptake is actually playing a key role in the imposition of *S. cerevisiae* or not, we compared the consumption profiles of nitrogen and carbon sources in single vs. co-inoculated fermentations.

a) Nitrogen uptake

Nitrogen source concentrations present in the medium (amino acids and ammonium) were measured by HPLC after 12 hours and 24 hours in the single and co-inoculated fermentations at 20 °C. After 12 hours, differences in consumption are almost inexistent among the samples, however, *S. cerevisiae*

had consumed after the first 24h (LEP) a larger amount of most nitrogen sources in the medium than S. *kudriavzevii* with the clear exception of tryptophan (**Figure III-7**). An interesting outcome of this analysis is the different pattern of nitrogen source preferences exhibited by the wine *S. cerevisiae* and the wild *S. kudriavzevii*. This way, there are clearly significant differences in the consumption of histidine, which is consumed by *S. cerevisiae* but not by *S. kudriavzevii*, tryptophan, one of the preferred amino acids for *S. kudriavzevii* but one of the less consumed by *S. cerevisiae*, and ammonium which is more preferable for *S. cerevisiae* than for *S. kudriavzevii*. Interestingly, nitrogen source consumption in co-inoculated cultures showed a very similar profile to that exhibited by *S. cerevisiae* in single cultures for all sources, including those that are differentially preferred. This is indicative that wine *S. cerevisiae* determines the amino acid uptake pattern because is faster consuming those nutrients present in the medium. This allows *S. cerevisiae* to outcompete *S. kudriavzevii* and dominate wine fermentations.

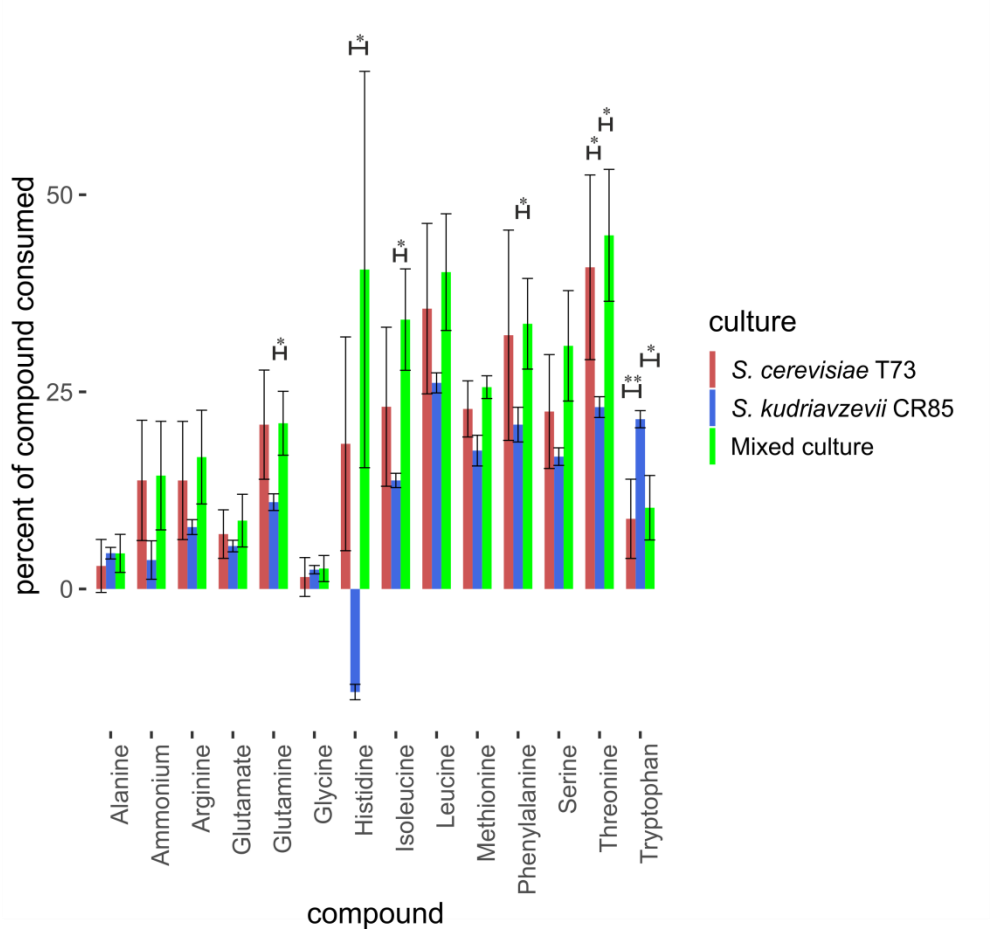


Figure III-7| Increment of **percentage of consumption of different nitrogen compounds between 24 hours and 12 hours in fermentation at 20ºC**. Values are the mean of three replicates. Error bars represent standard deviations. ANOVA analysis and Tuckey test were performed for significance evaluation (* = p-value < 0.05, ** = p-value < 0.01).

b) Sugar consumption

In a previous study (Tronchoni et al., 2009), we observed a different patterns of fructose and glucose consumption during fermentation in different *Saccharomyces* strains, including the two strains used in this study. Therefore, fructose and glucose concentrations were measured by HPLC along

fermentation to determine if the carbon source uptake rate is also accelerated during competence. Consumption kinetics of these compounds at 12 °C and 20 °C fits to a non-linear model (**Figure III-8**). In co-inoculated fermentations at 12°C, fructose consumption was clearly faster than in monocultures, which was statistically verified by the time necessary to consume 90% of the corresponding carbon source (**Table III-3**). Although differences are not statistically significant in the case of glucose consumption at 12ºC, a similar trend is observed. However, at 20 °C there were no differences in the fructose consumption between *S. cerevisiae* monoculture and the competition, but the difference is significant with respect to the single culture of *S. kudriavzevii*. Again, although differences are not statistically significant with respect to glucose consumption at 12ºC, consumption in the single culture of *S. cerevisiae* and in the competition are identical and different from consumption in *S. kudriavzevii* monoculture. *S. kudriavzevii* gene expression did not suffer major changes at this stage, so this sugar consumption acceleration was more likely due to *S. cerevisiae* activity.

2.3. Importance of cell contact in competitive fitness

As important part of the competitive response mechanism, we wanted to check whether direct cell-to-cell contact is necessary to trigger this process, we performed a set of fermentations in which a dialysis membrane was used to compartmentalize the cultures to avoid cell-to-cell contact. The *intrinsic growth rate* (*r*) parameter was calculated as a metric for fitness. Interestingly, whereas fermentations in which competitors were separated by membranes showed very similar fitness, co-cultures in the same compartment presented significantly lower values than the single cultures, with p-values of $2.32E^{-4}$ for

S. kudriavzevii and 2.01E^{-3} for *S. cerevisiae* (**Figure III-9**, *Sk_co_contact* and *Sc_co_contact* bars). This effect was clearer in *S. kudriavzevii*, agreeing with the stress response observed in competition with *S. cerevisiae T73*. These results indicate that cell-to-cell contact is a necessary, or at least important, condition for wine yeast to overcome their competitors.

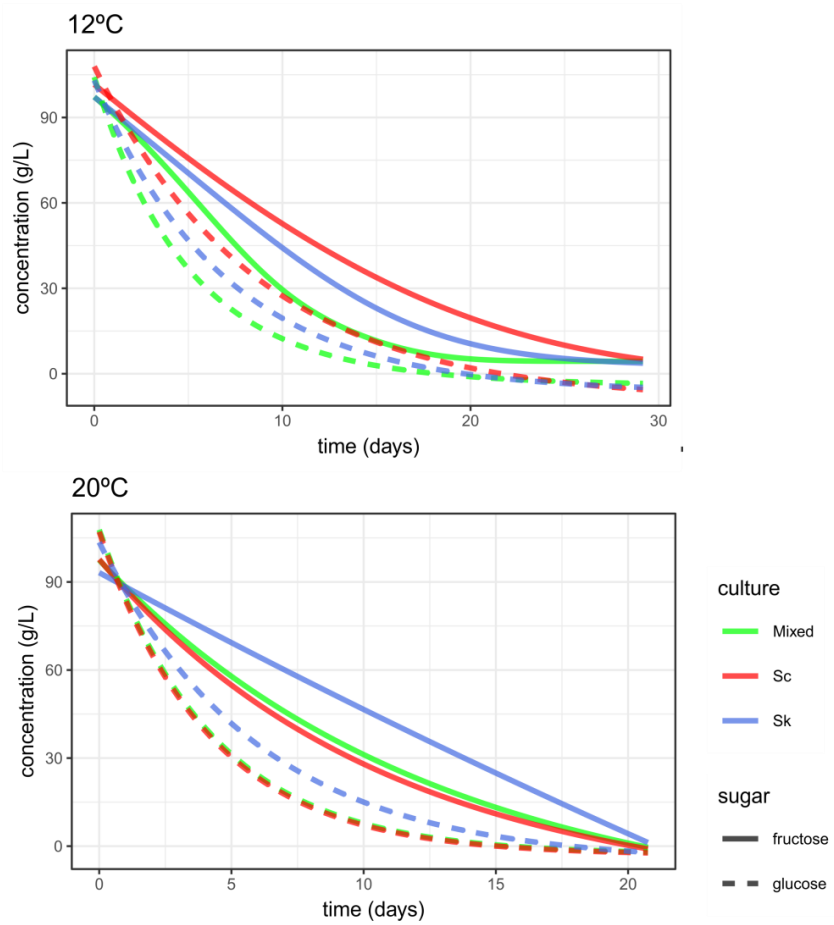


Figure III-8| Sugars consumption profiles during fermentations with single cultures of *S. cerevisiae* and *S. kudriavzevii* and co-cultures. Curves are the representation of sugars concentrations data fitted to different models (R2 > 0.9).

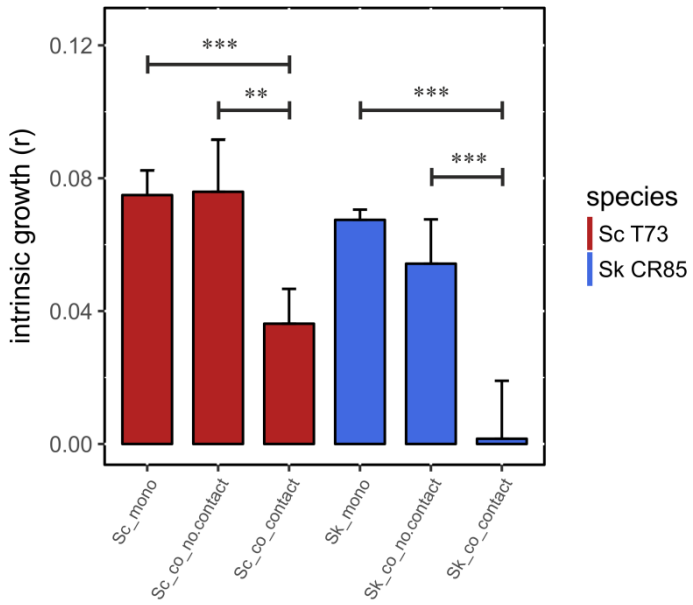


Figure III-9| Fitness after 60 hours in compartmentalized fermentations. Intrinsic growth rate (r) was calculated for inner and outer compartment in fermentations with only one of the yeast in both compartments (*Sc_mono* and *Sk_mono*), the two yeast separated in different compartments (*Sc_co_no.contact* and *Sk_co_no.contact*), and the two yeast mixed in both compartments (*Sc_co_contact* and *Sk_co_contact*). Values are mean of six replicates (both from inner and outer compartment taken together), and error bars represent standard deviation. ANOVA analysis and Tuckey test were performed for significance evaluation (** = p-value < 0.01, *** = p-value < 0.001).

Table III-3| Time (h) to consume 90% of glucose and fructose initially present in the media (*t90*). Values are given as mean ± standard deviation of three biological replicates and two HPLC detection runs. An ANOVA analysis was carried out.

Temperature	Sugar	$t_{90\ T73}$	$t_{90\ YPS128}$	$t_{90\ CR85}$	$t_{90T73\text{-}CR85}$	$t_{90YPS128\text{-}CR85}$
12 °C	**Glucose**	371.1 ± 10.3^{b}	382.6 ± 20.1^{b}	$316.0 \pm 4.0^{a,b}$	255.2 ± 61.2^{a}	361.0 ± 12.7^{b}
	Fructose	601.1 ± 11.9^{c}	623.5 ± 16.6^{c}	486.0 ± 59.1^{b}	346.3 ± 70.5^{a}	$548.6 \pm 8.4^{b,c}$
20 °C	**Glucose**	214.6 ± 16.2^{a}	191.4 ± 8.1^{a}	280.1 ± 0.9^{b}	220.1 ± 4.5^{a}	211.9 ± 23.5^{a}
	Fructose	$368.5 \pm 13.2^{a,b}$	342.9 ± 10.9^{a}	446.0 ± 8.0^{c}	387.7 ± 14.0^{b}	339.8 ± 25.0^{a}

The values followed by different superindexes in the same row are significantly different according to the Tukey HSD test ($\alpha = 0.05$).

2.4. Is the response to competition with *S. kudriavzevii* similar in wine and in wild *S. cerevisiae* strains?

To elucidate whether the response to competition is identical or different between wine and wild *S. cerevisiae* strains, we performed a similar study of the response to competition between *S. kudriavzevii* and a wild *S. cerevisiae* strain, YPS128, isolated from a Pennsylvanian Oak tree (Sniegowski et al., 2002). All fermentations were conducted at 20 ºC, a temperature at which both strains coexist (**Chapter I**).

