Isolation, characterization and adaptive laboratory evolution of baker's yeasts

Doctoral Thesis

Ciencias de la Alimentación

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El Dr. **José Antonio Prieto Alamán**, Investigador Científico de OPIs y la Dra. **Francisca Randez Gil**, Científica Titular de OPIs, ambos con destino en el Instituto de Agroquímica y Tecnología de Alimentos (IATA) del Consejo Superior de Investigaciones Científicas (CSIC),

CERTIFICAN:

Que Doña **Isabel Elena Sánchez Adriá**, Graduada en Ciencias Químicas por la Universidad de Valencia, ha realizado bajo su dirección el trabajo titulado "**Isolation, characterization and adaptive evolution of baker´s yeasts**", el cual reúne las condiciones necesarias para optar al grado de Doctora por la Universidad de Valencia. Asimismo, certifican haber dirigido y supervisado, tanto los distintos aspectos del trabajo, como su redacción.

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

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llegado el momento de plasmar con palabras el inmeso número de veces que me he sentido agradecida, se me ponen los pelos de punta. Y es que las situaciones vividas durante este último período de tiempo atañen a muchas personas de todos los aspectos de mi vida. Pero también a instituciones que han hecho posible que este proyecto de tesis doctoral tuviera lugar.

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panadería, cuyos conocimientos se extienden a todas las posibilidades de avance científico que estos pequeños seres nos ofrecen.

Me refiero al grupo de investigación de Biotecnología de la fermentación del laboratorio 317 del IATA. Habitado por personas extraordinarias. Erigido, dirigido y mantenido en base a la elevada (me quedo corta) capacidad de dedicación y superación de Paqui y Jose. Dedicación y superación *para y por la Ciencia* (conocimiento y personas, insisto). Me acogisteis por primera vez allá por el 2014 para que realizara mi trabajo de fin de Ciclo Formativo de Grado Superior. En ese momento no imaginaba el mundo de posibilidades que ibais a desbloquear ante mí. Pero así fue, hasta día de hoy, que me encuentro escribiendo estas líneas. No sólo a mí. A muchos otros estudiantes que, como yo, ven incrementados sus conocimientos y aptitudes laborales tras pasar por vuestro laboratorio. Desde vuestra cercanía y ganas de aprovechar y disfrutar la vida, nos enseñáis el valor del esfuerzo y la importancia de un trabajo *bien trabajado*. Continuaré dibujando una sonrisa cada vez que recuerde las expresiones denominación de origen Paqui y los canturreos de Jose por el laboratorio. Sois mis mentores. Colegas de Ciencia.

Desde 2017, este laboratorio está también co-habitado por una excepcional y maravillosa trabajadora y persona. Hablo de Gemma, quien ha sido, es y será (lo se) un apoyo en todos los sentidos. Me escuchas pacientemente, me tranquilizas y me ayudas. ¡Incluso haces de enfermera de los que te rodean! ...cualquier botiquín es pequeño. Me siento muy feliz de que pasases también a ser mi amiga, además de compi de trabajo y de clase.

Todas las personas con las que he coincidido en ese laboratorio han contribuído a mi evolución. También con las que he tenido oportunidad de interactuar dentro del IATA. Gerencia, dirección, informática, mantenimiento, limpieza, administración, el servicio de biblioteca, con Ana Veyrat al frente,

quien me ayudó a poner en marcha un curso de formación, el servicio de analítica, con Quique y Lola (entre otros) apoyándome en todo lo que he necesitado para realizar determinaciones de analitos varios; así como miembros de otros grupos de investigación. Siempre dispuestos a prestar ayuda a quien lo solicite. Casi como una levadura, he ido adquierendo cualidades, aptitudes y actitudes beneficiosas para todos los aspectos de mi vida. Por ello, os doy las gracias a todos vosotros.

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Alex… que compartas tu vida conmigo me hace sentir especial, valiosa. Como siento que eres tú. Eres capaz de *parar el tiempo para mí* y dibujar con detalle nuestra vida común, del mismo modo que has diseñado y plasmado todos los dibujos que aparecen en esta tesis. Me has apoyado desde la perspectiva más difícil, la de la convivencia día a día, año tras año. Gracias por estar siempre disponible, por animarme y por darme fuerzas. En estos momentos estás haciendo una paella para comer, aunque todavía no quieres que sepa de qué es, porque es una sorpresa. Así eres, una persona excepcional, dulce, diferente, …único.

A todos y cada uno de vosotros, continuaré en persona agradeciéndoos que forméis parte de mi vida.

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De aquí, a donde me lleve el viento, ¿verdad, Carol?

Siempre agradecida,
Isabel E. Sánchez Adriá

Por cierto, la paella era de marisco y estaba buenísima.

"Hay una fuerza motriz más poderosa que el vapor, la electricidad y la energía atómica: la voluntad"

Albert Einstein

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"Nunca desistas de un sueño. Sólo trata de ver las señales que te llevan a él"

Paulo Coelho

General Summary

Sourdough, a naturally fermented mixture of flour and water, is used in artisanal bread manufacture as a leavening agent and to increase the nutritional and organoleptic qualities of cereal-based goods. The production of this bread on an industrial scale is labour-intensive and time-consuming, as it requires daily refreshments of the dough to ensure a stable microbiota, process reproducibility and bread quality. As an alternative, the flour can be inoculated with a starter culture consisting of a mixture of lactic acid bacteria and yeasts (usually *Fructilactobacillus sanfranciscensis* and *Saccharomyces cerevisiae* or *Kazachstania humilis*), specifically selected for their ability to grow and ferment under stress conditions and contribute to typical bread characteristics.

In the frame of our collaboration with the Europastry, a global baking company, the first objective of this PhD work was to obtain information about the yeast diversity of sourdoughs produced in its facilities and select strains for the development of a *S. cerevisiae* starter. Analysis of the sourdough microbiota identified *S. cerevisiae* and *K. humilis* as the predominant yeast species. In addition, the volatile composition showed that these species are the main contributors, together with the type of flour, to the differential aromatic profile of the samples analysed. In parallel, the yeast strains isolated from the sourdoughs were classified according to their fermentative power and stress tolerance, which made it possible to identify the best strains of each species, generating a large collection of yeast isolates for future developments. These analyses allowed us to conclude that *K. humilis* performed better than *S. cerevisiae* in terms of leavening capacity, especially in the presence of lactic and/or acetic acid. In addition, the results showed that the presence of acetic acid reduces the CO_2 production of *S. cerevisiae* strains, but has no noticeable effect in the case of *K. humilis*.

The sensitivity of *S. cerevisiae* strains to the presence of acetic acid led us to the second objective of this PhD Thesis: to generate acetic-tolerant derivatives of sourdough isolates of *S. cerevisiae* through adaptive evolution strategies. These strategies resulted in heterogeneous populations being more tolerant to acetic acid but, in some cases, also more susceptible to lactic acid, suggesting that S. *cerevisiae* develops different responses to these acids. Nevertheless, individual clones resistant to both acids and showing high leavening activity were obtained. Genome sequencing and ploidy-level analysis of the parental and evolved strains revealed the existence of aneuploidies together with Single Nucleotide Polymorphisms (SNPs), which could explain the phenotypic heterogeneity. Studies of Copy Number Variations (CNVs) and SNPs confirmed the involvement of genes previously related to acid stress tolerance and maltose utilisation, as well as suggesting the possible role of new genes in these mechanisms.

Finally, as the third objective of this PhD Thesis, we decided to examine the use of sourdough isolates in other fermentation processes, such as brewing, encouraged by the rapid evolution of craft brewing companies. Although previous studies had shown that *S. cerevisiae* sourdough isolates can be used to ferment brewing wort, information regarding important properties such as fermentation rate or thermotolerance was scarce. Accordingly, the kinetic constants, fermentation activity and biochemical parameters of two *S. cerevisiae* sourdough strains isolated in our laboratory and two commercial brewers' strains, were determined in the fermentation of malt wort at 20 and 37ºC. The results showed the ability of the sourdough strains to reduce the fermentation time at both temperatures tested, a characteristic that provides microbiological stability to the process and flexibility to the brewer. In addition, the sourdough strains produce, in general, more aroma compounds than commercial strains, in particular esters and acids, a characteristic that is regulated by the fermentation temperature.

Resumen General

La masa madre, una mezcla de harina y agua fermentada de forma natural, se utiliza en la fabricación artesanal de pan como agente leudante y para aumentar las cualidades nutritivas y organolépticas de los productos a base de cereales. La elaboración de este pan a nivel industrial, se complica por la necesidad de realizar refrescos diarios de la masa en condiciones controladas para garantizar una microbiota estable, la reproducibilidad del proceso y la calidad del pan. Como alternativa, la harina puede inocularse con un cultivo iniciador formado por una mezcla de bacterias lácticas y levaduras (generalmente *Fructilactobacillus sanfranciscensis* y S*accharomyces cerevisiae* o *Kazachstania humilis*), específicamente seleccionadas por su capacidad para crecer y fermentar en condiciones de estrés y contribuir a las características típicas del pan.

En el marco de nuestra colaboración con Europastry, una multinacional de panadería, el primer objetivo de este trabajo de doctorado ha sido obtener información sobre la diversidad de levaduras de las masas madre producidas en sus instalaciones y seleccionar cepas para el desarrollo de un cultivo iniciador de *S. cerevisiae*. El análisis de la microbiota permitió identificar a *S. cerevisiae* y *K. humilis* como especies de levadura predominantes. Además, la composición volátil de las muestras demostró que estas levaduras son las principales responsables, junto con el tipo de harina, del perfil aromático diferenciador de las masas. Paralelamente, las cepas de levadura aisladas de las masas madre se clasificaron de acuerdo con su poder fermentativo y tolerancia a estrés, lo que posibilitó identificar las mejores cepas de cada especie, generado una amplia colección de aislados de levadura para futuros desarrollos. Estos análisis nos permitieron concluir que *K. humilis* mostró un mejor rendimiento que *S. cerevisiae* en términos de capacidad leudante, especialmente en presencia de ácido láctico y/o acético. Además, los resultados mostraron que la presencia de ácido acético reduce la producción de CO_2 de las cepas de *S. cerevisiae*, pero no tiene un efecto destacable en el caso de *K. humilis*.

La sensibilidad de las cepas de *S. cerevisiae* a la presencia de ácido acético nos llevó al segundo objetivo de esta Tesis Doctoral: generar derivados de aislados de masa madre de *S. cerevisiae* tolerantes a ácido acético mediante estrategias de adaptación evolutiva. Estas estrategias dieron como resultado la obtención de poblaciones heterogéneas más tolerantes a ácido acético y en algunos casos, también más susceptibles a láctico, lo que sugiere que *S. cerevisiae* desarrolla diferentes respuestas a estos ácidos. A pesar de ello, se obtuvieron clones individuales resistentes a ambos ácidos y que muestran una elevada actividad leudante. La secuenciación del genoma y el análisis a nivel de ploidía de las cepas parentales y evolucionadas reveló la existencia de aneuploidías junto con polimorfismos de un solo nucleótido (SNPs), que podrían explicar la heterogeneidad fenotípica. Los estudios de las variaciones en el número de copias (CNVs) y de los SNPs confirmaron la implicación de genes previamente relacionados con la tolerancia a estrés ácido y la utilización de la maltosa, así como sugirieron la posible función de nuevos genes en estos mecanismos.

Por último, como tercer objetivo de esta Tesis Doctoral, decidimos examinar el uso de aislados de masa madre en otros procesos fermentativos, como el cervecero, alentados por la rápida evolución de las empresas cerveceras artesanales. Aunque estudios anteriores habían demostrado que los aislados de masa madre de *S. cerevisiae* pueden emplearse para fermentar mosto cervecero, la información respecto a propiedades importantes como la velocidad de fermentación o la termotolerancia era escasa. De acuerdo a esto, se determinaron las constantes cinéticas, la actividad fermentativa y los parámetros bioquímicos de dos cepas de masa madre de *S. cerevisiae* aisladas en nuestro laboratorio y dos cepas cerveceras comerciales, en la fermentación de mosto de malta a 20 y 37ºC. Los resultados mostraron la capacidad de las cepas de masa madre para reducir el tiempo de fermentación a las dos temperaturas ensayadas, una característica que aporta estabilidad microbiológica y flexibilidad al proceso cervecero. Además, las cepas de masa madre producen, en general, más compuestos aromáticos que las cepas comerciales, en particular ésteres y ácidos, una característica regulada en función de la temperatura de fermentación.

Resum General

La massa mare, una barreja de farina i aigua fermentada de forma natural, s'utilitza en la fabricació artesanal de pa com a agent ludant i per augmentar les qualitats nutritives i organolèptiques dels productes a base de cereals. L'elaboració d'aquest pa a nivell industrial es complica per la necessitat de fer refrescos diaris de la massa en condicions controlades per a garantir una microbiota estable, la reproductibilitat del procés i la qualitat del pa. Com a alternativa, la farina pot inocular-se amb un cultiu iniciador format per una barreja de bacteris làctics i llevats, (generalment *Fructilactobacillus sanfranciscensis* i *Saccharomyces cerevisiae* o *Kazachstania humilis*), específicament seleccionades per la seva capacitat per a créixer i fermentar en condicions d'estrès i contribuir a les característiques típiques del pa.

En el marc de la nostra col·laboració amb Europastry, una multinacional panadera, el primer objectiu d'aquest treball de doctorat ha estat obtenir informació sobre la diversitat de llevats de les masses mare produïdes a les seves instal·lacions i seleccionar ceps per al desenvolupament d'un cultiu iniciador de *S. cerevisiae*. L'anàlisi de la microbiota va permetre identificar a *S. cerevisiae* i *K. humilis* com a espècies de llevat predominants. A més, la composició volàtil de les mostres va demostrar que aquests llevats són els principals responsables, juntament amb el tipus de farina, del perfil aromàtic diferenciador de les masses. Paral·lelament, els ceps de llevat aïllats de les masses mare es van classificar d'acord amb el seu poder fermentatiu i tolerància a estrès, fet que va possibilitar identificar els millors ceps de cada espècie, generant una àmplia col·lecció d'aïllats de llevat per a futurs desenvolupaments. Aquestes anàlisis ens van permetre concloure que *K. humilis* va mostrar un rendiment millor que *S. cerevisiae* en termes de capacitat ludant, especialment en presència d'àcid làctic i/o acètic. A més, els resultats van mostrar que la presència d'àcid acètic redueix la producció de CO_2 dels ceps de *S. cerevisiae*, però no té un efecte destacable en el cas de *K. humilis*.

La sensibilitat dels ceps de *S. cerevisiae* a la presència d'àcid acètic ens va portar al segon objectiu d'aquesta Tesi Doctoral: generar derivats d'aïllats de massa mare de *S. cerevisiae* tolerants a àcid acètic mitjançant estratègies d'adaptació evolutiva. Aquestes estratègies van donar com a resultat l'obtenció de poblacions heterogènies més tolerants a acètic però, en alguns casos, també més susceptibles a làctic, cosa que suggereix que *S. cerevisiae* desenvolupa diferents respostes a aquests àcids. Tot i això, es van obtenir clons individuals resistents a tots dos àcids i que mostren una elevada activitat ludant. La seqüenciació del genoma i l'anàlisi a nivell de ploïdia dels ceps parentals i evolucionats va revelar l'existència d'aneuploïdies juntament amb polimorfismes d'un sol nucleòtid (SNPs), que podrien explicar l'heterogeneïtat fenotípica. Els estudis de les variacions en el nombre de còpies (CNVs) i dels SNPs van confirmar la implicació de gens prèviament relacionats amb la tolerància a estrès àcid i la utilització de la maltosa, així com van suggerir la possible funció de nous gens en aquests mecanismes.

Per acabar, com a tercer objectiu d'aquesta Tesi Doctoral, vam decidir examinar l'ús d'aïllats de massa mare en altres processos fermentatius, com el cerveser, encoratjats per la ràpida evolució de les empreses cerveseres artesanals. Tot i que estudis anteriors havien demostrat que els aïllats de massa mare de *S. cerevisiae* es poden fer servir per fermentar most cerveser, la informació respecte a propietats importants com la velocitat de fermentació o la termotolerancia era escassa. D'acord amb això, es van determinar les constants cinètiques, l'activitat fermentativa i els paràmetres bioquímics de dos ceps de massa mare de *S. cerevisiae* aïllats al nostre laboratori i dos ceps cervesers comercials, en la fermentació de most de malt a 20 i 37ºC. Els resultats van mostrar la capacitat dels ceps de massa mare per reduir el temps de fermentació a les dues temperatures assajades, una característica que aporta estabilitat microbiològica i flexibilitat al procés cerveser. A més, els ceps de massa mare produeixen, en general, més compostos aromàtics que els ceps comercials, en particular èsters i àcids, una característica regulada en funció de la temperatura de fermentació.

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Lista de abreviaturas y acrónimos

AAB – bacteria ácido acética

AcH – ácido acético

ALE – evolución adaptativa en el laboratorio

ANF – factor anti-nutricional

ASV – amplicon sequencing variant

ATP – adenosina trifosfato

aw – actividad de agua

BLAST – Basic Local Alignment Search Tool

bp – pares de bases

CAR/PDMS – carboxeno/polidimetilsiloxano

cfu – unidades formadoras de colonias

Chr – cromosoma

CNV – copy number variation

DMSO – dimetilsulfóxido

DNA – ácido desoxirribonucleico

dw – peso seco

DY – rendimiento de masa

EDTA – ácido etilendiaminotetraacético

EFSA – European Food Safety Authority

e.g. – por ejemplo

EPS – exopolisacáridos

EtOH – etanol

FBD – flour-based dough

FDA – Food and Drug Administration

GC/MS – cromatografía de gases acoplada a espectrometría de masas

GMO – organismo modificado genéticamente

GRAS – generalmente reconocido como seguro

IR – índice de refracción

ITS – internal transcribed spacer

Kan – kanamicina

Kh – *Kazachstania humilis*

LA – ácido láctico

LAB – bacteria ácido láctica

LD – sistema modelo de masa líquida

LH – L'Hirondelle

LME – extracto líquido de malta

MAPA – Ministerio de Agricultura, Pesca y Alimentación

MM – masa madre

MP – masa panaria

Myr – miriocina

NAD – nicotinamida adenina dinucleótido

NIST – National Institute of Standards and Technology

OD – densidad óptica

PC – componentes principales

PCA – análisis de componentes principales

PCR – reacción en cadena de la polimerasa

PDA – detector de fotodiodos

PL – fosfolípido

POF – phenolic-off-flavour

PTFE – politetrafluoroetileno

QPS – qualified presumption of safety

RFLP – restriction fragment length polymorphism

RNA – ácido ribonucleico

RP-HPLC – cromatografía líquida de alta resolución en fase reversa

rpm – revoluciones por minuto

Sc – *Saccharomyces cerevisiae*

SCD – medio sintético completo

SCSIE – Servicio Central de Soporte a la Investigación Experimental

sd – desviación estándar

SNP – single nucleotide polymorphism

SL – esfingolípido

SPME – extracción en fase sólida

TCA – ciclo de los ácidos tricarboxílicos

Td – *Torulaspora delbrueckii*

TTA – acidez total titulable

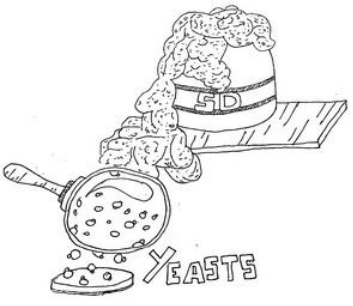
UV – ultravioleta

VOC – volatile organic compound

wt – wild type

YPD – yeast peptone dextrose medium

Introducción general



"Molino que estás moliendo el trigo con tanto afán, ¡Tú estás haciendo la harina y otros se comen el pan!"