Regarding the dynamics of differential expression between *S. cerevisiae* YPS128 in co-cultures with respect to monocultures, LEP was the only phase when *S. cerevisiae* YPS128 showed a certain level of differential gene expression, with 65 overexpressed genes and 2 repressed genes for cultures in competition (**Figure III-10a,** **Table III-S4**). This suggests that faster nutrient uptake did not take place in co-fermentation using a wild strain of *S. cerevisiae* as we had observed with the wine strain T73, which points to an important adaptation to fermentation by *S. cerevisiae* wine strains. This was confirmed by the HPLC analysis on sugar composition during the competition (**Table III-2**), and agrees with the lack of growth rate acceleration in co-cultures during competition (**Figure III-10b**), in contrast to the acceleration observed in T73 co-cultures (**Figure III-5b**).

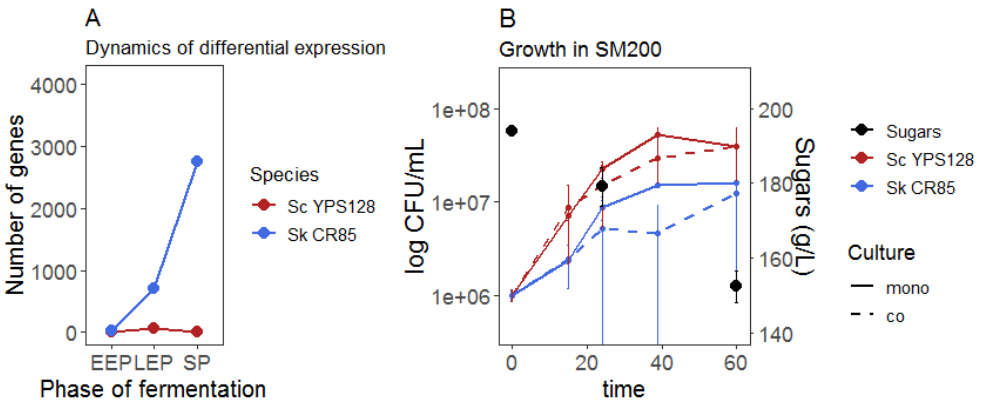


Figure III-10| (**A**) Number of differentially expressed genes in competition. (**B**) Growth curves of single (*Sc*, *Sk*) and mixed cultures (*Sc_co*, *Sk_co*) at 20 °C. Samples were taken at 15 h, 24 h, 39 h and 60 h. Values are mean of three to six replicates. Error bars represent standard deviation.

On the contrary, *S. kudriavzevii* level of response follows a similar pattern to that observed in its co-culture with the wine strain T73 (**Figure III-10a**). At EEP, overexpressed genes were related to sporulation, and others had a variety of functions such as glucose transport or nitrogen assimilation utilization (**Table III-S6**). There was an important gene expression regulation at LEP. The 494 overexpressed genes generated enriched GO terms *ammonium transport*, *fatty acid metabolic process*, *response to stress*, *protein refolding*, (**Table III-S5**). In addition, the MIPS categories *metabolism of nonprotein amino acids*, *oxidative stress response*, *C4-dicarboxylate transport* and *cell periphery* were found (**Table III-S5**). For the 213 repressed genes, every GO term enrichment result is related to vesicle transport, such as *ER to Golgi transport* or *membrane* (**Table III-S5**). Interestingly, a situation of stress and metabolism remodelling was taking place at this stage, despite the slight differential transcriptome regulation of *S. cerevisiae* YPS128. Finally, at SP stage, a huge transcriptome regulation change was observed, with around

1,500 overexpressed genes and 1,200 repressed genes in co-culture (**Table III-S6**). Involved processes showed nutrient limitation and metabolic profile remodelling. Processes that appeared to be diminished are *mitosis*, *cell cycle*, *mitochondrial translation*, *protein transport* and *ribosomal proteins* (**Table III-S5**). Thus, nutrients uptake and homeostasis together with response to toxicity seemed to be the main cell functions supported, reflecting a harsh situation for *S. kudriavzevii* in co-culture with respect to single culture.

Our results are compatible with a situation in which *S. cerevisiae* YPS128 did not change its behaviour during competition, and hence, is not able to reduce *S. kudriavzevii* up to the same extent as the industrial strain.

Furthermore, we showed above that cell-to-cell contact is important in the competition between *S. kudriavzevii* and a wine *S. cerevisiae* strain. However, when we assessed the performance of *S. cerevisiae* YPS128 in a compartmentalized fermentation, no significant differences were observed either in *S. cerevisiae* or in *S. kudriavzevii* fitness when cell contact is allowed or not (**Figure III-11,** S*k_co_contact* and *Sc_co_contact* bars). This result suggests that efficient competitive response in wine fermentation is a strain dependent trait in *S. cerevisiae*, and likely specific of the highly competitive wine yeasts. It also agrees with the lower differential gene expression in the wild strain compared to the wine one.

2.5. Transcriptomic analysis of co-fermentations by *S. cerevisiae* and different *S. uvarum* strains

We wanted to answer the question whether the response we observed in the case of our *S. cerevisiae* wine representative in competition with *S. kudriavzevii* was also present in the competition to other species of the genus.

To solve that question, we set up competition fermentations at 20 ºC using the wine strain *S. uvarum* BMV58 and the non-fermentative strain *S. uvarum* CECT12600 and analyzed gene expression at EEP, because was shown as the most relevant time point in our previous study. In addition, we were especially interested in this species because it is the other cryotolerant *Saccharomyces* species with a potential industry interest (**Tables I-3 and I-4**). Moreover, in our previous study, we observed that the competition fitness was clearly dependent on the origin on the strains (**Figure I-3**).

It was interesting to note that, as happened with *S. kudriavzevii*, *S. cerevisiae*'s response was stronger than *S. uvarum*; with 3293 and 2966 differential expressed genes during competition with *S. uvarum* BMV58 and *S. uvarum* CECT12600, respectively. In the case of *S. uvarum* strains, differential expression was observed in 544 genes for strain BMV58, and 927 for CECT12600. As depicted in **Figure III-11**, 2669 of the genes overexpressed in *S. cerevisiae* are common in both competitions against the two *S. uvarum* strains. However, the two *S. uvarum* strains seemed to have a clearly differentiated response with regard to the number and type of genes. All these genes can be found in **Table III-S7**.

Among the genes commonly overexpressed in *S. cerevisiae* during competition against both *S. uvarum* strains, we find terms related to lipid metabolism, sulfur amino acids and iron uptake (**Table III-4**). This terms are very similar to those found in the competition between *S. cerevisiae* and *S. kudriavzevii*. With respect to the enriched terms among the repressed genes in *S. cereviae*, we found some related to aerobic respiration and sporulation (**Table III-5**). The *Phenetics* analysis confirmed also notable similarities in central transcriptions

factors, with *CIN5* being one of the main nodes, and *SPT23* or *SPT12* also present.

Interestingly, overexpressed genes in the wine strain BMV58 are involved in metal ion homeostasis (*ZRT1, ZRT3, ZRT2, COT1, FIT3, PHO84, CCS1, CTR1, ATM1, ISU2*), as well as in sulfate and nitrogen resource uptake (*SUL1, GAP1, MEP1, VBA1, VBA2, UGA1*), sulfur metabolism (*CYS3, MET22*) and carbon metabolism *(GAL7, GAL10, GFA1, CRR1, TAL1, GAL80, YNR071C)*. Thus, nutrient utilization also seems to play an important role for this wine strain. With respect to the repressed genes, we obtained terms related to protein degradation, ribosome synthesis and translation (**Table III-6**). Nodal transcription factors obtained with *Phenetics* were numerous, among them, *YAP1*, involved in stress oxidative response; SMP1, encoding a regulator of osmotic stress response; *TEC1* and *STE12*; involved in filamentous growth; *PHD1*, recruited by Tup1p and involved in pseudohyphal growth; *DAL81*, involved in nitrogen sources degradation regulation; *YPR015C*, coding for a transcription factor that provokes cell cycle arrest; and *FLO8*, encoding an activator of the flocculines *FLO1* and *FLO11*.

However, for *S. uvarum* CECT12600, gene expression was extremely different. The only terms obtained from the overexpressed genes in competition are related to protein catabolism; *ubiquitin-dependent protein catabolic process* (p-value < $1e^{-14}$) *proteasomal ubiquitin-independent protein catabolic* (p-value = $4.733e^{-06}$) and transcription regulation; *regulation of transcription, DNA-dependent* (p-value = $3.383e^{-07}$). Within the repressed categories, we found *translation* (p-value = $1.571e^{-07}$), *thiamine biosynthetic process* (p-value = $1.252e^{-06}$) and *pyrimidine nucleotide biosynthetic process*

(p-value = 2.343^{e-06}). In addition, there were many genes related to nutrient homeostasis and stress response (**Table III-S7**). With respect to regulator nodes found with *Phenetics*, *YAP1* was one of the main factors; but also *RPN4*, involved in proteasome regulation; *SPT23*, regulator of *OLE1*; *CIN5*, also observed for *S. cerevisiae* and *S. kudriavzevii*; the involved in cell cycle regulation *FKH1*, *YHP1* and its paralog, *YOX1*; *THI2*, involved in thiamine biosynthesis; and *SOK2*, repressor of pseudohyphal differentiation.

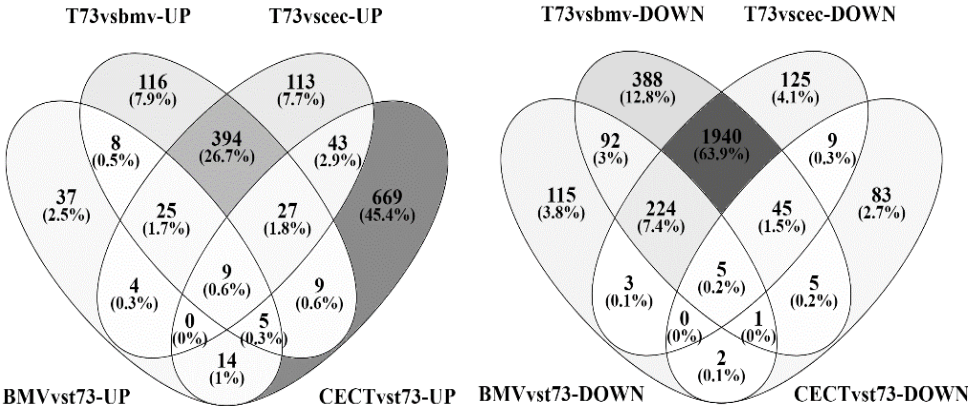


Figure III-11| Venn diagrams of the number over expressed (left panel) and repressed (right panel) genes for the different species in co-cultures in comparison to single cultures. The analyzed strain in each case is indicated in upper case in the labels and its competitor is indicated in lower case

Table III-4| Enriched Biological Process Go terms for the commonly overexpressed genes in *S. cerevisiae* T73

Category	p-value	In Category from Cluster
oxidation-reduction process [GO:0055114]	<1e-14	PDX3 LYS2 MET8 PDB1 LEU2 HBN1 MDH3 NDE2 PST2 HEM13 ARO1 TSA2 YDR506C HEM14 PRO3 ARG5,6 PDA1 MET10 YGL039W OLE1 YGL185C POX1 ADE3 GND2 PUT2 MSC7 NCP1 GRE3 FMO1 OYE2 CBR1 RNR3 IRC24 HYR1 MET5 HOM6 FAS1 FRE6 JLP1 FRE8 ERG3 ERG27 HMG2 HMG1 NDI1 ERG5 FMS1 CCS1 FET3 ADH3 HFD1 YMR315W YNL134C ZWF1 GOR1 ERG24 LYS9 FRE4 PRO2 FRE3 YPL088W GLR1 FAS2 YPR127W MET16
methionine biosynthetic process [GO:0009086]	2.293e-11	MET8 MET6 MET10 ADE3 MET30 MET3 MET5 HOM6 MET14 MET1 YLL058W MHT1 MET17 MET2 MET22 MET16
cysteine biosynthetic process [GO:0019344]	5.927e-09	CYS3 MET10 CYS4 MET30 MET3 MET5 MET14 MET17 MET16
lipid biosynthetic process [GO:0008610]	3.054e-08	ERG28 OLE1 NCP1 ELO1 LAC1 FAS1 ACP1 ERG3 ERG27 HMG2 ERG6 HMG1 ERG5 ERG24 MVD1 HES1 FAS2
iron ion homeostasis [GO:0055072]	3.946e-08	YDR506C SIT1 FTR1 ARN1 FRE6 SMF3 FRE8 FET3 FRE4 FRE3 FIT2 FIT3
sulfate assimilation [GO:0000103]	6.572e-08	MET8 MET10 MET3 MET5 MET14 MET1 MET22 MET16
steroid biosynthetic process [GO:0006694]	2.717e-07	ERG28 NCP1 ERG3 ERG27 HMG2 ERG6 HMG1 ERG5 ERG24 MVD1 HES1
ion transport [GO:0006811]	6.848e-07	VMA2 HSP30 CCC2 NHX1 YDR506C SIT1 FTR1 ARN1 YHK8 KHA1 ZRT3 FRE6 SMF3 FRE8 FET3 TOM40 FRE4 VMA4 FRE3 FIT2 FIT3 PMA2 VMA13
ergosterol biosynthetic process [GO:0006696]	1.107e-06	ERG28 NCP1 ERG3 ERG27 HMG2 ERG6 HMG1 ERG5 ERG24 ERG10
siderophore transport [GO:0015891]	2.578e-06	SIT1 ARN1 FRE4 FRE3 FIT2 FIT3

Table III-5| Enriched Biological Process Go terms for the commonly repressed genes in *S. cerevisiae* T73

Category	p-value	In Category from Cluster
mitochondrial translation [GO:0032543]	<1e-14	MRPL16 MRPS5 MRPL37 IMG1 MRPL1 RSM24 MRPL35 MRP1 MRP20 RSM18 RSM23 MRPS35 MRP4 RRF1 MSR1 MRPL6 RSM25 MRPL8 MRPL49 RSM7 MRP17 RSM22 MRPL38 MRPL13 MRPL20 MEF1 SLS1 MRPL15 MRPL4 YML6 MRPL24 MSK1 SWS2 MRPL19 MRPL10 MRPS18 PET123 TUF1 MRPL40 MRPL51
tricarboxylic acid cycle [GO:0006099]	2.696e-07	KGD2 SDH4 LSC2 KGD1 DAL7 YJL045W MDH1 SDH3 ACO1 IDP3 MLS1 CIT1 LSC1 FUM1 CIT3
aerobic respiration [GO:0009060]	2.671e-06	PET9 COR1 ETR1 MIC14 PET100 BCS1 RPO41 MNP1 RMD9 SHY1 MAM33 CBP1 MDH1 MBR1 SLS1 COQ9 AAC1 COX11 MRPL51 NCA2 QCR2
sporulation resulting in formation of a cellular spore [GO:0030435]	3.993e-06	SPO7 SEF1 MRPL37 SPS22 FMP45 ADY3 SPO71 DON1 XRS2 DIT2 DIT1 MEI4 SHC1 RMD9 IME4 RIM4 GSM1 IME1 SPO14 CDA1 CDA2 SPO20 OSW5 TGL5 SSP2 SPS4 SMA1 PEP4 CSM4
carbohydrate metabolic process [GO:0005975]	1.776e-05	GAL7 GAL10 ROT2 SOL2 YDR109C HXK1 XKS1 SOL4 SCW4 YHR210C PKP1 PCL7 YIR007W MDH1 CDA1 CDA2 FBP1 PGM2 CAT8 GLC8 SOL1 YNR071C PCL8 ATH1 GPH1 GDB1
ATP synthesis coupled proton transport [GO:0015986]	0.0003165	ATP16 ATP5 ATP17 ATP2 ATP7 ATP14 ATP4 ATP20

Table III-6| Enriched Biological Process Go terms for the repressed genes in *S. uvarum* BMV58

Category	p-value	In Category from Cluster
proteasomal ubiquitin-independent protein catabolic process [GO:0010499]	5.641e-13	PRE1 PUP3 PRE4 SCL1 PRE9 PUP2 PRE3 PRE8 PRE6 PUP1 PRE10 PRE2
proteolysis involved in cellular protein catabolic process [GO:0051603]	2.874e-12	CYM1 PRE1 PUP3 PRE4 SCL1 PRE9 PUP2 PRE3 PRE8 PRE6 PUP1 PRE10 PRE2
proteasomal ubiquitin-dependent protein catabolic process [GO:0043161]	2.768e-11	RAD23 PRE1 PUP3 RAD4 RPN11 PRE4 SCL1 RPT6 PRE9 PUP2 PRE3 PRE8 PRE6 PUP1 PRE10 PRE2
protein catabolic process [GO:0030163]	8.733e-08	RPT3 AFG3 RPT6 CDC20 UBR1 RPT1 YTA12 RPT5 RPT4 YME1
translation [GO:0006412]	3.716e-07	MRPL16 PET112 MRP21 RPL19A MRPS9 MRPL32 IMG1 RPP1A MRPL7 MRPS28 RPP2B TIF35 RML2 RPL12A RPL34A RPL24A RPS2 MRF1 RPL9A RPL26B VAS1 RPL24B RPS20 RPL27A MEF2 MRP17 MST1 RPL22A MEF1 RPS31 RPL37A MRPL4 RPS18B RPL6A MRPL24 TIF11 MSK1 RPS7B MRPL22 RPL18A RPS7A GCD1 RPS12 ISM1 MSD1 NAB3
proteasome regulatory particle assembly [GO:0070682]	5.784e-07	HSM3 RPT3 RPT6 NAS6 RPT1 RPT5 RPT4
positive regulation of RNA polymerase II transcriptional preinitiation complex assembly [GO:0045899]	5.784e-07	RPT3 GCN4 RPT6 SGF73 RPT1 RPT5 RPT4
cellular protein metabolic process [GO:0044267]	7.95e-07	CCT4 CCT6 TCP1 CCT2 CCT8 CCT3 CCT7 CCT5
mitochondrial translation [GO:0032543]	9.239e-07	MRPL16 PET112 MRP21 MRPS9 MRPL32 IMG1 MRPL7 MRPS28 MRF1 MRPS35 MRP17 MRPL13 MEF1 MRPL15 MRPL4 MRPL24 MSK1 MRPL22 MRPL17 ISM1

3. Discussion

Crabtree effect is a common to all *Saccharomyces* species ecological strategy and could explain how *Saccharomyces* yeasts could outcompete bacteria and non-*Saccharomyces* yeasts, but not how the ancestor of wine *S. cerevisiae* successfully occupied and outcompeted other *Saccharomyces* yeasts in the new ecological niche found in the crushed grape berries gathered by humans to produce the first fermented beverages. The simplest answer is that these yeasts have since then been exposed to selective pressures due to fluctuating stresses occurring during wine fermentation, such as osmotic stress due to high sugar concentrations, anaerobic stress, acid stress, nutrient limitations, ethanol toxicity or sulphite toxicity (Querol et al., 2003). As a result of this unaware domestication, wine *S. cerevisiae* yeasts are better adapted to this environment than other *Saccharomyces* yeasts (Arroyo-López et al., 2010b; Navarro-Tapia et al., 2016). This is supported by the fact that wine *S. cerevisiae* yeasts exhibit differential adaptive traits (Marsit and Dequin, 2015) and conform a genetically differentiated population (Almeida et al., 2015; Fay and Benavides, 2005; Liti et al., 2009).

In the last years, several studies tried to dissect in more detail yeast competition by using bottom-up approaches based on co-culturing different strain combinations in the laboratory, mainly wine *S. cerevisiae* and non-*Saccharomyces* yeasts due to their winemaking applications. This way, different possible, and up to some point, compatible mechanisms or phenotypes relevant for competitive interactions between *S. cerevisiae* and non-*Saccharomyces* have been identified. Although, in some studies, cell-to-

cell contact seemed to be unimportant in the competitive phenomenon, which would depend mostly on nutrient depletion or toxic metabolite release (Wang et al., 2015b), others indicated that interactions were clearly dependent on a cell-to-cell contact or a close proximity of the competitors (Nissen and Arneborg, 2003; Renault et al., 2013). In some studies, cell-to-cell contacts mediated a killer effect of the constitutive accumulation of GADPH-derived peptides in the cell wall of *S. cerevisiae*, which affects viability of non-*Saccharomyces* yeasts (Branco et al., 2018; Kemsawasd et al., 2015). Also, cell proximity was also required for a sulphite-sensitive *S. cerevisiae* strain to be affected by the toxic effect of the sulphite efflux produced by a tolerant strain (Pérez-Torrado et al., 2017b). In some cases, competition can be passive, such as the constitutive production of toxic compounds (Branco et al., 2016), but in other is regulated as a specific response to the presence of competitors. Active response to competitors can be mediated by indirect effector molecules (quorum-sensing signalling) or by cell-to-cell contact. As an example of the former, Rivero *et al* (Rivero et al., 2015) proposed that the detection of a competitor *S. cerevisiae* strain by a winery dominant *S. cerevisiae* strain was mediated by the altruistic autolysis and release of Hsp12p, which acts as a quorum sensing signal to stimulate killer activity and auto protection, encoded by the *PAU* genes. As an example of the latter, Perrone *et al.* (Perrone et al., 2013) suggested that dominant response of a strain only occurs when there is a detection of the competitor mediated by cell-to-cell contact.

In the present study, we also reported how a wine *S. cerevisiae* strain was able to dominate the fermentation niche after the detection of a *S. kudriavzevii* competitor mediated by cell-to-cell contact. *S. cerevisiae* T73 extensively

reprogrammed gene expression, which lead to a more efficient nutrient consumption and apparent growth anticipation. This behaviour had been previously observed in the case of competitions against bacteria and non-*Saccharomyces* yeast (Tronchoni et al., 2017). This seems to be linked to the modification of the plasma membrane composition. Ergosterol modifies the fluidity of the yeast membrane, which allows a more efficient activity of membrane transporters and increases tolerance to ethanol, characteristics related with a higher fermentation performance. According to the transcription factors found to be most likely the central nodes in this genetic acclimation, stress like response also seems to take place during competition. However, once *S. cerevisiae* accelerated nutrient uptake and accumulation in the first fermentation stages, the expression remodelling response decreased in the subsequent periods. Interestingly, *HSP30* was found to be the only gene downregulated in the three different time points. Hsp30p is a chaperone involved in the correct folding of certain membrane proteins, among which Pmp1p is one of the most important. Pmp1p is a basic element in intracellular pH regulation and is directly involved in processes such as stress response (Dong et al., 2017) and aging. Pmp1p accumulates in the plasmatic membrane after every budding event, and its accumulation determines cell aging by impeding further cell divisions (Henderson et al., 2014). Thus, the repression of HSP30 expression would imply defects in Pmp1p folding, which could constitute a mechanism for cell division deregulation.

S. kudriavzevii also exhibits a response to competition in which nutrients uptake seems to be important. High affinity sugar transporters were overexpressed in a moment when sugar was still at elevated concentration in

the medium, as well as the oligopeptide transporter coding gene *OPT1*, which have been recently identified among the upregulated genes of wine *S. cerevisiae* in co-culture with *Oenococcus oeni* (Rossouw et al., 2012). Oligopeptides transporters activation could be a mechanism for nitrogen resources increased acquisition (Marsit et al., 2016). Moreover, the nodes obtained for the control of gene expression in response to competition at 20 °C are very similar to those found for *S. cerevisiae*, pointing to a similar response which would be temperature dependent. However, this response in *S. kudriavzevii* is delayed and weaker than in *S. cerevisiae,* which acquires and accumulates nutrients in a faster way, and hence, this response could be activated as a consequence of the progressive reduction of nutrients available in the medium, especially limiting nitrogen sources, rather than by the presence of a competitor. In fact, when *S. kudriavzevii* detected the nutrient depletion caused by *S. cerevisiae*, increasingly triggers stress response mechanisms to cope with it in the later stages of fermentation.

Similarly, in competitions where *S. uvarum* strains were present, *S. cerevisiae* again showed a stronger response than its competitors regardless of the *S. uvarum* strain involved. The response mechanism seems to be the same than in competition with *S. kudriavzevii*, because includes processes such as lipid and ergosterol biosynthesis for membrane remodelling, sulphur amino acid biosynthesis, and sulphur and metals uptake.

Regarding the expression regulation during competition with *S. uvarum*, we identified several central transcription factors present in most conditions of the competition between *S. cerevisiae* T73 and *S. kudriavzevii*. The most frequently found was *CIN5*. Cin5p belongs to the Yap protein family, and is

involved in protein degradation (Sollner et al., 2009), salt tolerance (Ni et al., 2009), and diverse stress response (Nevitt et al., 2004). Noteworthy, its paralog Yap6p, also involved in salt tolerance (Mendizabal et al., 1998) is present among the central factors in *S. kudriavzevii* during the SP of fermentations at 20 °C. Another YAP family member, Yap1p, involved in oxidative stress response, appeared as a central node of transcription regulation in *S. uvarum*. Therefore, the role of this family of proteins may be key to understand the competence response in *Saccharomyces*.

Moreover, we found different transcription factors involved in pseudohyphal growth regulation. For instance, Sok2p, a repressor of the enhancer Phd1p, was also present in our results. *PHD1* was found for both *S. cerevisiae* and *S. uvarum* wine strains at EEP, and in the last fermentation stages for *S. kudriavzevii*. Swi5p, another of the transcription regulators found, is also involved in this process (Pan and Heitman, 2000). Pseudohyphal growth occurs under nitrogen limitation conditions, mediated by the heterodimers Tec1p and Ste12p (Gavrias et al., 1996), also present in our analysis. Cin5p, Yap6p, and Phd1p regulate expression under changing environmental conditions, such as stress by nutrient limitation, by recruiting the transcription repressor Tup1p (Hanlon et al., 2011). Mga1p has also been related to pseudohyphal growth (Lorenz and Heitman, 1998).

The general stress response transcription factors Msn2p and Msn4p, were also present in an important part of the *S. cerevisiae* competition against *S. kudriavzevii* datasets, indicating that cells are responding to stress. However, we did not find similar results in fermentations were *S. uvarum* participated. In this case, we only analysed samples from EEP, 20 ºC, perhaps the analysis

of samples from other stages or at other temperatures could reveal a role for this regulators. However, we cannot discard a differential response for this species, or even a difference due to the experimental conditions for each batch.

We used a wine *S. cerevisiae* strain for the present study because the main goal was to understand the mechanisms that allow a wine strain to outcompete strains from other *Saccharomyces* species not present in wine. In fact, the wine strain T73 was selected for commercialization as a dry yeast due to its good performance during wine fermentation, and is widely used at industrial level (Querol et al., 1992b). However, despite other *S. cerevisiae* strains isolated from diverse fermentative and wild environment are variable with respect to fermentation capability, osmotic and ethanol tolerances, they generally show better characteristics than strains from other *Saccharomyces* species (Arroyo-López et al., 2010b) and, depending on the fermentation temperature, they can also outcompete them, as reported in **Chapter I**. Here, we report two different competitive phenotypes in *S. cerevisiae*. We hypothesize that the wine strain exhibits a strong response including enhanced nutrient uptake abilities based on an active conditional response to the presence of the competitor *S. kudriavzevii*. These results are congruent with the observation mentioned above that *S. cerevisiae* T73 response to competition consisted on a deep gene expression remodelling which would switch the cells into a more active nutrient-uptaking state. On the contrary, the wild isolate YPS128 showed a passive constitutive response in the presence of the same competitor. Similar adaptation events seem to have independently occurred for the species *S. uvarum*. We showed how the behaviour of the wine strain BMV58 during competition was oriented to nutrient uptake, in a similar way than the wine *S.*

cerevisiae strain, but *S. uvarum* CECT12600 response is a nitrogen metabolism modulation trough protein degradation, likely due to the nitrogen depletion generated by its *S. cerevisiae* competitor.