Melchor de Palau

Las necesidades e inquietudes del ser humano cambian con el contexto histórico en el que se encuentra y la manera como se alimenta no es una excepción. Actualmente, no sólo nos preocupa el qué, cómo y la procedencia de los ingredientes con los que ha sido elaborado lo que ingerimos. Nuestra preocupación se extiende hasta el bienestar animal y la salud ambiental del planeta. Un ejemplo claro es el compromiso conocido como 'etiqueta verde' (del inglés, 'clean label'), que cada vez más empresas adoptan de manera voluntaria.

En general, existe una tendencia a consumir alimentos adaptados al estilo de vida de cada uno, lo que conduce a la elección de cierto tipo de alimentos, como ecológicos, de proximidad, sin aditivos o bajos en grasa y sal (Silow *et al*., 2018). Además, crece la demanda de alimentos poco procesados, que se elaboren siguiendo métodos cercanos a lo tradicional y/o que se demuestre su condición de alimento funcional. Es decir, que sean saludables también por contener compuestos activos que ayuden, por ejemplo, a prevenir enfermedades.

Desde hace miles de años, el pan representa una manera cómoda y satisfactoria de consumir cereales. Debido a su moderado coste y a que provocan sensación de saciedad al aumentar de volumen en el intestino, la ingesta de cereales está recomendada con frecuencia diaria en las pirámides nutricionales. De este modo, el pan constituye una fuente rica en energía que nos aporta variedad de nutrientes, como proteína vegetal, fibra, calcio, hierro y vitaminas (Koehler y Wieser, 2013). Según datos del Ministerio de Agricultura, Pesca y Alimentación (MAPA, 2021), durante el año 2020 se produjo una importante redistribución del gasto intra- y extra-doméstico, motivada por el aislamiento domiciliario a raíz de la pandemia por el SARS-CoV-2. El pan alcanzó el 4,6% del gasto total y recibió tal grado de atención, que se ha convertido en un icono de la vuelta a lo artesanal y de la correlación popular "tradicional igual a saludable". De hecho, la tercera receta más buscada a nivel mundial en Google

fue 'masa madre' (Google Trends Global, 2020); mientras que 'pan casero', 'masa madre' y 'levadura fresca' ocuparon el primer, segundo y cuarto puesto en las recetas más buscadas desde España, respectivamente (Google Trends España, 2020).

1. El proceso de panificación

En su sentido más simple, el pan es el producto de hornear una mezcla compuesta por harina, agua y sal. A pesar de que existe una amplia variedad de recetas que introducen otros ingredientes (mantequilla, yogur o fruta, entre otros), estos tres componentes básicos son suficientes para desarrollar productos finales muy diferentes variando el tipo de harina (cereal, fuerza, hidratación) y parámetros tecnológicos específicos (Cauvain, 2014).

Durante el mezclado (o amasado), se pone en contacto la harina con el resto de ingredientes, dando paso a la formación de la red de gluten, compuesta por gliadinas y gluteninas (Wieser *et al*., 2006). El objetivo es desarrollar una estructura en forma de malla, capaz de retener gas y otros compuestos volátiles de interés durante el proceso (Haegens, 2014). A continuación, durante la etapa de fermentación, se genera el gas (CO_2) responsable del aumento de volumen de la masa, lo que confiere a la miga su esponjosidad. Se trata de un proceso bioquímico, mediante el cual, los microorganismos presentes obtienen energía para llevar a cabo sus funciones vitales a partir de los azúcares disponibles (Randez-Gil *et al*., 2014). Debido a que las masas constituyen en sí un ambiente microaerófilo, la fermentación es alcohólica, generándose EtOH, CO_2 y otros subproductos, como ésteres, ácidos orgánicos, carbonilos y alcoholes, que pueden contribuir, por sí mismos o como precursores de otros, a proporcionar los rasgos sensoriales que caracterizan al pan y que tanto valoran los consumidores (Pico *et al.*, 2015; Arora *et al*., 2021). La fermentación durante 2-

3 horas de la masa panaria (MP) impulsada por la adición de levadura comercial y su posterior cocción, da lugar al pan directo, el más comercializado. Por otro lado, es posible elaborar pan añadiendo masa madre (MM) como ingrediente (**Figura 1**). En este caso, la fermentación se prolonga (12-24 horas), dando lugar a una masa ácida capaz de albergar una microbiota estable y exclusiva, específica de su proceso de producción y mantenimiento, que aporta cualidades diferenciales al pan elaborado con ella (pan con MM).

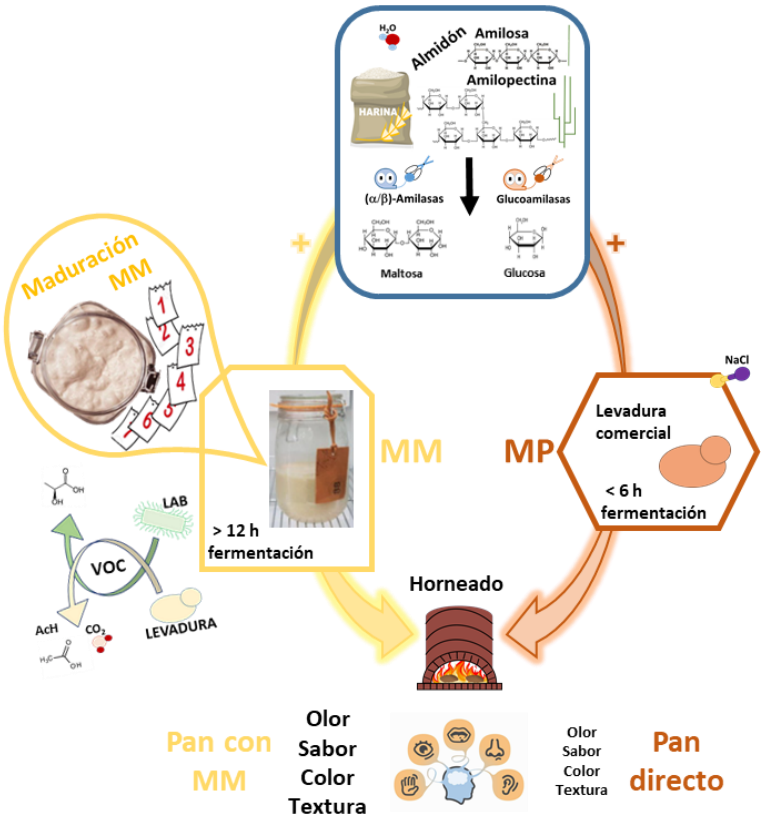


Figura 1. Principales diferencias entre el proceso de elaboración del pan directo y el pan con masa madre. La incorporación de masa madre (MM) a la formulación del pan (pan con MM) permite reducir la adición de levadura comercial y enriquecer el producto final con compuestos orgánicos volátiles (VOCs), ácido láctico (LA) y ácido acético (AcH). MP: masa panaria; LAB: bacterias ácido lácticas; CO_2: dióxido de carbono; NaCl: cloruro sódico; H_2O: agua.

La Norma Nacional de Calidad del Pan, que entró en vigor en 2019 (BOE-A-2019-6994; Real Decreto 308/2019, de 26 de abril), garantiza a los consumidores la compra de productos de calidad perfectamente caracterizados y etiquetados, estableciendo qué se considera masa madre y pan con masa madre, entre otros.

2. La masa madre

El uso de MM como medio para levar la MP es uno de los métodos de fermentación más antiguos (Cappelle *et al*., 2013). Desde este punto de vista, una MM es el conjunto de comunidades simbióticas de bacterias y hongos endógenos de la harina empleada para su elaboración, que perduran tras sucesivos refrescos hasta alcanzar una composición ecológica estable, propia de la MM madura (Landis *et al*., 2021; De Vuyst *et al*., 2023). Se trata de una microbiota que se ha adaptado eficazmente al entorno y a las condiciones del proceso (Gänzle y Ripari, 2016; Comasio *et al*., 2020a).

2.1. Tipos de masa madre

Si bien en inglés se emplea el término 'sourdough' para resaltar su acidez, en castellano se la denomina 'masa madre' para referenciar su uso como masa inicial (o madre) que da lugar a las subsiguientes, al mezclar periódicamente porciones de la misma con más harina y agua. Dependiendo de cómo se inicie y del proceso de mantenimiento, las MM adquieren entidad propia, convirtiéndose en fuentes exclusivas de comunidades microbianas con características diferenciales. En general, se acepta una clasificación en tres tipos (De Vuyst *et al*., 2023).

- **Tipo 1**: Se refiere a la MM tradicional, en la que bacterias y levaduras endógenas de la harina o vinculadas al entorno de elaboración, colonizan y

se desarrollan en una mezcla de harina y agua que se mantiene, mediante una serie de refrescos a intervalos regulares, hasta obtener una MM madura (Gobbetti *et al*., 2016). Se emplean como agente leudante de la MP, a la que también aporta sabores y aromas específicos.

- **Tipo 2**: La mezcla de harina y agua es inoculada con bacterias y/o levaduras adaptadas para iniciar rápidamente la fermentación (starters o iniciadores) y proporcionar propiedades organolépticas o tecnológicas deseables (Calvert *et al*., 2021). Aunque compiten con la microbiota endógena y ambiental, acaban prevaleciendo en la MM madura. Son comunes en sistemas de producción a gran escala, puesto que la inoculación inicial permite reducir el tiempo de maduración (Siepmann *et al*., 2019). Se comercializan como preparaciones semilíquidas, tratadas térmicamente o liofilizadas para su uso como aromatizantes o acidificantes. Excepto en el caso de utilizar MM semilíquidas de tipo 2, se requiere la adición de levadura comercial para garantizar el correcto levado de la MP (Calvert *et al.*, 2021).

- **Tipo 3**: Son una versión híbrida de la 1 y 2, en las que la mezcla de harina y agua se inocula con un cultivo iniciador, pero posteriormente se madura mediante sucesivos refrescos como la MM de tipo 1 (De Vuyst *et al.*, 2014; Siepmann *et al*., 2018).