These results are of especial relevance from an evolutionary point of view because they indicate that wine strains acquired new active mechanisms of response to competition during their adaptation to fermentation environments, such as the general acceleration of nutrient uptake and accumulation during competition. The appearance of this trait is compatible with the acquisition of other specific mechanisms based on the production of toxic compounds (Pérez-Torrado et al., 2017b). For example, sulphite production is used by sulphite-tolerant strains, a trait that has appeared at least twice in wine *S. cerevisiae* strains (Pérez-Ortín et al., 2002; Zimmer et al., 2014).

As reported, the active response of the wine *S. cerevisiae* strain to competition depends of a direct contact or a close proximity to the competitor *S. kudriavzevii*. However, additional research is required to unveil the mechanisms triggering this response. The recent description of the *S. cerevisiae* pangenome based on 1,011 genomes (Peter et al., 2018) reported 2,856 variable (present/absent) ORFs, being *cell-cell interaction* one of the most enriched functional categories. Indeed, some of the genes found in this study as differentially expressed during competition, which functions remain unknown, could be specifically involved in such microbial interactions. Among them, the *FLO* gene family could be a clear candidate as the main function of these genes is self-recognition and flocculation interaction with other cells (Goossens et al., 2015). In fact, a study in which *FLO1*, *FLO5*, *FLO9* and *FLO10* expression was controlled in co-cultures of *S. cerevisiae* and

several non-*Saccharomyces* yeast, aggregation-flocculation and yeast competitive fitness varied depending on the competitor species and the overexpressed flocculin, which implies a species or strain-specific mechanism of cell-to-cell interaction (Rossouw et al., 2015). Strikingly, one of the main nodes in the *Phenetics* net of our wine *S. uvarum* strain was Flo8p, the transcription factor that regulates FLO1 and FLO11, implicating that we might have found a competition response mechanism in which this family of proteins enact. Whatever the role of the *FLO* gene family is, the recognition mechanism involved in the competition interactions must be not only species dependent, but also strain dependent according to our results.

Transcriptomic analysis is broadly considered a good first approach to understanding the state of a given cell population or its response to a stimulus. In fact, its use is becoming wider in the study of the physiology of *Saccharomyces* from industrial or other origins, particularly the species *Saccharomyces cerevisiae* (Carvalho-Netto et al., 2015; Nielsen et al., 2017; Sardi et al., 2016; Yang et al., 2017; Zhang et al., 2018). However, different authors demonstrated a notable lack of correlation with proteomic or metabolomic data that cannot be diminished (Chen et al., 2002; Ghazalpour et al., 2011; Gygi et al., 1999; Pascal et al., 2008; Yeung, 2011). Thus, we tried to confirm the most relevant features of the extensive response observed by physiological or metabolic experiences. Moreover, we intend to conduct future research to obtain a more accurate and informed prediction on the nature of interactions in the wine microbiota; *i. e.* whether the recognition of a strange species could depend on physical interactions among cell wall proteins or on the detection of another kind of interacting molecules. This can have many

important implications in the management and design of the inoculation process to improve wine fermentations according to the producer's and consumer's demands.

GENERAL DISCUSSION

Throughout practically the entire winemaking history, human intervention in microorganism selection was an unconscious process. Despite so, wine production expanded worldwide, increasing its economic importance until today, when around 250 million hL are produced every year. Only in the last decades, the development of modern technologies for winemaking included the large scale production of dry yeast, to assure greater control on the fermentation kinetics and the proliferation of spoilage microorganisms.

Back then, the yeasts selected for industrial use were those with an optimal performance, mostly with respect to fermentation kinetics. However, in the last years, the new determining challenges and demands of the market, such as global warming, low temperature fermentations, and the development of more variated and rich aroma in wines, fostered the investigation of new yeast strains and species for winemaking. Most of these efforts have been pointed to the utilization non-*Saccharomyces* yeast that had been previously isolated from natural musts or environments close to fermentation. In fact, some of them are available as commercial dry yeast for the industry (Hansen, 2004b, 2004a; Laffort, 2013; Lallemand, 2012).

However, the *Saccharomyces* genus is composed by several species apart from *S. cerevisiae*. Some of them has been characterized and proven to be able to carry out wine fermentation, and demonstrated to provide wine with valuable improvements in composition (Gamero et al., 2013; Naumov et al., 2000b, 2002; Pérez-Torrado et al., 2016; Peris et al., 2016; Sniegowski et al., 2002; Tosi et al., 2009). Thus, one of the main objectives of our group is to take advantages of their good features to implement them in wine productions.

A major concern is the implantation of alternative yeast in the fermentations because in most cases *S. cerevisiae* is able to outcompete the rest of yeast present in the must, including those belonging to the *Saccharomyces* genus (Arroyo-López et al., 2011; Jolly et al., 2014; Querol et al., 2018; Williams et al., 2015). This implies that these organisms of interest are not able to remain in the process enough to provide the wine with the wanted characteristics. Thus, in this thesis aimed to gather deeper knowledge of the factors influencing the phenomenon of competition among the *Saccharomyces* species, and to better understand it from a molecular point of view.

Improving the fermentation by the use of alternative *Saccharomyces* species

The first of our objectives was to study the behavior of several *Saccharomyces* species of potential interest for winemaking; *S. paradoxus*, *S. kudriavzevii*, *S. uvarum* and *S. eubayanus*, when they had to compete with a *S. cerevisiae* wine representative strain during synthetic must fermentation. These species come from a diversity of ecological niches, meaning that they are adapted to different conditions. One of the differential traits among them is temperature adaptation; *S. cerevisiae* and *S. paradoxus* optimal growth temperature is close to 30 °C, whereas *S. kudriavzevii*, *S. uvarum* and *S. eubayanus* prefer temperatures around 20 °C-25 °C (Salvadó et al., 2011b). As we mentioned, fermentation temperature is a factor of matter nowadays, because white and rosé wines producers, in some cases, tend to lower the temperature of fermentation as low as 12 °C to achieve wines with higher content in aromatic compounds, which are volatile.

In our assays, we included competition fermentations at low temperatures

(8 °C, 12 °C, and 20 °C) and at control temperature (25 °C) to assess the effect of low temperature on the competitive performance of the different species. We reported the relation between temperature and competitive performance among the *Saccharomyces* species; the higher the temperature, the better competitive fitness showed by *S. cerevisiae*. This was in concordance with previous works with non-*Saccharomyces* yeasts (Ciani and Comitini, 2006; Gao and Fleet, 1988).

However, *S. cerevisiae* was able to dominate the fermentation even at low temperatures in most cases, defining domination as the situation where a strain presents a higher cell proportion than its competitor (Pérez-Torrado et al., 2017b). Although Arroyo-López et al. (2011) observed something similar in the case of competitions of *S. cerevisiae* and *S. kudriavzevii*, this was up to some extent unexpected, since growth kinetics parameters of some of those strains under the same fermentation conditions were faster than *S. cerevisiae*'s. Thus, we conclude that there must be something else than simply adaptation to the media that allows *S. cerevisiae* to dominate the fermentation.

In addition, it is worth to say that a *S. uvarum* wine strain was able to dominate the fermentation over *S. cerevisiae* at 12 °C and coexist even at 20 °C. However, a non-fermentative *S. uvarum* strain was totally outcompeted at 20 °C. In line with this result, are those obtained for a wild *S. cerevisiae* strain, which was not able to outcompete the cryotolerant species *S. kudriavzevii*, except in fermentations at 25 °C, in comparison to the *S. cerevisiae* wine strain, which was able to do it even at 12 °C. Thus, adaptation seems be a determining factor, and consequently, wine strains are going to be able to better compete in the fermentative environment in comparison to strains from other origins.

Moreover, we showed how the combinations of certain strains were good for fermentation kinetics or final wine composition, whereas other combinations resulted in completely the opposite. That means that the output of fermentations carried out by a combination of yeasts cannot be predicted based on the characterization of fermentations performed by these organisms alone.

In our case, the most promising results were achieved for the competition of *S. kudriavzevii* and the wine *S. cerevisiae* strain, with a reduction of more than 20% in the time to consume 90% of the sugars from the media, plus a reduction of 2 degrees in the ethanol content, and an also a notable increase in the glycerol concentration. Ethanol reduction up to this extent is a very positive result in comparison to previously reported values using co-inoculation of *S. cerevisiae* and an alternative yeast, only comparable with the results published Contreras et al. (2014) (Goold et al., 2017). Thus, we propose that the combination of *S. cerevisiae* and *S. kudriavzevii* for low temperature fermentations could be a good resource for winemakers to reduce ethanol, and what is more, increase ethanol and accelerate fermentation at industrial level. Also, the use of the *S. uvarum* strains BMV58 and CECT12600 produced promising results.

The approach of simply mixing two yeast strains in the inoculum is not the only one proposed for improving wine characteristics. For instance, decreasing the proportion of *S. cerevisiae* with respect to the strain of interest, either by unbalancing their proportion in the initial inoculum, or by sequential inoculation of the strain of interest before de *S. cerevisiae* (Goold et al., 2017; Querol et al., 2018). In addition, metabolic engineered strains have been proven to be successful for ethanol glycerol or aroma synthesis modulation,

although sometimes with unexpected side products such as too much acetic acid (Goold et al., 2017). However, the main problem with this kind of approaches is that GMOs are not allowed for food production in many countries, such as the EU. Hybridization by techniques considered as non GMO, such as rare mating has been used to this purpose. Based on the characterization of the natural interspecific *Saccharomyces* hybrids present in natural musts, that contained profitable characteristics from both their parents, artificial hybrids development attempts have been carried out (Bizaj et al., 2012; Origone et al., 2018; Pérez-Través et al., 2012). At last, oxygenation of the fermentative medium has also been used. Normally, alternative species used for winemaking, both *Saccharomyces* or non-*Saccharomyces*, are characterized for presenting a carbon metabolism more inclined to respiration than *S. cerevisiae*, this is why a controlled oxygen flow can benefit their yield in co-culture (Gonzalez et al., 2013; Querol et al., 2018; Quirós et al., 2014; Varela et al., 2016).

Once we achieved the improvement of the parameters of fermentations at low temperatures by the use of *S. kudriavzevii*, we wanted to do it as well as regular red winemaking temperature conditions, more close to 25 °C. This is why we chose the described approaches of increasing the proportion of *S. kudriavzevii* in the inoculum, the sequential inoculation and the application of controlled oxygenation to the fermentation. Our results showed that increasing the initial proportion did not show to be enough to improve final product competition. Furthermore, they showed that the sequential inoculation allowed *S. kudriavzevii* to dominate the fermentation until the end; however, this was not enough to get statistically significant values for improved wine composition. At last, the combination of increased proportion of *S. kudriavzevii* in the

inoculum plus the aeration of the system, improved both the performance of *S. kudriavzevii* and the final composition of wine, with lower ethanol concentration and higher glycerol content. The ethanol reduction was comparable to those of previous results published where oxygenation was utilized (Morales et al., 2015; Shekhawat et al., 2016; Varela et al., 2016), presenting also the counterpart of a notable increase in the acetic acid concentration, which could be due to the use to synthetic must for our experiments (Beltrán et al., 2008; Moruno et al., 1993).Anyway, oxygenation is already used in some wine productions despite its costs, so it could expand to be applied to processes where *S. kudriavzevii* participated.

In the near future, the issue of acetic acid, among others, will be probably possible to be controlled thanks to predictive modeling. In the last years, modelling of metabolite consumption, especially sugars and nitrogen compounds, and production, such as CO2 or ethanol, has gained substantial importance (Coleman et al., 2007; David et al., 2010; Malherbe et al., 2004; Marín, 1999; Sainz et al., 2003; Varela et al., 2004). Of particular interest for us is our recently published work in collaboration with experts in dynamic modelling of biological processes, in which growth, sugar consumption, ethanol and glycerol production, as well as fermentation kinetics, of the strains used in the present thesis, *S. kudriavzevii* CR85 and *S. cerevisiae* T73, have been dissected by predictive modelling (Henriques et al., 2018). Our results demonstrate that, for instance, final concentration of those metabolites of interest can be calculated as a function of the initial inoculum size and the fermentation temperature. That constitutes a powerful tool for winemakers as it opens the possibility to modulate the concentration of any metabolite of interest by this approach, of course, based on previous empiric data, suitable

for each case. In fact, currently, we keep developing this line of research with more complex models for dealing with data from competitions and metabolic fluxes.

Competition mechanism among *Saccharomyces* yeasts

Going back to competitions at low temperature fermentations, we observed how *S. cerevisiae* was able to dominate the culture in most cases despite sharing the media with species presenting better growth parameters under these temperature conditions. This motivated our transcriptomics analysis of *S. cerevisiae* and *S: kudriavzevii* in competition at very low temperature, 12 °C, and at temperature close to wine production, 20 °C, so that we could look for common factors being expressed at both temperatures in competition by *S. cerevisiae* to explain its domination in fermentation.

High levels of ethanol production due to the vigorous fermentative capacity of *S. cerevisiae* in what is called *the crabtree effect* has been considered the main agent allowing its imposition over the rest of the naturally present organisms in fermentation (Crabtree, 1928; Piškur et al., 2006; Thomson et al., 2005). However, in the recent past years, researches have gone in depth in the different possible mechanisms that make this possible. That has come, in part, together with the necessity of knowing the behavior of yeast species of interest for industry, which would allow for better designing of starter cultures. Maybe the most studied factors are killer toxins, in the case of *S. cerevisiae/S. cerevisiae* interactions and GADPH derived peptides in the case of *S. cerevisiae*/non-*Saccharomyces* interactions (Albergaria et al., 2010; Branco et al., 2014; Ciani et al., 2016a; Pérez-Nevado et al., 2006). Another of the elements proposed has to do with *S. cerevisiae* engineering its environment,

for instance, by increasing the temperature in microscopic regions within the fermentation, which would affect its closer relatives' fitness, and explains *S. cerevisiae* colonizing winemaking instead of other species (Buser et al., 2014; Goddard, 2008).

However, what seemed to be more relevant in the cases we studied, according to gene expression, is the acceleration of the consumption of certain nutritive resources and of the metabolism. As reviewed by Ghoul and Mitri (2016), these are two of the main mechanisms for competition among microbes. In fact, competition for nutrients has been investigated in the case of *S. cerevisiae* (Liu et al., 2015; Medina et al., 2012).

Sugar consumption acceleration was one of the main traits found in our results. A similar behavior was observed in a case competition between *S. cerevisiae* and *Starmerella bombicola* (Milanovic et al., 2012). More recently, is has been described as part of the transcriptomics response of *S. cerevisiae* to the presence of different non-*Saccharomyces* yeasts (Curiel et al., 2017; Tronchoni et al., 2017). However, that is the first time that this was experimentally demonstrated among *Saccharomyces* species.

Another of the main nutrients found in our dataset analysis was iron. The principal way of acquiring iron from the medium of *S. cerevisiae* is to convert it to its reduced form before transporting it into the cell. Another mechanism is to capture sideophores, chelating agents capable of the transported together with the Fe^{3+} without previous reduction into the cells. No siderophore synthesis capability has ever been reported by *Saccharomyces*, however, it is able to capture a diversity of this compounds bound to iron (Philpott and

Protchenko, 2008). Precisely, siderophore uptake activation has been reported as a competitive trait for different organisms (Cordero et al., 2012; Kummerli et al., 2009; Lesuisse et al., 1998; Morris, 2015). Here, we reported for the first time how *S. cerevisiae* and *S. uvarum* also activated genes related to iron homeostasis and iron and siderophore uptake when they share the fermentative niche with a competitor.

Furthermore, we observed this response to be strain dependent. In our analysis, competition for nutrients in the initial phase of the fermentation was only found for wine strains. We hypothesize that this competition trait evolved very recently, in environments linked to human activities. The acquisition of this trait would have been occurred independently for these species, since strains from other niches do not present it. Moreover, the regulation behind the expression of these genes, according to the set of transcription factors obtained for our experiments, does not seem to be the same in both species. Despite so, of course, further research is needed to be able to affirm the existence of these independent events.

One of the basics elements in the response to any abiotic or biotic stimulus is the sensing. Yeast-yeast interactions have been object of many studies (Albergaria and Arneborg, 2016), however, it is not clear how the response to competition is triggered, or even if there are more than one possible mechanism in *S. cerevisiae*. In fact, most of the studied competitive interactions correspond to constitutive processes such as ethanol production, killer toxin secretion, or GADPH derived peptides anchored to the cell wall (Branco et al., 2014; Ciani and Comitini, 2015; Pretorius, 2000).

The nature of these trigger molecules could correspond to, either soluble molecules secreted by the competitor organisms, or elements in the surface of yeast cells such as proteins or glycans. Those would be identified as strange molecules belonging to a competitor by the yeast, which would initiate the response. In our case, this is an important piece of the puzzle that remains still unknown. However, we proved that cell-to-cell contact was necessary to observe a decrease of fitness in the competitions between *S. cerevisiae* and *S. kudriavzevii*, meaning, according to our hypothesis, that the molecules responsible to the recognition and triggering of the response must be part of the cellular surface.

Recently, Rossouw et al. (2015) demonstrated the important role of the *FLO* family of proteins as drivers of microbial population dynamics during wine fermentation. The presence and genic expression levels of different *FLO* proteins marked the ability to flocculate, recruiting other yeast strains of interest, and improving its fitness in fermentation. Also, they hypothesized that the existence of different proteins with apparently the same function, and the important variation in their genomic sequence depending on the strain, might respond to the evolution under selective pressures linked to the competitive relationship among the organisms being part of interactions in an ecological niche.

Moreover, *FLO* proteins are linked to glycans that are important for cell adhesion (Bojsen et al., 2012; Kobayashi et al., 1998). Recently, a study revealed that glycan-glycan interactions are important for cellular self-recognition, playing an important role in mating (Goossens et al., 2015). Thus, we propose that, as a mechanism of yeast self-recognition, glycan-glycan and

glycan-*FLO* interactions could as well constitute a mechanism for the recognition of a strange yeast, or even distinguish whether this strange yeast is desirable for co-flocculation or, by the contrary, needs to be outcompeted from the niche.

In the future, the identification of the molecular interactions responsible for the yeast population dynamics in fermentation could be a powerful tool for the designing of starter cultures in wine industry. Together with the predictive modelling of certain fermentation systems, we could be able to choose among unlimited combinations of yeast strains, in order to obtain the desired wine composition with regard to certain compounds, or even to the duration or the costs of a fermentative process.

CONCLUSIONS

The most relevant conclusions drawn from the results obtained in this thesis are:

1. Temperature remarkably affects competitive fitness. *S. cerevisiae* fitness increases with temperature under the studied range. Consequently, cryotolerant species show better fitness at low temperature, especially *S. uvarum*, which is able to dominate the fermentation at 12 °C despite presence of *S. cerevisiae*. That agrees with the fact that *S. uvarum* is found as the main responsible for the fermentation of some wines from cold regions. However, *S. cerevisiae* is able to outcompete *S. paradoxus*, *S. eubayanus* and *S. kudriavzevii* even at temperatures to which cryotolerant species are better adapted.
2. There is a notable difference on the competitive performance of strains from a species depending on their origin. That was observed both for *S. cerevisiae* and *S. uvarum*. Wine strains were able to dominate the fermentation under conditions where non-fermentative strains were not. That implies recent evolution of this trait in strains from human-driven environments.
3. Growth kinetics parameters not always correlate with competitive fitness. That means there are other elements than temperature or adaptation to media abiotic stresses influencing yeast interactions.
4. Fermentations carried out by *S. cerevisiae* in co-culture with *S. kudriavzevii* or *S. uvarum* improved kinetics parameters of the fermentation and generally yield lower levels of ethanol and higher of glycerol. Co-fermentation of *S. cerevisiae* and *S. kudriavzevii* at 12 °C was especially promising; with about 2 degrees of ethanol reduction

and 2 g/L increase in glycerol concentration at the end of the fermentation. On the contrary, co-fermentations including *S. paradoxus* or *S. eubayanus* presented comparatively less favorable composition than the wine *S. cerevisiae* reference.

5. *S. kudriavzevii* gained an enhanced competitive performance against *S. cerevisiae*; at regular winemaking temperature, when it was inoculated at higher proportions (9:1), and when *S. cerevisiae* was inoculated 24 hours after the initial inoculation of *S. kudriavzevii* alone.
6. Air supply notably enhanced biomass production of both *S. kudriavzevii* and *S. cerevisiae* in fermentation.
7. Air supply of 20 VVH synergistically improved *S. kudriavzevii* competitive fitness when combined with higher proportions in the initial inoculum.
8. All these strategies to improve *S. kudriavzevii* performance also achieved reductions of the ethanol yield and increases of glycerol concentration during fermentation. Moreover, air supply promoted a notable acceleration of fermentation kinetics. However, acetic acid concentration increased dramatically, presumably due to respiration.
9. *S. cerevisiae* showed a stronger and more efficient transcriptomic response than its competitor *S. kudriavzevii* at the early exponential phase, regardless of the temperature of fermentation. This response consisted on a deep remodeling of the transcriptome, with respect to single culture, to accelerate nutrient uptake and cell proliferation. This allowed *S. cerevisiae* to dominate the fermentation and turn off the response in later stages.
10. *S. kudriavzevii* response to competition at the early exponential phase

was much lighter than that exhibited by *S. cerevisiae*. Multiple genes involved in *S. kudriavzevii* response to starvation and stress appeared at late exponential and stationary phase. This agrees with its severely decreased fitness when sharing the fermentative environment with *S. cerevisiae*.

11. HPLC analysis confirmed the role of nutrient utilization as driver of the domination of *S. cerevisiae* over *S. kudriavzevii*. Nitrogen sources consumption was led by *S. cerevisiae* during the competition. In addition, an accelerated sugar uptake at 12 °C was observed, probably due to the strong response showed by *S. cerevisiae*, involving deep remodeling of metabolism and plasma membrane, concordant with the observed transcriptomic response.
12. The response to the presence of a competitor is mediated by direct cell-to-cell contact. Only when placed in the same compartment of fermentation, *S. cerevisiae* and especially *S. kudriavzevii* showed a decreased fitness. This did not happen when both species were in compartments separated by a dialysis membrane.
13. Nutrients uptake is also part of the *S. cerevisiae* response to the presence of *S. uvarum*.
14. The wine strain *S. uvarum* BMV58 response to competitors also seems to rely on an enhanced nutrient uptake, especially heavy metals and sulfur compounds, as observed for the wine *S. cerevisiae* yeast.
15. Therefore, response to competition during fermentation is a strain dependent trait both in *S. cerevisiae* and *S. uvarum*.

MATERIAL AND METHODS

1. Yeast strains

Seven different *Saccharomyces* strains were used in our experiments. We chose a commercial strain, T73 (Lalvin T73 from Lallemand Montreal, Canada), as our wine *S. cerevisiae* representative. We also included YPS128, a *S. cerevisiae* strain isolated from Pennsylvania woodlands, which is a well characterized and genome sequenced (BioProject reference PRJEB7245) strain; *S. paradoxus* strain 54, isolated from Croatian vineyards; two *S. uvarum* strains, BMV58, selected in our laboratory and commercialized for winemaking (VELLUTOBMV58^{TM} from Lallemand), and CECT12600, isolated from a non-fermented beverage (*mistela*) in Alicante, Spain; *S. eubayanus* strain NPCC1292 is a natural isolate from North Patagonian *Mudai*, traditional fermentation made with *Araucaria araucana* seeds; and *S. kudriavzevii* strain CR85, a natural isolate from oak tree bark in Agudo, Ciudad Real, Spain, characterized by being closely related to the parent of the wine hybrids *S. cerevisiae x S. kudriavzevii* and by its good performance in microvinification (Peris et al., 2016). Its Genome is available at NCBI repository (BioProject reference PRJNA480800).

2. Competition experiments set up

Overnight precultures were grown in GPY (2% glucose, 2% peptone, 1% yeast extract) medium at 25 °C. After that, cells were inoculated to synthetic must (SM, **Table 2**), which is frequently used in microvinification experiments (Riou et al., 1997). Fermentations were performed in 3x or 6x replicates in 250 mL flasks that contained 200mL of SM. Flasks were incubated at a fixed temperature (8, 12, 20 or 25 °C) with agitation at 100 rpm. Müller valves were

used to monitor fermentation stage through weight loss, until it reached a constant weight, when it was considered to be over.

Table 2| SM composition (1L)

SUGARS	g
Glucose	100.00
Fructose	100.00
ACIDS	
Malic	5.00
Citric	0.50
Tartaric	3.00
SALTS	
KH2PO4	0.75
K2SO4	0.50
MgSO4 · 7 H2O	0.25
CaCl2 · 2 H2O	0.16
NaCl	0.20
NITROGEN SOURCE	
NH4Cl	0.46
Aa stock buffer	13.09
OLIGOELEMENTS	
Oligoelements stock solution	1
VITAMINS	
Vitamin stock solution	10

AMINO ACID STOCK SOLUTION	g
Tyrosine (Tyr)	1.5
Tryptophan (Trp)	13.4
Isoleucine (Ile)	2.5
Aspartic Acid (Asp)	3.4
Glutamic Acid (Glu)	9.2
Arginine (Arg)	28.3
Leucine (Leu)	3.7
Threonine (Thr)	5.8
Glycine (Gly)	1.4
Glutamine (Gln)	38.4
Alanine (Ala)	11.2
Valine (Val)	3.4
Methionine (Met)	2.4
Phenylalanine (Phe)	2.3
Serine (Ser)	6
Histidine (His)	2.6
Lysine (Lys)	1.3
Cysteine (Cys)	1.5
Proline (Pro)	46.1

OLIGOELEMENTS STOCK SOLUTION	g
$MnSO_4$ H_2O	4
$ZnSO_4$ $7H_2O$	4
$CuSO_4$ $5H_2O$	1
Kl	1
$CoCl_2$ $6H_2O$	0.4
H_3BO_3	1
$(NH_4)_6Mo_7O_{24}$	1

VITAMIN STOCK SOLUTION (1 L)	g
Myo-inositol	2
Calcium pantothenate	0.15
Thiamine hydrochloride	0.025
Nicotinic acid	0.2
Pyridoxine	0.025
***Biotin (stocking solution 100 mg L-1)**	0.0003

In our competition experiments, we included a *S. cerevisiae* strain, either T73 or YPS128, and a non-*cerevisiae* one, and measured their relative abundance at different fermentation time points either by quantitative polymerase chain reaction (QPCR) or by plating under selective conditions as explained below. As controls, we monitored the growth of each strain in monocultures under the same conditions as the competitions experiments.

In experiments with an initial ratio of strains 1:1, initial cell density of each of the strains was 10^6 cells/mL. For fermentations with different *S. cerevisiae* / *S. kudriavzevii* ratio (3:7 and 1:9) total cell density was $2 \cdot 10^6$ cells/mL. At last, the condition in which *S. cerevisiae* was inoculated after 24 hours in a proportion of 1% with respect to *S. kudriavzevii*, initial concentration of *S. kudriavzevii* was 10^6 cells/mL.

Yeast cells were collected at different moments during fermentation and kept at -20 °C to determine the proportion of the involved yeast strains. Supernatants of the samples were also stored at -20 °C for the analysis of wine composition by HPLC. In the case of extraction for RNA isolation and sequencing, cells were stored at -80 °C.