En general, los sucesivos refrescos de la MM proporcionan tiempo para que se acumulen compuestos del metabolismo secundario de, principalmente, levaduras y bacterias ácido lácticas (del inglés, LAB), muchos de los cuales son indetectables en masas de fermentación directa. Por tanto, cabe esperar que los panes que los incluyan en su formulación proporcionen al consumidor, en mayor o menor medida, los beneficios que derivan de ellos.

2.2. Propiedades tecnológicas

El interés que generan las MM entre los consumidores no ha pasado desapercibido para los productores de alimentos fermentados. Ello ha suscitado la publicación de numerosos artículos, tanto científicos como divulgativos, que atribuyen multitud de beneficios al pan con MM, destacando los relativos a mejoras tecnológicas y nutricionales (Gobbetti *et al*., 2019).

2.2.1. Reproducibilidad y diversidad

A pesar de ser un sustrato rico en nutrientes y, por tanto, susceptible de ser contaminado por la microbiota ambiental, las MM maduras constituyen nichos ecológicos estables si se mantienen adecuadamente (Landis *et al*., 2021; De Vuyst *et al*., 2023). Cuando se utilizan como agente leudante, permiten eliminar o reducir el uso de levadura comercial o agentes químicos. Esto se traduce en un catálogo de productos más diverso y acorde, entre otros factores, a la composición microbiológica específica de cada MM madura, y repercute tanto en la reproducibilidad del proceso, como en la calidad de los productos elaborados con ellas (Calvert *et al*., 2021).

2.2.2. Vida útil del pan

Las causas más comunes del deterioro del pan son su degradación química y el crecimiento de mohos (Garcia *et al*., 2019). Algunos tratamientos de conservación van encaminados a reducir el número de esporas fúngicas presentes en el pan, mediante vaporización con etanol, irradiación con luz UV o la adición de ácidos (acético, sórbico y/o propiónico) a la MP (Kavková, 2019). Sin embargo, todos estos métodos son de aplicación limitada o incluyen aditivos que los alejan del objetivo "*clean label"* y provocan el rechazo del consumidor.

En este contexto, el uso de MM es una alternativa eficaz. Por una parte, el pH ácido de la masa inhibe las lipasas del cereal que contribuyen al deterioro estructural (Rizzello *et al*., 2010). Por otra, los ácidos orgánicos derivados del metabolismo de las LAB inhiben la proliferación de hongos superficiales productores de toxinas, como *Aspergillus*, *Penicillium* o *Fusarium* (Guimarães *et al*., 2018; Sadeghi *et al*., 2019; Liu *et al*., 2022). A esta actividad antifúngica y antitoxigénica también contribuye la proliferación de especies de levaduras, tanto del género *Saccharomyces*, como non-*Saccharomyces*, que generan acetato, succinato y citrato (Heitman *et al*., 2015) o feniletilalcohol y 2-fenilacetato (Mo y Sung, 2014), entre otros. Finalmente, compuestos producidos por LAB y/o levaduras, como fenilos sustituidos, dipéptidos cíclicos o ácidos grasos hidroxilados, pueden contribuir de manera sinérgica a la actividad antifúngica de los ácidos orgánicos (Quattrini *et al*., 2019; Liu *et al*., 2022).

2.2.3. Propiedades reológicas

El estudio reológico del pan incluye varios atributos (dureza, adhesividad, cohesión, masticabilidad, elasticidad y gomosidad), que confieren propiedades reológicas al producto final. Los panes elaborados con MM se aproximan más a valores óptimos de algunos de estos atributos de interés (Galle, 2013). Por ejemplo, el carácter ácido induce cambios fisicoquímicos en la red de proteínas que aumentan la capacidad de expansión de la MP (Clarke *et al*., 2004). Por otra parte, las LAB producen exopolisacáridos (EPS) que retienen agua y reducen la dureza de la miga (Di Monaco *et al*., 2015). Además, algunos de estos EPS, como los β-glucanos, promueven la viscoelasticidad de la masa, contribuyendo a que incluso las elaboradas con harinas sin gluten, adquieran y mantengan su volumen (Galle *et al*., 2012).

2.2.4. Características organolépticas

Junto con el aspecto visual, el aroma es la primera percepción que tiene el consumidor de los productos de panadería, siendo clave en la decisión de compra. Arora *et al*. (2021) enumeran hasta 27 atributos sensoriales que confieren a los panes con MM mayor grado de aceptación que a los panes directos. Sabor y aroma ácidos, intenso post-gusto y aroma, aspecto atractivo, color distintivo de la corteza y la miga, corteza crujiente, frescura y alta porosidad son algunos de ellos.

La generación de compuestos que proporcionan aroma y/o sabor vía retronasal depende, tanto de parámetros biológicos, incluyendo enzimas endógenas del cereal o metabolitos producidos por las levaduras y LAB (Thiele *et al*., 2002; Di Cagno *et al*., 2014); como tecnológicos, desde la harina (Cho y Peterson, 2010; Moskowitz *et al*., 2012), temperatura y tiempo de horneado (Torner *et al*., 1992; Bianchi *et al*., 2008; Heenan *et al*., 2009), hasta las condiciones de enfriamiento y almacenamiento del pan (Jensen *et al*., 2011). Incluso, de si se encuentran en la miga o en la corteza (Bianchi *et al*., 2008; Cho y Peterson, 2010; Birch *et al*., 2013). Los compuestos orgánicos volátiles (del inglés, VOCs) detectados, incluyen prácticamente todas las familias químicas; desde ésteres, carboxilos, carbonilos y alcoholes, hasta éteres, pirazinas e hidrocarburos (Paraskevopoulou *et al*., 2012). Sin embargo, no todos contribuyen de manera olfativa y/o gustativa, sólo aquellos que superan las concentraciones perceptibles por el ser humano (Quílez *et al*., 2006).

2.3. Propiedades nutricionales

Debido a la creciente demanda de alimentos con contenido nutricional de calidad, se ha incrementado el número de estudios científicos encaminados a dilucidar los beneficios en términos de nutrición y salud del uso de MM, destacando los descritos a continuación.

2.3.1. Biodisponibilidad de fibra alimentaria y minerales

La acidez de la MM estimula la actividad de la fitasa endógena de los cereales en la harina, que cataliza la hidrólisis del ácido fítico (myo-inositol hexafosfato), impidiendo que se asocie con minerales libres (calcio, sodio, magnesio, hierro y cinc) y, en última instancia, que reduzca su biodisponibilidad en el pan (Di Cagno *et al*., 2008). Por otra parte, aumenta la fracción soluble de fibra en masas elaboradas con harinas de trigo integral y legumbres (Chinma *et al*., 2016) y permite añadir hasta un 20% de salvado sin comprometer las propiedades reológicas y organolépticas del producto final (Salmenkallio-Marttila *et al*., 2001).

2.3.2. Degradación de compuestos antinutricionales

En general, los pseudocereales, como el trigo sarraceno, la quinoa o el amaranto, y las legumbres, contienen más proteínas y fibra que el trigo común, por lo que su uso en la fabricación de productos de panadería se ha incrementado en los últimos años (Paucar-Menacho *et al*., 2022). Además, no contienen gluten, siendo una buena alternativa para la población celíaca. Sin embargo, contienen factores antinutricionales (FAN), como rafinosa, taninos condensados e inhibidores de tripsina, cuya ingesta puede reducir la digestibilidad y/o absorción de los propios nutrientes. Para evitar estos efectos indeseados, se recomienda, entre otros tratamientos, la fermentación de este tipo de harinas mediante su incorporación a la MM. Por ejemplo, la fermentación dirigida por especies

seleccionadas de LAB durante los sucesivos refrescos de una MM, reduce el contenido en FAN, aumentando el valor nutricional y las propiedades funcionales de estos ingredientes (Montemurro *et al*., 2019; De Pasquale *et al*., 2020; Gobbetti *et al*., 2020).

2.3.3. Beneficios en enfermedades metabólicas

Diferentes estudios han evaluado los posibles efectos del consumo de pan con MM en la salud humana. En particular, en sujetos con enfermedades metabólicas o gastrointestinales (Pérez-Alvarado *et al*., 2022). Entre ellos, la reducción en la respuesta glucémica frente a la observada por consumo de pan blanco común (Scazzina *et al*., 2009; Bondia-Pons *et al*., 2011). Por su parte, el pan elaborado con MM presenta niveles superiores de almidón resistente y los ácidos orgánicos producidos por su microbiota retrasan el vaciado gástrico. Asimismo, la ingesta de pan con MM aumenta los niveles de triptófano en el plasma sanguíneo, lo que reduce el apetito (Bondia-Pons *et al*., 2011). Por último, el consumo de pan elaborado con MM se ha asociado a una menor respuesta insulínica en pacientes con sobrepeso, resistentes a insulina o hiperglicémicos/hiperinsulinémicos, sugiriendo un papel funcional en la prevención de ciertos trastornos metabólicos asociados a la diabetes tipo 2 y al riesgo cardiovascular (Najjar *et al*., 2009; Lappi *et al*., 2010; MacKay *et al*., 2012).

No obstante, hasta la fecha no se han demostrado, mediante criterios de valoración clínica (reducción del riesgo de padecer diabetes, mejoras en el control del peso, reducción de intolerancias al gluten o aumento de la densidad ósea), los beneficios nutricionales derivados de la inclusión de MM en la formulación del pan (Korem *et al*., 2017). Además, existen discrepancias entre los resultados *in vitro* e *in vivo* (tanto en modelos animales, como en humanos), posiblemente debido al uso de condiciones de control poco representativas de

situaciones reales y/o a la falta de una metodología unificadora de los ensayos (diferentes formulaciones y métodos de elaboración de MM y pan). Frente a esta heterogeneidad en los métodos y resultados experimentales, ni la "Food and Drug Administration" de Estados Unidos (FDA), ni la Autoridad Europea de Seguridad Alimentaria (EFSA), han aprobado hasta la fecha incluir ninguna declaración de propiedad saludable en el etiquetado o la publicidad de productos derivados de MM o que incorporen MM en su formulación (EUR-Lex - 32006R1924, 2020; D'amico *et al*., 2023).