3. Determination of different strains abundance through QPCR

Primer design

Alignments of homologous chromosomes from *S. cerevisiae* S288c, *S. paradoxus*, *S. kudriavzevii*, and *S. uvarum* were carried out by the Mauve alignment tool (Darling et al., 2004). Genomic sequences were downloaded from the *Saccharomyces Sensu Stricto Resources* website (Scannell et al., 2011) and *Saccharomyces Genome Database* (Engel, 2013). By way of example, a SNPs map of the gene *BUD3* of *S. paradoxus*, *S. kudriavzevii*, and *S. uvarum* individually aligned against *S. cerevisiae* is shown in **Figure 9**. This highly conserved single copy gene was selected to look for strain-specific pairs of primers (**Table 3**). All the resulting amplicons were approximately 100 bp in length and had a similar melting temperature when detected with our *LightCycler© 480 II* instrument.

Specificity of the PCR assay

Total DNA samples were extracted from yeasts as described below. PCRs were carried out in a 20 µL final volume, including 1 µL of the DNA template, 0.25 µM of each primer, 200 µM of each dNTP, 2.5 mM of $MgCl_2$, 10X buffer and 0.75 U of *Taq DNA polymerase* (Takara, Bio, Shiga, Japan). For each case, total DNA from the competitor strain was used as a crossed amplification control.

The PCR program consisted of an initial denaturalization step at 94 °C for 5 min, followed by 30 cycles of a denaturalization step at 94 °C, an annealing step at either 53 °C or 54 °C for 1 min, an extension step at 72 °C for 10 sec, and a final extension step at 72 °C for 5 min. PCR products were analyzed by

electrophoresis on a 1.5% (w/v) agarose gel stained with *RealSafe™ nucleic acid staining solution (20,000X)*(Chembio Diagnosis Systems, Medford, NY, USA) in 1x TAE buffer, and were visualized under UV light. A 100-bp DNA ladder marker (Invitrogen™, Carlsbad, CA, USA) was used as the size standard.

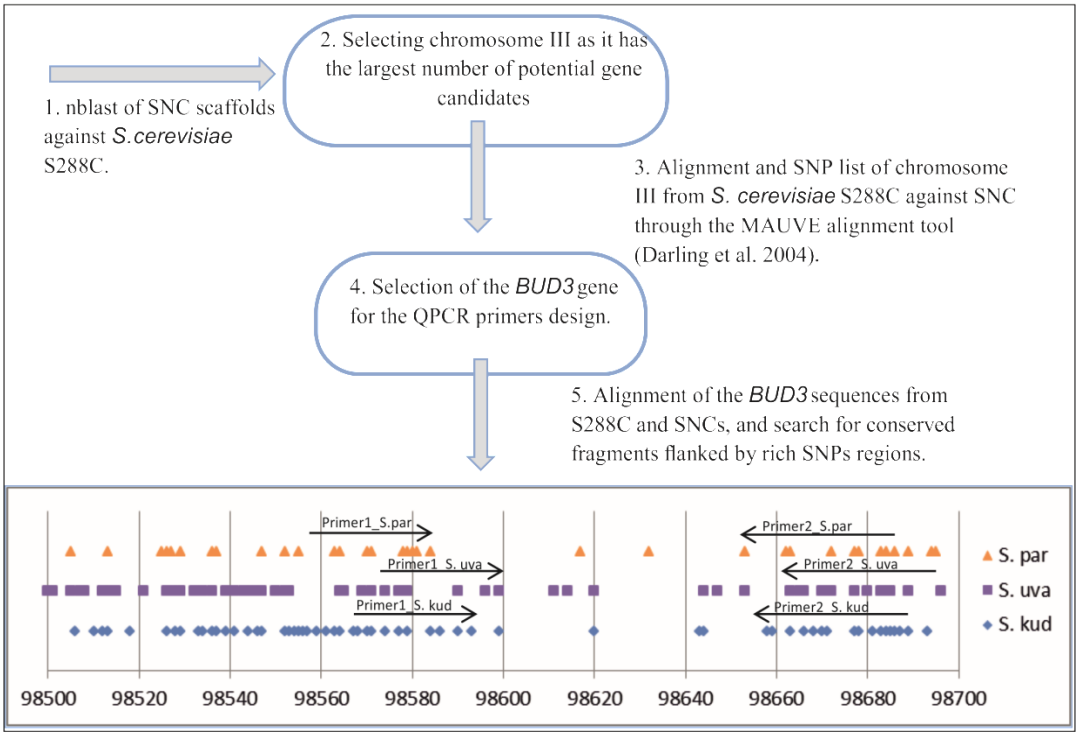


Figure 9| Scheme used for QPCR primers design.

DNA extraction and sample preparation for QPCR

Total DNA samples from all the yeasts were extracted as described elsewhere (Querol et al., 1992a). The concentration of the DNA samples was measured in a Nanodrop spectrophotometer ND-1000 (Nanodrop Technologies™, Wilmington, DE. USA) and adjusted to 20 ng/µL.

Table 3| List of primers used for QPCR experiments according to their target strains. The field *Group* indicates which primers have been used together in our qPCR reactions due to lack of cross amplification between them.

Primer	Target strains	Group	Sequence (5' → 3')
Sc-F	*S. cerevisiae* T73	A	CGATTTCGATGCTACTCACG
Sc-R	*S. cerevisiae* T73	A	TTATCGCCTGATGGACTGTC
Sk-F	*S. kudriavzevii* CR85	A/B	AATTTAGGTGCCACCCACG
Sk-R	*S. kudriavzevii* CR85	A/B	TTATCTGCCGGTGAACTCAC
Su-F	*S. uvarum* BMV58/CECT12600	A	ACAACCGTATAGTGGCAGG
Su-R	*S. uvarum* BMV58/CECT12600	A	AATCTTCTCACAACGGTGGC
Sc-F2	*S. cerevisiae* T73	B	GATTTCGATGCTACTCACGAG
Sc-R2	*S. cerevisiae* T73/YPS128	B	TTACTATTATCGCCTGATGGAC
ScYPS128-F	*S. cerevisiae* YPS128	B	CTGATTTCGATGCTACTCACG
Se-F	*S. eubayanus* NPCC1292	B	AGA CCG GCT GAT CTA CTG G
Se-R	*S. eubayanus* NPCC1292	B	GAC GCT ACT TTG ATG TCA TCC
Sp-F	*S. paradoxus* 54	B	GAAGACGACGGAATCATCAC
Sp-R	*S. paradoxus* 54	B	TCACCAGTCAGAATTGCAGG

QPCR analysis

PCR amplification was performed in a 10 µL final volume that contained 2.5 µL of the DNA template, 1.5 µL MilliQ water, 0.2 µM of each primer, and 5 µL of *LightCycler© 480 SYBR Green I Master* (Roche). Reactions were performed in 96-well plates in an *LightCycler© 480 (II)* PCR amplification and detection instrument with an initial denaturalization step at 95 °C for 5 minutes, followed by 45 cycles of 95 °C for 10 seconds, either 53 °C or 54 °C for 10 seconds and 72 °C for 4 seconds. The C_T values were automatically calculated by the instrument.

Plates were always divided into two symmetric halves. In each one, a different reaction mix was used where the pair of primers was specific for one of the two strains. For each half, an internal standard curve was included, made of six serial

dilutions of the mixed total DNA from both competing strains in 1:1 proportions, the total DNA from the strain amplified in this half as a positive control, the total DNA from the other strain in competition as a control for cross amplification, and a negative control with PCR grade water instead of the template DNA. Three to six biological replicates were used.

The relative concentration of both strains in each biological replicate was given by the ratio of the means of the technical replicates concentrations calculated by the *LightCycler 480 instrument software 1.5* (Roche Diagnosis, Darmstadt, Germany).

Method sensitivity

For every competition experiment, the following test was performed to assess the reliability of our method. The mix of cells of the corresponding strains was prepared from overnight GPY precultures in known proportions (10:90, 30:70, 50:50, 70:30, 90:10). The QPCR analysis was carried out using total DNA extraction samples from the mixes of cells. The relative concentration of both strains in each sample was given by the ratio of the means of the concentrations of the replicates given by the *LightCycler 480 instrument software 1.5* (Roche Diagnosis, Darmstadt, Germany). Three biological replicates were included.

Linear model adjustments were made for the cell proportions estimated with each used pair of primers against the theoretical values, and for all the collected data as a whole. The function *lm()* from R (Team, 2018) was used for this purpose.

4. Relative intrinsic growth rate determination

The intrinsic growth rate (*r*) can be calculated as in a previous work of Williams et al. (2015). Here the same method was followed with some modifications:

$$N_t = N_0 e^{rt}$$

where N_t is cell density at a given time point, N_0 corresponds to the initial cell density, and *t* is the time (in hours) when both strains reached their highest cell density in both competition and monoculture.

The effect that competition has on the involved strains can be assessed as the difference in their intrinsic growth rate in single culture and in competition ($\Delta r = r_{single} - r_{competition}$). For the sake of better clarifying the results, the relative intrinsic growth rate ($R\Delta r = \Delta r / r_{single}$) was determined.

5. Determination of growth kinetics parameters for each strain

On day 1, the precultures of all the used strains were grown o/n at 25 °C in GPY medium. On day 2, cells were harvested by centrifugation, washed, suspended in dH_2O and diluted to an OD_{600} of 2.7. Next 10 µL from each dilution were pipetted into one well of a 96-well plate, previously filled with 260 µL of SM (10 replicates). Four wells were filled with only sterile SM as a blank for the OD_{600} measurements. Four plates were set, one for each assayed temperature: 8 °C, 12 °C, 20 °C and 25 °C.

OD_{600} was monitored in a SPECTROstar Omega instrument (BMG Labtech, Offenburg, Germany). Frequency of measurements varied according to temperature in order to obtain sufficient data points for a statistically significant

adjustment to the reparametrized Gompertz equation proposed by Zwietering et al. (1990), which takes this expression:

$$\boldsymbol{y = D \times e\text{^}\{-e\text{^}[((\mu max \times e)/D) \times (\lambda - t) + 1]\}}$$

where y = ln (OD_t/OD_0), OD_0 is the initial OD and OD_t is the OD at time t, D is the asymptotic maximum, the equivalent to ln (OD_{max}/OD_0), μ_{max} is the maximum specific growth rate (h^{-1}) and λ is the lag phase period (h). An adjustment was made using a nonlinear regression procedure of minimizing the sum of the squares of the difference between the experimental data and the fitted model. This was done using version 7.0 of the Statistica software (Stat-Soft, Inc., Tulsa, OK, USA).

Strains were tested for the significant differences among them with an ANOVA using the one-way ANOVA module of the Statistica 7.0 software. Growth parameters μ_{max} and λ were introduced as dependent variables. Means were grouped using the Tukey HSD test ($\alpha = 0.05$).

6. Correlation of relative intrinsic growth rate and growth kinetics parameters

Linear regression models ($y = Ax_1 + B$ and $y = Ax_2 + B$) were constructed, where y = $R\Delta r$ for the non-*cerevisiae* strain, $x_1 = (\mu_{\max_{competitor}} - \mu_{\max_{S.cerevisiae}})/\mu_{\max_{competitor}}$ ($R\Delta\mu$) and $x_2 = (\lambda_{S.cerevisiae} - \lambda_{competitor})/\lambda_{competitor}$ ($R\Delta\lambda$). This was done using the R function *lm* (Team, 2018).

7. Determination of the fermentation kinetics

Here a distinction between **Chapter I** and **Chapter III** for kinetics analysis of fermentation needs to be made. In **Chapter I**, the end point concentrations of glucose and fructose were obtained by HPLC. The recorded mass loss of the fermentation flasks due to CO_2 release correlates with sugar consumption, which was taken into consideration to fit our curve to Gompertz equation (Zwietering et al., 1990) and obtain fermentation parameters *m* (maximum sugar consumption rate, g L^{-1} h^{-1}), *l* (lag phase period, h) and t_{90} (time taken to consume 90% of sugars, h) as in Pérez-Través *et al.* (2014a) for the fermentations in **Chapter I**.

In **Chapter III**, glucose and fructose concentrations were measured by HPLC at different time points along the fermentation. This data was fitted by means of the three following mathematical equations as in (Tronchoni et al., 2009):

1. A linear decay function:

$$Y = S_0 - K * t$$

Where *Y* is the percentage of glucose or fructose still present in must, *t* is the time (hours), S_0 is the value of interception in the origin, and *K* is the kinetic constant.

2. An exponential decay function:

$$Y = D + S * e^{-K * t}$$

Where *Y* is the percentage of glucose or fructose still present in must, *t* is the time, *D* is a specific value when *t* tends to infinity, *S* is the estimated value of change, and *K* is the kinetic constant.

3. A sigmoid or altered Gompertz decay function:

$$Y = A + C * e^{-e^{(K*(t-M))}}$$

Where *Y* is the percentage of glucose or fructose still present in must, t is the time, *A* is the lower asymptote when *t* tends to infinity, *K* is the kinetic constant, *C* is the distance between the upper and lower asymptote, and *M* is the time when the inflection point is obtained.

Equations were fitted by means of linear and nonlinear regression procedures with the R function *nls* (Team, 2018), minimizing the sum of squares of the difference between the experimental data and the fitted model. Fit adequacy was checked by the proportion of variance explained by the model (R^2) respect to the experimental data. For each yeast and temperature, the three equations were tested, but only the function with the highest R^2 was chosen. Subsequently, these equations were used to calculate the time necessary to consume 90% of the initial sugar concentration present in must (t_{90}).

Finally, kinetics parameters were tested for significant differences among by ANOVA using the one-way ANOVA module of the Statistica 7.0 software. Mean values were grouped using the Tukey HSD test ($\alpha = 0.05$).