2.3.4. Prebióticos y postbióticos

Los microorganismos de la masa, inactivados por el calor durante el horneado, son fuente de envolturas celulares, como EPS, membranas o metabolitos, incluyendo péptidos bioactivos o ácidos grasos de cadena corta, con potencial efecto beneficioso para la salud humana, ya que pueden tener actividad como prebióticos o postbióticos (Salminen *et al*., 2021). Esto ha motivado un creciente interés por incorporar nuevas LAB y/o levaduras capaces de proliferar en MM (Lynch *et al*., 2018; Păcularu-Burada *et al*., 2020). En consecuencia, la caracterización de microorganismos de MM debe considerar también su contribución a este respecto (Fernandez-Pacheco Rodríguez *et al*., 2018; Hernández-Alcántara *et al*., 2022).

3. El microbioma de las masas madre

Cada MM es única debido a la variedad de ingredientes empleados en su formulación, entorno en el que se desarrolla y condiciones de refresco y fermentación, ya que determinan la presencia inicial de microorganismos, su interacción y adaptación. Las MM albergan una amplia gama de especies de bacterias, levaduras y otros organismos infrarrepresentados que evolucionan constantemente hasta alcanzar el equilibrio ecológico en la MM madura. Esta se

consigue normalmente después de 7-10 refrescos de la masa inicial (Corsetti y Settani, 2007; Ercolini *et al*., 2013) y en ella cohabitan a menudo de una a tres especies de bacterias con una especie de levadura (Landis *et al*., 2021; Chiva *et al*., 2021), frecuentemente con una proporción bacteria:levadura de 100:1 o, incluso, 1000:1 (Lhomme *et al*., 2015; Urien *et al*., 2019; Comasio *et al*., 2020a; Lau *et al*., 2021; De Vuyst *et al*., 2023).

3.1. Bacterias ácido lácticas

De los más de 50 géneros de bacterias detectados en MM, 10 pertenecen al grupo dominante de LAB y el resto a otros tipos de bacterias catalogadas como subdominantes o satélites del género bacteriano predominante, *Lactobacillus* (Arora *et al*., 2021). Así, aunque la mezcla inicial de harina y agua puede contener LAB de otros géneros (*Enterococcus*, *Lactococcus* o *Leuconostoc*) o incluso bacterias Gram negativas (*Pseudomonas* sp.), aerobias (*Enterobacteriaceae)* o de la familia *Acetobacteraceae* [bacterias ácido-acéticas (del inglés, AAB)], estas no se consideran típicas de la MM, ya que su número se reduce a lo largo de los sucesivos refrescos; si bien contribuyen a la acidificación inicial de la mezcla de harina y agua (Minervini *et al*. 2014; Ripari *et al*., 2016).

En cuanto a la composición de las LAB, al predominio inicial de los géneros citados le sucede, con el tiempo, el de especies adaptadas a las condiciones de la MM madura, como *Lactobacillus*, *Pediococcus* y *Weissella*; y, por último, especies con mayor tolerancia a la presencia de altas concentraciones de ácido (Huys *et al*., 2013), entre ellas, especies heterofermentativas obligadas (*L. brevis*, *L. fermentum*, *L. reuteri*, *L. rossiae* y *Fructilactobacillus. sanfranciscensis*), heterofermentativas facultativas (*L. alimentarius*, *L. paralimentarius* y *L. plantarum*) y homofermentativas obligadas (*L.*

delbrueckii). En términos de abundancia, *F. sanfranciscensis*, *L. plantarum, L. brevis*, *L. paralimentarius* y *P. pentosaceus* son los aislados más frecuentes en MM producidas en ambientes panaderos, mientras que, en entornos de laboratorio, donde el único componente no estéril es la harina, prevalecen *L. plantarum*, *L. fermentum*, *L. brevis*, *P. pentosaceus* y *L. citreum* (Rocha *et al*., 2023). Si se amplía la búsqueda, se encuentran otras LAB menos abundantes, como *W. cibaria, W. confusa, P. acidilactici* y *L. mesenteroides* (De Vuyst *et al*., 2017). Por último, indicar que *F. sanfranciscensis* y *L. paralimentarius* se han aislado casi exclusivamente de MM, mientras que otras especies, como *L. brevis* y *L. plantarum* se asocian en un sentido más amplio con alimentos fermentados (Gobbetti *et al*., 2016; Martino *et al*., 2016).

3.1.1. Propiedades tecnológicas de las bacterias lácticas

El interés tecnológico de las LAB va estrechamente ligado al metabolismo predominantemente heterofermentativo de las hexosas, que se caracteriza por la producción equilibrada de ácido láctico y acético (acidez fresca y fuerte, respectivamente). Además, pueden metabolizar pentosas y generar CO_2, así como EtOH y acetaldehído (Lau *et al*., 2021). Esto, unido a su elevada actividad proteolítica, al metabolismo secundario de compuestos nitrogenados y a que transforman aminoácidos en aldehídos y cetonas asociados a sabores y fragancias, contribuye a mejorar las propiedades organolépticas y la textura del pan. También su capacidad para generar otros compuestos, como la ornitina, que tras la cocción se transforma en 2-acetil-1-pirrolina, uno de los principales responsables del aroma característico de la corteza del pan (Limbad *et al*., 2020; Pico *et al*., 2015), es clave en la intensificación del sabor. La reducción del pH de la masa, la acumulación de ácidos débiles y la generación de compuestos con propiedades antifúngicas determinan su funcionalidad para ralentizar el deterioro del pan (Axel *et al*., 2016) y, como se ha mencionado anteriormente, son

productoras de prebióticos y una vez inactivadas por el calor tras la cocción, pueden tener actividad postbiótica.

3.2. Levaduras

Debido a su elevada capacidad fermentativa y tolerancia a ambientes ácidos, las levaduras aisladas de MM pertenecen, casi exclusivamente, al Phylum Ascomycota y, en concreto, a la familia *Saccharomycetaceae* (De Vuyst *et al*., 2016; Chiva *et al*., 2021), siendo *Saccharomyces cerevisiae* y *Kazachstania humilis* las especies predominantes, tanto por separado como cohabitando una misma masa (Viiard *et al*., 2016; Van Kerrebroeck *et al*., 2017). Destacan por ser tolerantes a acidez, pero difieren en el metabolismo de la maltosa, el carbohidrato mayoritario en las MM. Mientras que *S. cerevisiae* es maltosa positiva, *K. humilis* es incapaz de asimilar el disacárido (Brandt *et al*., 2004), por lo que las MM que contienen estas especies presentan componentes bióticos diferenciales (véase más adelante). Otras especies de levaduras aisladas de MM son *Torulaspora delbrueckii*, *Wickerhamomyces anomalus*, *K. exigua*, *Pichia kudriavzevii* y *Candida glabrata* (De Vuyst *et al*., 2017; Chiva *et al*., 2021). Aunque representan, en muchos casos, una parte minoritaria de la microbiota específica, algunas de estas levaduras presentan características que las pueden hacer adecuadas para formar parte de cultivos iniciadores mixtos. Por ejemplo, *T. delbrueckii* muestra buena capacidad fermentativa en masas dulces, así como tolerancia a estrés osmótico y a congelación (Hernandez-López *et al*., 2003). Respecto al aislamiento de estas especies, cabe indicar que *C. glabrata* y *W. anomalus* son especies más frecuentes en MM de laboratorio, que en MM procedentes de panaderías (Vrancken *et al*., 2010). Esto suele atribuirse a las condiciones semiestériles de su procesado en el laboratorio y al tipo de harina utilizada, que podría contener determinados flavonoides y/o taninos con actividad antimicrobiana, que afectarían de manera diferencial a las levaduras de

la MM, favoreciendo o dificultando la imposición de unas sobre otras (De Vuyst *et al*., 2016; Moroni *et al*., 2011; Lhomme *et al*., 2016).

3.2.1. Propiedades tecnológicas de las levaduras

Las levaduras, generalmente cepas de *S. cerevisiae* y *K. humilis* son, junto con las LAB heterofermentativas, los agentes leudantes naturales del pan, debido a su capacidad para producir CO_2. Su actividad fermentativa está estrechamente ligada al metabolismo de los azúcares disponibles en la masa, principalmente maltosa (pero también glucosa y fructosa) y a su transformación eficiente a través de la ruta glicolítica. De este proceso, las levaduras obtienen energía, poder reductor (NADH) y dos moléculas de piruvato, que luego degradan para producir CO_2 y EtOH. Además, la producción de glicerol a partir de dihidroxiacetona y la generación de succinato a través del ciclo TCA, contribuyen a la regeneración del NAD^+ consumido durante la glicólisis (van Maris *et al*., 2006).

Como se ha mencionado, la liberación de CO_2 contribuye al levado de la masa, pero también aumenta el volumen de la miga, mejora la textura del pan, favorece la retención de aromas y aporta esponjosidad. La síntesis de glicerol, ácido succínico y EtOH contribuye de manera sinérgica a la formación de la red de gluten, la cual determina las propiedades reológicas de la masa y del pan (Jayaram *et al*., 2014a, 2014b; Aslankoohi *et al*., 2016; Rezaei *et al*., 2016). Paralelamente, los compuestos derivados de otras vías metabólicas, principalmente alcoholes superiores y ésteres, contribuyen a la percepción gustativa y olfativa de los productos de panadería. Por ejemplo, la síntesis de 3-metil-butanol, a partir del aminoácido L-leucina, juega un papel importante en la percepción aromática de la miga (Birch *et al*., 2014; Pétel *et al*., 2017). Por último, la condensación alcohólica de ácidos (especialmente acético), da lugar a ésteres apreciados por los consumidores por su contribución al aroma del pan.

Algunos ejemplos son el afrutado del acetato de etilo, las notas de miel y rosa del acetato de feniletilo, y una mezcla de manzana verde con tonos florales del octanoato de etilo (De Vuyst *et al*., 2023).

3.3. Interacciones entre levaduras y bacterias ácido lácticas

A lo largo de los años, numerosas investigaciones han analizado la influencia de factores bióticos y abióticos en la estructura y composición ecológica de la MM. Entre ellos, se han descrito el impacto de las materias primas, el equipo, la ubicación y las condiciones del proceso (De Vuyst *et al*., 2017; Chiva *et al*., 2021). No obstante, en un estudio con más de 500 MM de todo el mundo, Landis *et al*. (2021) apuntan a un predominio de factores bióticos como las interacciones microbianas, metabolismo de los microorganismos, la transferencia horizontal de genes y la expresión diferencial de genes relacionados con el estrés (adaptación), sobre los abióticos. También ponen de manifiesto la necesidad de mejorar nuestra comprensión acerca de cómo afectan estos factores, en particular la relación entre las LAB y las levaduras, a la calidad del producto final.

Los microorganismos que habitan la MM establecen relaciones estímulo-dependientes con el resto de integrantes de la comunidad, lo que modifica sus ciclos vitales y posibilita que algunas especies se perpetúen y diversifiquen en entornos en los que no sobrevivirían de manera aislada. Mientras que las levaduras presentan gran capacidad de adaptación a medios relativamente sencillos y con disponibilidad variable de oxígeno [efecto Crabtree (De Deken, 1966)], las especies de LAB son más sensibles al tipo de atmósfera y a la disponibilidad de nutrientes, especialmente aminoácidos y vitaminas. Ciertas especies de *Lactobacillus*, como *L. lactis* y *L. plantarum*, muestran, bajo ciertas condiciones, dependencia de crecimiento con la levadura *S. cerevisiae*; la

cual, en medios ricos en nitrógeno, responde excretando metabolitos (aminoácidos y/o pequeñas moléculas hidrofílicas no identificadas) que las LAB aprovechan para implantarse en la MM (Ponomarova *et al*., 2017). No obstante, las principales asociaciones LAB/levadura descritas hasta la fecha, tienen su origen en diferencias en la capacidad de metabolizar carbohidratos. Alrededor del 55-70% del trigo es almidón (Koehler y Wieser, 2013), por lo que, en la mezcla inicial de harina y agua, hay una baja disponibilidad de azúcares fermentables. A medida que el pH disminuye por la acción de las LAB, las amilasas de los cereales liberan gradualmente glucosa y, sobre todo, maltosa (**Figura 1**). Esto hace que el crecimiento de las especies microbianas se establezca a medida que avanza la fermentación.

La hidrólisis de la sacarosa (actividad invertasa) y la degradación de fructanos del trigo, llevadas a cabo por las levaduras, proporcionan fructosa a las LAB (Sainz-Polo *et al*., 2013), que la utilizan como aceptor de electrones para producir manitol y ácido acético, en lugar de EtOH (von Weymarn *et al*., 2002). La mayoría de los lactobacilos heterofermentativos, como *F. sanfranciscensis*, emplean fructosa de manera preferente al piruvato como aceptor de electrones, ya que de esta manera obtienen ATP y poder reductor adicionales (De Vuyst *et al*., 2017). Por su parte, *S. cerevisiae* utiliza glucosa frente a maltosa debido a su regulación mediante represión por catabolito (Gancedo, 1998), lo que reduce su competencia con otras especies maltosa positivas. Por el contrario, las LAB heterofermentativas metabolizan desde el inicio el disacárido, ya que expresan constitutivamente la enzima maltosa fosforilasa (Gobetti *et al*., 2005). Concretamente, escinden la maltosa en glucosa-1-P, que continúan metabolizando; y glucosa, que se libera al medio en condiciones de estrés. Tradicionalmente, se ha aceptado que esta glucosa es la que permite prosperar a otras especies, como *L. plantarum*, que crece mejor cuando hay glucosa presente (Sieuwerts *et al*., 2018), o a levaduras maltosa-negativas, como *K. humillis* y *K.*

exigua que, a cambio, proporcionarían a las LAB vitaminas y aliviarían su auxotrofia para ciertos aminoácidos (Vogel *et al*., 2011). Sin embargo, se ha descrito que este mutualismo es óptimo a pHs dentro del rango de 4,2 a 7,0 y entre 25 y 30°C (Brandt *et al*., 2004). Además, en un estudio reciente, Carbonetto *et al*. (2020) señalan que esta relación parece ser dependiente de cepa, tanto para las LAB, como para las levaduras.

De este modo, las interacciones entre los géneros de microorganismos predominantes en la MM van más allá de la especie, debiendo considerar la cepa concreta que se describe. En el caso de *S. cerevisiae*, una de las levaduras más ubicuas del planeta, esto adquiere una importancia aún mayor. Por ejemplo, en su estudio con 9 aislados de MM de esta especie, Marongiu *et al*. (2015) descubrieron que todos ellos diferían a nivel genómico, por ejemplo, en la secuencia de genes relacionados con la pared celular, y fenotípicamente, ya que tres de ellos resultaron ser maltosa-negativos. En este sentido, las MM constituyen sumideros de estirpes de levaduras, tanto *Saccharomyces* como non-*Saccharomyces*, algunas de las cuales han obtenido el estatus de "Presunción Cualificada de Seguridad" o QPS (Koutsoumanis *et al*., 2020). Además de su uso tradicional, estas cepas podrían ser claves para reforzar la producción de diversos alimentos fermentados, con el fin de adaptar la industria alimentaria a las demandas de los consumidores, así como a los retos medioamabientales que afronta.

4. Cultivos iniciadores

La inoculación de diferentes sustratos con cultivos iniciadores permite dirigir la fermentación hacia la obtención de productos con características controlables, reproducibles y deseables. Un cultivo iniciador contiene una o más especies (cultivos simples o mixtos) de levaduras que han sido específicamente seleccionadas por su capacidad para crecer y fermentar en condiciones de estrés y contribuir a las cualidades tecnológicas y organolépticas del producto final (De Vuyst *et al*., 2017). Estos cultivos están disponibles comercialmente en forma de levadura líquida, granulada, prensada (en bloque), seca o congelada (Guerzoni *et al*., 2013).

Aunque la mayoría de los estudios sobre cultivos iniciadores versan sobre el uso de *S. cerevisiae*, la demanda de una cartera de productos más diversificada ha despertado el interés por otras levaduras, como *K. exigua*, *K. humilis* y *T. delbrueckii*. Ciertas cepas de las dos primeras, que se consideran microorganismos típicos de MM, son osmotolerantes (10% de NaCl y 5% de glucosa) y su perfil metabólico se caracteriza por su incapacidad para utilizar la maltosa (Vigentini *et al*., 2014). Por su parte, *T. delbrueckii* se encuentra más dispersa en la naturaleza y se utiliza como cepa productora de compuestos aromatizantes en la producción de pan y vino, así como en la producción de masas dulces y congeladas, debido a su tolerancia a estos tipos de estrés (Hernandez-Lopez *et al*., 2003, 2006, 2007; Fernandes *et al*., 2021). Cabe señalar que estas levaduras también muestran un alto grado de biodiversidad intraespecífica, aunque sólo unos pocos estudios se han dirigido a comprender los mecanismos de divergencia.

4.1. Divergencia evolutiva de las levaduras de panadería

La levadura *S. cerevisiae* se encuentra en hábitats naturales diferentes, como suelo, agua y árboles (corteza y raíz). A lo largo de la historia, el ser humano la ha domesticado para elaborar una amplia gama de alimentos (pan, chocolate o aceitunas) y bebidas (vino, cerveza, sake, café o leche fermentada). En particular, la comercialización del pan condujo a la selección y propagación de linajes heterogéneos de cultivos iniciadores. La introducción de levaduras comerciales para acortar los tiempos de fermentación, llevó a la selección guiada por el hombre de cepas de levadura con mayor tamaño celular, buen rendimiento de propagación en medios industriales de melaza (fuente limitada de carbono) y capacidad de generar más CO_2 en fermentaciones cortas. En cambio, la producción artesanal de pan con MM, ha llevado a la selección de cepas que se han perpetuado en este medio, sometidas a fuerte competencia con otros microorganismos y en condiciones hostiles de alta presión osmótica, deficiencia de oxígeno y acumulación de ácidos orgánicos. No es de extrañar, por tanto, que presenten características filogenéticas, bioquímicas y fenotípicas propias, diferentes tanto de sus parientes silvestres, como de las cepas comerciales de panadería (**Figura 2**).

Como describen Bigey *et al.* (2021), las levaduras comerciales de panadería son tetraploides y muestran homologías filogenéticas con las levaduras utilizadas por el hombre para otros procesos de fermentación, como la elaboración de vino, sake y cerveza. Por el contrario, la mayoría de las levaduras de MM son diploides y se agrupan en un segundo cluster que incluye genomas de diversos orígenes, como frutas y entornos naturales. Además, al igual que sus parientes cerveceros, presentan un alto índice de genes implicados en la

utilización de la maltosa y tienen una capacidad fermentativa inferior a la de sus parientes comerciales (**Figura 2).**

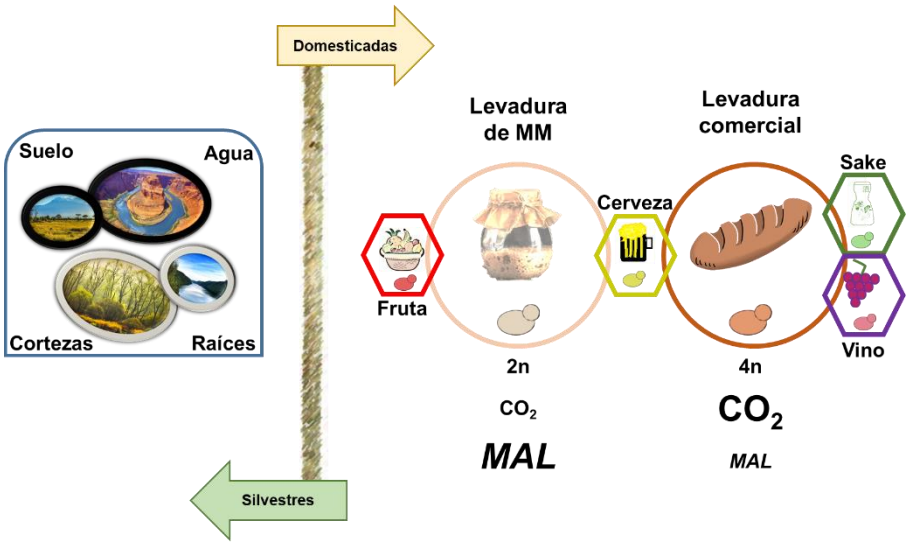


Figura 2. Divergencia genómica entre levaduras. Siglos de uso continuado de las levaduras en diferentes procesos biotecnológicos ha conducido a su domesticación diferencial. En su estudio, Bigey *et al.* (2021), muestran que el genoma de las levaduras comerciales de panadería contiene importantes contribuciones del de otras levaduras utilizadas en la producción de vino y sake, mientras que el genoma de las levaduras de masa madre (MM) está más influenciado por especies que habitan en ambientes menos controlados por el ser humano, como las frutas. Por último, ambos tipos de levaduras de panadería, de MM y comerciales, contienen partes importantes del genoma de levaduras destinadas a la elaboración de cerveza.