8. Determination of metabolites by means of HPLC

Glucose, Fructose, Ethanol, Glycerol and Acetic Acid concentrations along the fermentation were determined by HPLC (Thermo Fisher Scientific,Waltham, MA, USA) using a refraction index detector and a HyperREZTM XP Carbohydrate H + 8µm column (Thermo Fisher Scientific) equipped with a HyperREZTM XP Carbohydrate Guard (Thermo Fisher Scientific). Samples were appropriately diluted, filtered through a 0.22-µm nylon filter (Symta, Madrid, Spain), and injected in two technical replicates. The analysis conditions

were: eluent, 1.5mM of H2SO4; 0.6ml min-1 flux and an oven temperature of 50◦C.

Amino acids and ammonium were determined by High Performance Liquid Chromatography (HPLC, Thermo Scientific Dioned ultimate 3000 series, Waltham, MA, USA). Separation was made in a Thermo Scientific Accucore C18 column (4.6mm*150mm particle size 2.6um) following the method described in Gómez-Alonso *et al.* (2007).

Fermentations were tested for the significant differences among metabolites concentrations by ANOVA using the one-way ANOVA module of the Statistica 7.0 software. Mean values were grouped using the Tukey HSD test (α=0.05).

9. Microaerated fermentations

Aeration system is composed of a compressed air generator, 3.1 mm internal diameter silicon tubes, 0.2 µm pore-size filters, a flow meter and a set of flow regulators (one for each bottle) as depicted in **Figure 10**. All the experiments were conducted in triplicate at 25 °C with gentle shaking (100 rpm).

Four different flow rates conditions were applied: 1 VVH, 5 VVH, 10 VVH and 20 VVH to determine the best aeration condition. These values were chosen according to previous a previous publication (Morales et al., 2015). To the different set of competitions in different proportions we applied a flow rate of 20 VVH during the first 48 hours of fermentation, after what we stopped aeration.

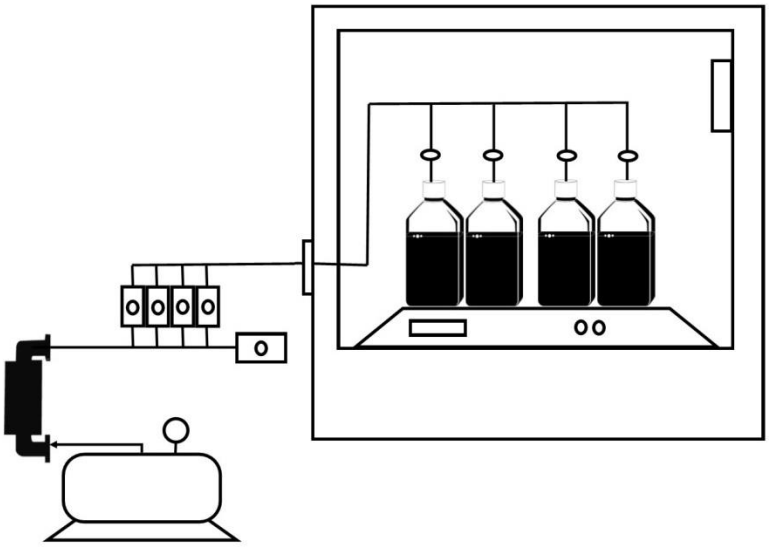


Figure 10| Scheme of aeration system. (1) Compressed air generator; (2) Silicon tubes; (3) Filters; (4) 515 Flow meter; (5) Set of flow regulators; (6) Shaker; (7) Incubator with temperature control; (8) 516 Thermometer; (9) Bottle bioreactor.

A bottle containing distilled water and another one with water and 5% (v/v) ethanol were set as control for evaporation and ethanol loss due to aeration. Water and ethanol losses were considered as lineal with respect to time. Deviation factors were dimensioned in bottles with 5% (w/v) ethanol in 400 mL water, and bottles with 400 mL of water, all them with air supply (20 VVH). Water mass loss followed a lineal equation (R^2 = 0.99569):

$$\boldsymbol{y = 0.1684t} \text{ (}\textit{Equation 1}\text{)}$$

where *y* refers to weight loss due to H_2O evaporation in bottles with only water and *t* refers to time.

$$\boldsymbol{y = 0.2532t}$$ (*Equation 2*)

where *y* refers to weight loss due to H_2O and ethanol evaporation in bottles with 5% (w/v) ethanol and *t* refers to time. HPLC measures of the last were taken at different time points. We observed that ethanol loss followed a lineal function, and that a subtraction of the equation for ethanol bottle minus the one for water bottle, very precisely predicted HPLC results. The calculation was done following equations 3, 4 and 5:

$$\boldsymbol{F_1 = \frac{((a_1 - a_2) \times 100)}{20}}$$ (*Equation 3*)

where F_1 is factor 1 for ethanol correction (% h^{-1}), a_1 is the slope of *Equation 1*, a_2 is the slope of *Equation 2*, and 20 is the value for the total mass of ethanol weighted for 400 mL of solution.

$$\boldsymbol{F_2 = \frac{(F_1 \times t) \times E_{HPLC}}{20}}$$ *(Equation 4)*

where F_2 is factor 2 for ethanol correction (%), *t* is the time corresponding to an assessed value and E_{HPLC} is the HPLC measure for ethanol concentration.

$$\boldsymbol{E_C = \frac{(F\_2 + E\_HPLC).[V\_T - (a2 \times t)]}{V_T}}$$ *(Equation 5)*

where E_C is corrected ethanol concentration (%).

The rest of compounds in our system were assumed as nonvolatile, however, their concentration values were considered as affected by water and ethanol volume losses. To calculate this concentration factor, the density of must was

considered to be equal to the density of water. HPLC values for glucose, fructose, glycerol and acetic acid were corrected using the following equation:

$$C_C = \frac{C_{HPLC} \times 1000}{(1000 + (a_2 \times t) + [(E_C - E_{HPLC}) \times 10]} \quad \textit{(Equation 6)}$$

where C_C is the corrected concentration for the compound.

10. RNA Sequencing

Sample collection for gene expression profiling was done at three different fermentation times: early exponential phase (EEP), late exponential phase (LEP) and stationary phase (SP). These correspond to 24, 90 and 135 h, respectively, for 12 °C fermentations and to 12, 24 and 50 h, respectively, for 20 °C fermentations. Cells where centrifuged and stored at 80 °C. RNA isolation was performed with the High Pure RNA Isolation kit (Roche Applied Science, Germany). After oligo (dT) mRNA purification, RNAseq libraries were generated with the TruSeq Stranded mRNA Library Preparation Kit (Illumina, CA, USA). A pool of the libraries from the samples of the single *S. cerevisiae* T73 and *S. kudriavzevii* CR85 fermentations, and the *S. cerevisiae* T73 / *S. kudriavzevii* CR85 co-cultures was sequenced on a NextSeq Sequencing System from Illumina (2 x 150 bp). Another pool including the single *S. cerevisiae* YPS128 culture and the *S. cerevisiae* YPS128 / *S. kudriavzevii* CR85 co-culture was also sequenced on a separate batch. All raw reads have been deposited under the BioProject PRJNA487511. In this second batch, we also included the samples of the competitions and single cultures of the strains from *S. uvarum* BMV58 and CECT12600.

Pair end and read length sequencing allowed to effectively separate sequences coming from genomes with high identity. The large amount of reads that were generated in the process, granted obtaining enough data from the less represented transcriptomes in competitions.

11. RNAseq and Differential Gene Expression Analysis

Sequence reads from the *S. cerevisiae* T73 and *S. kudriavzevii* CR85 experiment were mapped to a combined reference of both genomes using Bowtie2 v. 2.2.9 (Langmead, 2013). Similarly, sequences from *S. cerevisiae* YPS128 and *S. kudriavzevii* CR85 experiment were mapped to a combined reference of those two genomes. The same approach was applied to the competitions of *S. cerevisiae* T73 and both *S. uvarum* strains. The genomes of *S. cerevisiae* T73 and *S. kudriavzevii* CR85 were previously sequenced and annotated in our laboratory (M. Morard, unpublished), and contained 6009 and 5537 genes, respectively, according to the RATT tool (Otto et al., 2011), refined by manual editing. Of them, 5414 genes were orthologous in both species. The genomes of *S. uvarum* BMV58 and *S. uvarum* CECT12600 were also sequenced and annotated by our laboratory members by the same procedures. 5541 and 5540 genes were annotated for them, respectively. The reference genome sequence of *S. cerevisiae* YPS128 was obtained from Liti *et. al* (2009), but the annotation was revised in our laboratory. Read counts for each gene were obtained using HTSeq-Count (HTSeq-0.6.1p1, -m intersection-nonempty) (Anders et al., 2015). We obtained on average 13.5 million reads per strain and sample, with a range of 1.3 to 28.5 million after removing one expression outlier replicate of the sample *S. cerevisiae* T73 / *S. kudriavzevii* CR85, 12 C, EEP. We observed a median of 1034 reads per gene across all 130 samples.

Differential gene expression was estimated by using the R package *DESeq2* (Love et al., 2014), based on fitting genes to a generalized linear model (GLM) to obtain maximum-likelihood estimates for the log fold changes (LFCs), to then acquire *maximum a posteriori values* from a second GLM round, which correspond to the final LFC values. Then, Wald tests were performed for differential expression by contrasting two groups, mono- vs. co-cultures at each sampling time and temperature in most of the cases, as detailed in the Results section. Subsequently, Wald test p-values were adjusted for multiple testing, using the approach of Benjamini and Hochberg (1995). The resulting adjusted p-values were used as our reference p-values in the Results section.

Functional enrichment analyses were performed with the web tool *FunSpec* (Robinson et al., 2002), specially designed for yeast datasets. This tool calculates the probability that a *Gene Ontology* (GO) or *Munich Information Centre for Protein Sequences* (MIPS) term is enriched in a given list of genes using the hypergeometric distribution. A Bonferroni correction was applied to compensate the problem of multiple comparisons.

For principal component analyses (PCA), a variance stabilizing transformation dataset from the $\log_2$fold scale normalized data given by *DESeq2* was used. This function, included in the same R package, allows a more efficient clustering of samples into groups.

The expression matrix for heatmap building was obtained with the function *getVarianceStabilizedData* from the *DESeq2* package. Data was scaled to study the variation of each gene in the given set of samples. The *war.D* clustering method for Euclidean distance matrices was performed. We summarize the

main biological processes with a reasonable number of categories that included all the analysed genes.

12. Compartmentalized fermentations

Dialysis tubes (VISKING® dialysis tubing RC diameter 28 mm, cut-off MWCO = 12.000-14.000, SERVA Electrophoresis GmbH, Germany) were used to create an inner compartment of 20 mL of synthetic must located inside 250-mL capacity screw cap bottles with 180 mL of SM (outer compartment), as described elsewhere (Wang et al., 2015b). This way, metabolite and other solute can be exchanged between two yeast populations, inoculated and cultured in the separated compartments. The different fermentation inoculation patterns are shown in **Table 4**. Each compartment was inoculated to reach an initial concentration of 10^6 cells/mL of each strain. Bottles were incubated at 20 ºC and 100 rpm. Cell viability was measured by plating into GPY-agar plates at different time points up to 60 hours of fermentation. After that, cell deposition in the bottom of the membrane prevented us from obtaining decent reproducibility among replicates. In the case of fermentations with both cell types in contact in contact, two technical replicates were done; one of them incubated at a non-selective temperature (25 ºC), and the other at a selective temperature (37 ºC) at which only *S. cerevisiae* can grow. This way, selective temperature cultures can be used to determine the CFU for *S. cerevisiae*, and subtracting this value from the total CFU obtained in the non-selective plates, we can estimate CFU for *S. kudriavzevii* CR85.

To measure fitness, we calculated the intrinsic growth rate (*r*) using the exponential growth equation (Williams et al., 2015):

$$N_t = N_0 * e^{r*t}$$

Where N_t is final cell density (CFU/mL), N_0 is initial cell density (CFU/mL), and t is time in hours. Fermentation time 60 hours was used to estimate the intrinsic growth rate because it showed the lowest deviation among replicates.

Table 4| Summary of compartmentalized fermentations performed.