Por otra parte, las levaduras industriales de panadería presentan una tolerancia limitada a condiciones de estrés, lo que se traduce en un pobre rendimiento fermentativo, por ejemplo, en masas dulces o congeladas, donde las células están expuestas a estrés osmótico severo y estrés por frío, respectivamente (Hernández-López *et al*., 2007). Este comportamiento podría atribuirse a condiciones selectivas homogéneas, a partir de las cuales, se han producido sucesivos cruces entre linajes silvestres que han generado cepas con baja diversidad genética y alto nivel de ploidía (Aguilera *et al*., 2010), en comparación con levaduras de otros usos tecnológicos, como levaduras vínicas

y cerveceras (Liti *et al*., 2009) o levaduras aisladas de MM (Bigey *et al*., 2021). La relación inversa entre el nivel de ploidía y la tolerancia a estrés se pone de manifiesto en experimentos de adaptación evolutiva, en los que se demuestra que la adquisición de este fenotipo correlaciona con la pérdida de cromosomas (Aguilera *et al*., 2010; Randez-Gil *et al*., 2020). En definitiva, el diseño biológico de las levaduras comerciales dificulta, tanto su mejora genética por técnicas tradicionales (ver más adelante), como su utilización en entornos industriales y procesos biotecnológicos que requieran un alto grado de tolerancia a estrés.

Ante esta situación, la MM se considera un ecosistema estresante adecuado para seleccionar cepas de levadura adaptadas a este entorno (De Vuyst y Vancanneyt, 2007). Se trata de una matriz semisólida con valores de pH en el rango de 3-4 (debido a la acumulación de ácidos orgánicos), baja actividad de agua, limitación de oxígeno y composición variable de azúcares (De Vuyst *et al*., 2014, Van Kerrebroeck *et al*., 2016). No obstante, las levaduras de la MM son también sensibles a este ambiente, llevando a que, bajo ciertas circunstancias, pueda verse mermada su viabilidad y capacidad de fermentación. De hecho, se ha descrito una correlación positiva entre el tiempo de fermentación y la producción de ácidos (Calvert *et al*., 2021), un efecto que depende de la especie e, incluso, de la cepa. Esta sensibilidad a estrés tiene un impacto tecnológico evidente sobre las MM, en particular cuando se utilizan como único agente leudante, ya que se necesitan fermentaciones más largas. La mayor parte de los panaderos solucionan este problema añadiendo levadura comercial, aunque los efectos positivos de la MM sobre la elasticidad, viscosidad y extensibilidad de la masa, así como su contribución al desarrollo del aroma y sabor del pan, pueden verse mitigados (Bender *et al*., 2018; Gaglio *et al*., 2018; Yildirim-Mavis *et al*., 2019). Además, existe una normativa que limita el uso de levaduras comerciales en panes con MM (BOE-A-2019-6994; Real Decreto 308/2019, de 26 de abril).

Por otro lado, existen evidencias de la relación entre la producción de compuestos aromáticos, la exposición a estrés y la interacción LAB-levaduras (Guerzoni *et al*., 2007). Así pues, una selección adecuada de levaduras tolerantes a estrés puede repercutir en el aroma del pan y, al mismo tiempo, reducir el tiempo de fermentación. El uso de estas levaduras debería favorecer su proliferación frente a las LAB al aumentar la competencia por los azúcares disponibles, reduciendo así la relación LAB/levadura y la acumulación de ácidos. En este sentido, cabe señalar que el exceso de acidez tiene un impacto significativo en el sabor, lo que limita su aceptabilidad por parte de los consumidores, especialmente en el mercado español, como se desprende de un gran número de blogs y artículos en redes sociales que sugieren trucos o formas de reducir la acidez de este tipo de pan (Un pedazo de pan, 2023). Por tanto, es evidente que las propiedades tecnológicas y la calidad del pan con MM podrían beneficiarse del desarrollo de cultivos iniciadores compuestos por levaduras más robustas, capaces de enfrentarse de forma más eficiente a este entorno competitivo y hostil.

5. Mejora genética de levaduras

La microbiología y la biotecnología modernas abordan el reto de establecer métodos experimentales capaces de seleccionar y proporcionar a la industria levaduras con propiedades tecnológicas a medida, en función del proceso biotecnológico al que se apliquen. En la actualidad, las levaduras, y en particular las cepas de *S. cerevisiae*, se utilizan en numerosos procesos industriales que contribuyen a la salud humana, la sostenibilidad medioambiental y al consumo y producción responsables (Ballet *et al*., 2023). Además de ser el principal agente fermentativo en alimentos y bebidas, la levadura *S. cerevisiae* es la plataforma celular más explotada en biotecnología industrial, producción

de biocombustibles y biorremediación (Parapouli *et al*., 2020). Durante su aplicación industrial, *S. cerevisiae* se enfrenta a condiciones extremas, diferentes de las que soportan sus congéneres de laboratorio y/o silvestres. Principalmente, los procesos industriales demandan levaduras con alta capacidad metabólica en ambientes de pH bajo y acumulación de ácidos, donde deben mostrar fuerte competencia frente a microorganismos contaminantes (Brodeur *et al*., 2011); pero también exhibir un buen rendimiento de biomasa en medios de propagación industrial y resistir tratamientos térmicos y de secado, para favorecer su comercialización. Aunque las levaduras aisladas de MM cumplen algunos de estos requisitos, hacerlas competentes para aplicaciones industriales, no sólo de panificación, sino también de otros procesos biotecnológicos, requiere, en algunos casos, que mejoremos algunas de sus características intrínsecas.

5.1. La diversidad natural y su generación artificial

Desde que se introdujo el uso de cultivos iniciadores en la industria alimentaria, se han dedicado numerosos esfuerzos encaminados a su optimización. A este respecto, existen muchas estrategias dirigidas a la selección y/o construcción de nuevas estirpes con mayor capacidad tecnológica o más adecuadas a un proceso concreto. La más inmediata es, sin duda, explotar la diversidad genética que ofrece la naturaleza o las colecciones de cultivo. Varios estudios revelan que la biodiversidad fúngica natural es enorme y en su mayor parte inexplorada, mientras que las levaduras industriales utilizadas actualmente representan solo una pequeña fracción de ésta (Liti *et al*., 2009; Wang *et al*., 2012). Esto sugiere que algunas de estas cepas silvestres pueden presentar propiedades relevantes para la industria. Sin embargo, el aislamiento de cepas silvestres con un fenotipo particular requiere un arduo y a menudo infructuoso trabajo de cribado, identificación y caracterización. *S. cerevisiae*, aunque abundante en determinados nichos, muestra una presencia en la naturaleza de

sólo 1:20.000, en comparación con otras especies fúngicas (Taylor *et al*., 2014). Además, la aplicación industrial de un cultivo iniciador a veces requiere una combinación de fenotipos que podrían no encontrarse en la naturaleza, por no mencionar que su aplicación no debe suponer un riesgo para la seguridad de las personas, los animales o el medio ambiente.

Frente a esta opción, algunas técnicas permiten obtener artificialmente variantes genéticas (Steensels *et al*., 2014). El desarrollo de la tecnología del ADN recombinante, junto con un conocimiento más profundo de la biología molecular de *S. cerevisiae*, ha permitido obtener levaduras con características específicas de interés tecnológico, como consumo constitutivo de maltosa, osmo y/o criorresistencia o mayor producción de biomasa (Codón *et al*., 2003; Dueñas-Sánchez *et al*., 2010). Sin embargo, su uso práctico ha quedado aparcado en la industria alimentaria debido a restricciones normativas y al rechazo de los consumidores. Por otro lado, los métodos clásicos de mejora genética implican mutagénesis aleatoria, hibridación o entrecruzamiento (*mating)* sexual (por ejemplo, *rare-mating o genome shuffling*) o asexual (fusión de protoplastos) y posterior selección del fenotipo más adecuado. Estas técnicas combinan las características de dos células parentales y han sido fundamentales en la obtención de híbridos para su uso en vinificación (Pérez-Través *et al*., 2012; Bellon *et al*., 2013), elaboración de cerveza (Tubb *et al*., 1981), producción de biocombustibles (Benjaphokee *et al*. 2012) o panificación (Oda y Ouchi, 1990). A pesar de estos éxitos, su empleo también tiene limitaciones. A diferencia de las cepas de laboratorio, muchas cepas industriales son poliploides y/o aneuploides y muestran baja capacidad de esporulación o viabilidad de las esporas (Codón *et al*., 1995), lo que limita el uso de la hibridación sexual. También son homotálicas gran parte de ellas, lo que reduce la eficacia de esta metodología. La hibridación por fusión de protoplastos genera derivados poliploides, muchos de los cuales son inestables, con pérdida de cromosomas

que dificultan la predicción del fenotipo de los híbridos resultantes (Attfield y Bell, 2003). No obstante, su uso es interesante para la adquisición de fenotipos cuantitativos que dependen directamente del número de copias de genes y, por tanto, del nivel de ploidía, como el tamaño celular. Sin embargo, las levaduras comerciales de panificación, en su mayoría tetraploides, emplean, como se mencionó anteriormente, mecanismos de adaptación en sentido contrario (Aguilera *et al*., 2010; Randez-Gil *et al*., 2020), perdiendo cromosomas y acercando su constitución genómica a la de las cepas aisladas de MM, en las que predominan la aneuploidía y diploidía (Bigey *et al*., 2021). Por lo tanto, cabe pensar que las mejoras conseguidas de esta forma terminen perdiéndose con el uso continuado de estas cepas. Además, los híbridos obtenidos por fusión de protoplastos se consideran OMGs en algunos países, incluído el nuestro.