	MONOCULTURE			Separated				IN Contact	
INNER COMPARTMENT	ScT73	ScYPS128	SkCR85	ScT73	SkCR85	ScYPS128	SkCR85	ScT73, SkCR85	ScYPS128, SkCR85
OUTER COMPARTMENT	ScT73	ScYPS128	SkCR85	SkCR85	ScT73	SkCR85	ScYPS128	ScT73, SkCR85	ScYPS128, SkCR85

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ANNEX I: ABBREVIATIONS

λ: growth lag phase

μ_{max}: maximum growth rate

AAB: Acetic Acid Bacteria

ABC: ATP-binding Cassette

ATP: Adennosine Triphosphate

CFU: Colony Former Unit

co: competition culture

C_T: Cycle Threshold

DNA: Deoxyribonucleic Acid

dNTP: deoxinucleoside triphosphate

dsRNA: double stranded Ribonucleic Acid

EEP: Early Exponential Phase

ESR: Environmental Stress Response

FSR: Fermentation Stress Response

GLM: General Lineal Model

GMO: Genetically Modified Organism

GO: Gene Ontology

GPY: Glucose Peptone Yeast extract

GRAS: Generally Recognized As Safe

HPLC: High Performance Liquid Chormatography

HSP: Heat Shock Protein

***l*:** fermentation lag phase

LAB: Lactic Acid Bacteria

LEP: Late Exponential Phase

***m*:** maximum growth rate of sugar consumption

MIPS: Munich Information Center for Protein Sequences

MLF: Malolactic Fermentation

mono: single culture

NGS: New Generation Sequencing

OD_{600}: Optical density at 600 nm wavelength

ORF: Open Reading Frame

PCA: Principal Component Analysis

QPCR: Quantitative Polymerase Chain Reaction

r: Intrinsic growth rate

RΔλ: Relative increase of lag phase

RΔμ: Relative increase of maximum growth rate

RΔr: relative increase of intrinsic growth rate

R^2: coefficient of determination

RNA: Ribonucleic Acid

RNAseq: RNA sequencing

ROS: Reactive Oxygen Species

SM: Synthetic Must

SNC: *Saccharomyces* non-*cerevisiae*

SNP: Single Nucleotide Polymorphism

SP: Stationary Phase

***t_{90}*:** time to consume 90% of initial sugar content

TOR: Target Of Rapamincin

UPR: Unfolded Protein Response

VVH: 10 gas volumes/culture volume/hour

ANNEX II: PUBLICATIONS

frontiers in Microbiology

ORIGINAL RESEARCH
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Effect of Temperature on the Prevalence of *Saccharomyces* Non *cerevisiae* Species against a *S. cerevisiae* Wine Strain in Wine Fermentation: Competition, Physiological Fitness, and Influence in Final Wine Composition

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Saccharomyces cerevisiae is the main microorganism responsible for the fermentation of wine. Nevertheless, in the last years wineries are facing new challenges due to current market demands and climate change effects on the wine quality. New yeast starters formed by non-conventional *Saccharomyces* species (such as *S. uvarum* or *S. kudriavzevii*) or their hybrids (*S. cerevisiae* x *S. uvarum* and *S. cerevisiae* x *S. kudriavzevii*) can contribute to solve some of these challenges. They exhibit good fermentative capabilities at low temperatures, producing wines with lower alcohol and higher glycerol amounts. However, *S. cerevisiae* can competitively displace other yeast species from wine fermentations, therefore the use of these new starters requires an analysis of their behavior during competition with *S. cerevisiae* during wine fermentation. In the present study we analyzed the survival capacity of non-*cerevisiae* strains in competition with *S. cerevisiae* during fermentation of synthetic wine must at different temperatures. First, we developed a new method, based on QPCR, to quantify the proportion of different *Saccharomyces* yeasts in mixed cultures. This method was used to assess the effect of competition on the growth fitness. In addition, fermentation kinetics parameters and final wine compositions were also analyzed. We observed that some cryotolerant *Saccharomyces* yeasts, particularly *S. uvarum*, seriously compromised *S. cerevisiae* fitness during competences at lower temperatures, which explains why *S. uvarum* can replace *S. cerevisiae* during wine fermentations in European regions with oceanic and continental climates. From an enological point of view, mixed co-cultures between *S. cerevisiae* and *S. paradoxus* or *S. eubayanus*, deteriorated fermentation parameters and the final product composition compared to single *S. cerevisiae* inoculation. However, in co-inoculated synthetic must in which *S. kudriavzevii* or *S. uvarum* coexisted with

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The Use of Mixed Populations of *Saccharomyces cerevisiae* and *S. kudriavzevii* to Reduce Ethanol Content in Wine: Limited Aeration, Inoculum Proportions, and Sequential Inoculation

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Saccharomyces cerevisiae is the most widespread microorganism responsible for wine alcoholic fermentation. Nevertheless, the wine industry is currently facing new challenges, some of them associate with climate change, which have a negative effect on ethanol content and wine quality. Numerous and varied strategies have been carried out to overcome these concerns. From a biotechnological point of view, the use of alternative non-*Saccharomyces* yeasts, yielding lower ethanol concentrations and sometimes giving rise to new and interesting aroma, is one of the trendiest approaches. However, *S. cerevisiae* usually outcompetes other *Saccharomyces* species due to its better adaptation to the fermentative environment. For this reason, we studied for the first time the use of a *Saccharomyces kudriavzevii* strain, CR85, for co-inoculations at increasing proportions and sequential inoculations, as well as the effect of aeration, to improve its fermentation performance in order to obtain wines with an ethanol yield reduction. An enhanced competitive performance of *S. kudriavzevii* CR85 was observed when it represented 90% of the cells present in the inoculum. Furthermore, airflow supply of 20 VVH to the fermentation synergistically improved CR85 endurance and, interestingly, a significant ethanol concentration reduction was achieved.

Keywords: *Saccharomyces* yeast, wine fermentation, ethanol reduction, fermentation oxygenation, starter cultures

INTRODUCTION

Wine composition is the product of complex interactions among yeast and bacteria that take place in vineyards and wineries, although one yeast species, *Saccharomyces cerevisiae*, is generally the main microorganism responsible for winemaking process (Pretorius, 2000). Its vigorous fermentative capacity, even in the presence of oxygen (Crabtree effect), makes *S. cerevisiae* a very efficient ethanol producer, strategy that allows its imposition over the rest of the microbiota during fermentation due to the toxicity of this compound (Thomson et al., 2005; Piškur et al., 2006.)

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ORIGINAL RESEARCH
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Saccharomyces cerevisiae and *S. kudriavzevii* Synthetic Wine Fermentation Performance Dissected by Predictive Modeling

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Wineries face unprecedented challenges due to new market demands and climate change effects on wine quality. New yeast starters including non-conventional *Saccharomyces* species, such as *S. kudriavzevii*, may contribute to deal with some of these challenges. The design of new fermentations using non-conventional yeasts requires an improved understanding of the physiology and metabolism of these cells. Dynamic modeling brings the potential of exploring the most relevant mechanisms and designing optimal processes more systematically. In this work we explore mechanisms by means of a model selection, reduction and cross-validation pipeline which enables to dissect the most relevant fermentation features for the species under consideration, *Saccharomyces cerevisiae* T73 and *Saccharomyces kudriavzevii* CR85. The pipeline involved the comparison of a collection of models which incorporate several alternative mechanisms with emphasis on the inhibitory effects due to temperature and ethanol. We focused on defining a minimal model with the minimum number of parameters, to maximize the identifiability and the quality of cross-validation. The selected model was then used to highlight differences in behavior between species. The analysis of model parameters would indicate that the specific growth rate and the transport of hexoses at initial times are higher for *S. cervisiae* T73 while *S. kudriavzevii* CR85 diverts more flux for glycerol production and cellular maintenance. As a result, the fermentations with *S. kudriavzevii* CR85 are typically slower; produce less ethanol but higher glycerol. Finally, we also explored optimal initial inoculation and process temperature to find the best compromise between final product characteristics and fermentation duration. Results reveal that the production of glycerol is distinctive in *S. kudriavzevii* CR85, it was not possible to achieve the same production of glycerol with *S. cervisiae* T73 in any of the conditions tested. This result brings the idea that the optimal design of mixed cultures may have an enormous potential for the improvement of final wine quality.

Keywords: *Saccharomyces* species, temperature, wine fermentation, dynamic modeling, parameter estimation, cross-validation

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Expression of heterologous transporters in *Saccharomyces kudriavzevii*: A strategy for improving yeast salt tolerance and fermentation performance

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ABSTRACT

S. kudriavzevii has potential for fermentations and other biotechnological applications, but is sensitive to many types of stress. We tried to increase its tolerance and performance via the expression of various transporters from different yeast species. Whereas the overexpression of *Z. rouxii* fructose uptake systems (*Zr*Ffz1 and *Zr*Fsy1) or a glycerol importer (*Zr*Stl1) did not improve the ability of *S. kudriavzevii* to consume fructose and survive osmotic stress, the expression of alkali-metal-cation exporters (*Sc*Ena1, *Sc*Nha1, *Yl*Nha2) improved *S. kudriavzevii* salt tolerance, and that of *Sc*Nha1 also the fermentation performance. The level of improvement depended on the type and activity of the transporter suggesting that the natural sensitivity of *S. kudriavzevii* cells to salts is based on a non-optimal functioning of its own transporters.

1. Introduction

The demand for products of high quality, with novel taste properties and good effects on human health has been steadily increasing in recent years. Thus a lot of effort has been spent on studying non-conventional yeast species, including sequencing their genomes, on characterizing their specific properties related to fermentation processes, such as the production of ethanol and aroma compounds or the use of various sugars as carbon sources (Borneman and Pretorius, 2015; Johnson, 2013; Jolly et al., 2014; Kurtzman et al., 2010).

Recently, non-*cerevisiae Saccharomyces* species have attracted attention as a barely exploited resource of yeast biodiversity with interesting traits not present in *S. cerevisiae*, the dominant species in alcoholic fermentation (Jolly et al., 2014; Marsit and Dequin, 2015; Radecka et al., 2015). *Saccharomyces kudriavzevii* is one of them. Besides being one of the parental strains of several interesting natural hybrids (Gonzalez et al., 2006), it has interesting oenological properties itself, including a higher glycerol and lower ethanol production than *S. cerevisiae* during wine must fermentation (Gamero et al., 2013; Gonzalez et al., 2007). Its production of aroma-active compounds, which surpasses *S. cerevisiae* (Stribny et al., 2015; Stribny et al., 2016a; Stribny et al., 2016b), is highly interesting and can be exploited e.g. in the biotechnological production of aroma compounds for the food industry. Though *S. kudriavzevii* has a better growth profile and fructose consumption rate than *S. cerevisiae* at low temperatures (Salvado et al., 2011; Tronchoni et al., 2009), it is highly sensitive to various stresses (Zemancikova et al., 2017) and is probably less adapted to stressful fermentation conditions (Perez-Torrado et al., 2016). Although *S. kudriavzevii* exhibits very similar basic physiological parameters (such as membrane potential, intracellular pH and the degree of quick Pma1 H^+-ATPase activation upon glucose addition) to the other *Saccharomyces* species (*S. cerevisiae*, *S. bayanus*, *S. paradoxus*), it has a low ability to proliferate in media with a limited concentration of potassium, very low osmotolerance and tolerance to toxic cations and cationic drugs, and minimal capacity to survive anhydrobiosis (Zemancikova et al., 2017). The difference in stress-survival among the *Saccharomyces* species seems to be based on their ability to quickly accommodate their cell size and metabolism to changing environmental conditions, and to adjust their portfolio of available transporters.

S. kudriavzevii also lacks competitiveness at higher fermentation temperatures (Arroyo-Lopez et al., 2011). Although it has been defined as a psychrotrophic yeast exhibiting a growth temperature range of 6–32 °C, its optimal growth temperature is 23–26 °C (Arroyo-Lopez et al., 2009; Arroyo-Lopez et al., 2011; Perez-Torrado et al., 2016; Salvado et al., 2011; Sampaio and Goncalves, 2008).

The aim of our work was to improve the fitness and performance of *S. kudriavzevii* under various stresses as it might help in using this species in biotechnology applications, e.g. the production of aroma compounds. We used the approach of the heterologous expression of various transporter genes from other, more stress-tolerant yeast species

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CHAPTER FIVE

New Trends in the Uses of Yeasts in Oenology

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Contents

Abstract

The most important factor in winemaking is the quality of the final product and the new trends in oenology are dictated by wine consumers and producers. Traditionally the red wine is the most consumed and more popular; however, in the last times, the wine companies try to attract other groups of populations, especially young people and women that prefer sweet, whites or rose wines, very fruity and with low alcohol content. Besides the new trends in consumer preferences, there are also increased concerns on the

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Dominance of wine *Saccharomyces cerevisiae* strains over *S. kudriavzevii* in industrial fermentation competitions is related to an acceleration of nutrient uptake and utilization

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Summary

Grape must is a sugar-rich habitat for a complex microbiota which is replaced by *Saccharomyces cerevisiae* strains during the first fermentation stages. Interest on yeast competitive interactions has recently been propelled due to the use of alternative yeasts in the wine industry to respond to new market demands. The main issue resides in the persistence of these yeasts due to the specific competitive activity of *S. cerevisiae*. To gather deeper knowledge of the molecular mechanisms involved, we performed a comparative transcriptomic analysis during fermentation carried out by a wine *S. cerevisiae* strain and a strain representative of the cryophilic *S. kudriavzevii*, which exhibits high genetic and physiological similarities to *S. cerevisiae*, but also differences of biotechnological interest. In this study, we report that transcriptomic response to the presence of a competitor is stronger in *S. cerevisiae* than in *S. kudriavzevii.* Our results demonstrate that a wine *S. cerevisiae* industrial strain accelerates nutrient uptake and utilization to outcompete the co-inoculated yeast, and that this process requires cell-to-cell contact to occur. Finally, we propose that this competitive phenotype evolved recently, during the adaptation of *S. cerevisiae* to man-manipulated fermentative environments, since a non-wine *S. cerevisiae* strain, isolated from a North American oak, showed a remarkable low response to competition.

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Introduction

In most natural environments, a vast diversity of microorganisms coexists and compete for space and resources. In many aspects, microbial habitats resemble ecological battlegrounds where microorganisms fight until domination or utter destruction of the opponent. Grape must is sugar-rich habitat for a complex microbiota of yeasts and bacteria that are usually replaced by just one or a few *Saccharomyces cerevisiae* strains after the first stages of wine fermentation (Querol *et al*., 1994; Fleet, 2003). In this study, we understand the concept of dominance as the phenomenon that is observed in mixed microbial populations when one individual (strain) is outnumbered by another (Pérez-Torrado *et al*., 2017).

Competitive interactions between *S. cerevisiae* and other naturally present microorganisms in wine must, mostly non-*Saccharomyces* yeast, have been the subject of diverse studies (Fleet, 2003; Bagheri *et al*., 2016; Ciani *et al*., 2016). This interest has recently been propelled due to the fact that, in the last years, the use of alternative yeasts in winemaking has become a widespread trend to respond to the new demands of the wine industry (Jolly *et al*., 2014; Pérez-Torrado *et al*., 2018). These demands come from, first, the effect of global warming on vines, which produces an uncoupling of sugar content and phenolic maturity in grapes resulting in higher ethanol yields; and two, an increasing market demanding wines with lower ethanol content and with diverse flavours and aroma.

S. cerevisiae yeasts are characterized by their high capability to ferment simple sugars into ethanol even in the presence of oxygen, known as Crabtree effect (Crabtree, 1928). Although, alcohol fermentation is energetically much less efficient than aerobic respiration, it provides with a selective advantage to these yeasts to outcompete other microorganisms: sugar resources are consumed faster and the ethanol produced during