Si nos fijamos en la naturaleza e intentamos recrear los procesos que han conducido a la amplia variabilidad ecológica actual, la evolución dirigida (Butler *et al.*, 1996), también llamada evolución experimental o evolución adaptativa en el laboratorio (del inglés, ALE), es una alternativa potente y de bajo coste para generar diversidad genética en cualquier cepa parental y que, además, no provoca rechazo entre los consumidores.

5.2. Evolución adaptativa en el laboratorio

Mediante diferentes mecanismos adaptativos, los microorganismos adquieren rasgos diferenciados en respuesta a la percepción de señales físico-químicas cambiantes del entorno que les rodea. Si el nuevo estímulo se mantiene en el tiempo, los fenotipos mejor adaptados terminan seleccionándose e imponiendo sus genotipos a la mayor parte de la población, promoviendo que la especie evolucione y perdure. De hecho, la domesticación de microorganismos se basa en la adaptación evolutiva llevada a cabo durante un largo periodo de

tiempo. La alternativa simplificada viene dada por el método ALE. La mejora de las cepas mediante ALE imita los fenómenos naturales aplicando una presión selectiva artificial en el laboratorio para obtener un microorganismo mejorado (evolucionado) hacia el fenotipo deseado (Shepelin *et al*., 2018; Phaneuf *et al*., 2020).

Debido a la relativa facilidad de simular diferentes condiciones ambientales y/o nutricionales a escala de laboratorio y a la manejabilidad y tiempos cortos de generación de las levaduras, el método ALE constituye una herramienta valiosa para mejorar fenotipos de relevancia industrial. El cultivo puede llevarse a cabo mediante transferencias secuenciales de las células a un nuevo medio de cultivo, de modo que las células se mantengan en fase de crecimiento exponencial; o de forma continua, siendo más común el primero (Sandberg *et al*., 2019). A pesar de que el cultivo en biorreactores confiere ventajas (presión selectiva de forma constante y más controlada), debe tenerse en cuenta que el crecimiento prolongado en condiciones estresantes y/o de limitación nutrientes puede conducir a que las células inviertan su respuesta, pudiendo adquirir dificultad de crecimiento en situaciones dinámicas no estresantes y/o con abundancia de nutrientes (Jansen *et al*., 2005). Además, es importante que las cepas evolucionen en un entorno similar al de su uso industrial, para evitar una mejora del rasgo seleccionado a expensas de otras propiedades relevantes (cepas *"crippled").* La estrategia de evolución adaptativa también permite confrontar a la población parental con una combinación de diferentes estreses (Gibson *et al*., 2007), lo que puede dar lugar a clones evolucionados con múltiples adaptaciones a estas condiciones. (**Figura 3**).

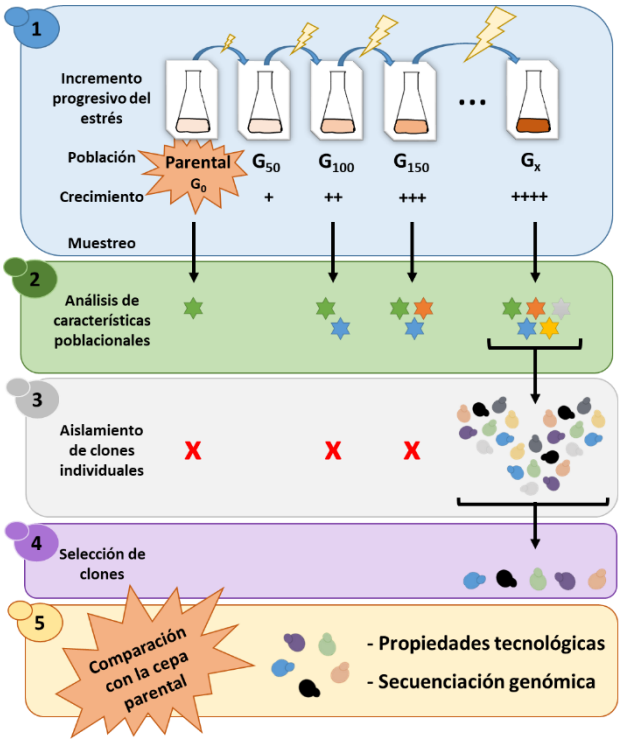


Figura 3. Esquema de las principales etapas del método ALE aplicado a levaduras. En la etapa 1, la presión selectiva se aumenta progresivamente para incrementar la velocidad de crecimiento en cultivos sucesivos. El análisis de características de interés (etapa 2) permite seleccionar poblaciones para aislar clones individuales (etapa 3) y elegir aquellos que muestren un crecimiento más activo en las condiciones de estrés impuestas (etapa 4). Tanto las cepas parentales como las poblaciones evolucionadas y los clones individuales se almacenan en glicerol a -80oC a lo largo del proceso, y están disponibles para análisis adicionales (etapa 5) como pruebas tecnológicas y/o genómicas que ayuden a esclarecer los mecanismos moleculares subyacentes a la adaptación.

A diferencia de los métodos de ingeniería genética, en los que se requiere un amplio conocimiento de las rutas metabólicas implicadas y en las que surgen limitaciones de manipulación de microorganismos poliploides, el método ALE presenta la ventaja de enfocarse hacia la adquisición de fenotipos contraintuitivos (Lee y Kim, 2020), donde el desafío consiste en identificar el tipo y magnitud de presión selectiva a ejercer sobre las células (acidez, presencia de ácidos orgánicos, temperatura y/o fármacos que alteren su metabolismo), con

el fin de conseguir clones evolucionados en los que se haya adquirido un número suficiente de mutaciones, como para trasladar la ventaja observada en el laboratorio a la situación real. Sin embargo, el método ALE se utiliza a menudo para ajustar o afinar un fenotipo específico, ya existente en una cepa silvestre o construida artificialmente, a las necesidades de su uso tecnológico. Por ejemplo, para maximizar tasas de crecimiento, aumentar la resistencia a estrés, modificar la capacidad de emplear sustratos o lograr mayor rendimiento de compuestos de interés. Algunos ejemplos son la adaptación de cepas silvestres a procesos de fermentación comerciales (Tian *et al*., 2020), la obtención de cepas resistentes a temperatura (Caspeta *et al*., 2014; Randez-Gil *et al*., 2020; García-Rios *et al*., 2021) o a ambientes ácidos (Fletcher *et al*., 2017).Por último, el análisis ómico de clones parentales y evolucionados, por ejemplo, mediante la secuenciación masiva de sus genomas, permite arrojar luz sobre los mecanismos moleculares subyacentes a las adaptaciones ocurridas durante la evolución (Gibson *et al*., 2020; Randez-Gil *et al*., 2020; García-Ríos *et al*., 2021; Mavrommati *et al.*, 2022; Menegon *et al*., 2022; Fernandes *et al.*, 2023; Jia *et al*., 2023).

6. Otros usos de las levaduras de panadería

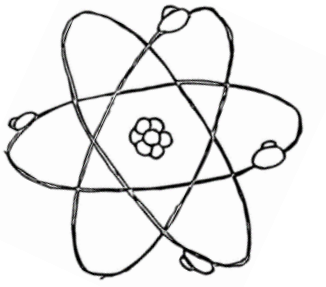
Las levaduras se utilizan desde hace miles de años en fermentaciones tradicionales para elaborar cerveza, pan o vino. *S. cerevisiae*, *S. bayanus* y *S. pastorianus* son las especies predominantes en la elaboración de estos productos, debido, principalmente, a su adaptación intrínseca a las condiciones particulares de estas fermentaciones y a la recurrente selección humana de fermentos activos. La domesticación, base de la diversificación genética de las levaduras industriales actuales (Liti *et al*., 2009; Wang *et al*., 2012; Gallone *et al*., 2018; Bigey *et al*., 2021), ha permitido acortar los tiempos de fermentación y mejorar la reproducibilidad e higiene del proceso, así como aportar calidad y valor añadido al producto. Prueba de ello, es el enorme uso actual de cepas comerciales

y la aparición de gigantes industriales que basan su negocio en la fermentación. Sin embargo, la industria no siempre utiliza las cepas mejor adaptadas, con los mayores rendimientos o las mejores propiedades organolépticas (Steensels *et al*., 2014). Esto se debe a que, en muchos casos, las cepas se utilizan por razones históricas, bien porque han sido seleccionadas para un fin específico, bien por la reticencia de las empresas industriales a cambiar sus protocolos. Un claro ejemplo son las levaduras comerciales de panadería, seleccionadas, como se ha mencionado en esta Introducción, principalmente en base a criterios de los productores de levadura, como el tamaño celular o alta producción de biomasa, olvidando en parte propiedades como la tolerancia a estrés o el aporte aromático, más cercanas a las necesidades de los usuarios y consumidores. En consecuencia, el número de cepas comerciales en estos sectores es bastante reducido (Lengeler *et al*., 2020), lo que limita sus posibilidades de hacer frente a las actuales demandas de los consumidores de productos innovadores, de mayor calidad o, simplemente, de propiedades organolépticas diferentes. En el sector cervecero, por ejemplo, la aparición incesante de cervecerías artesanales ha puesto en guardia a las grandes empresas, que ven cómo aumenta la competencia y peligra su importante negocio por la dificultad de responder rápidamente a la necesidad de diversificación de aromas, sabores, estilos, etc.

Como ya se ha mencionado, la naturaleza es una enorme fuente de diversidad genética, aunque la aplicación industrial de nuevas cepas con este origen tiene sus limitaciones. Una alternativa, además de la generación artificial de variantes, es utilizar cepas de un tipo fermentativo diferente, pero no demasiado alejado del que se quiere diversificar. Por ejemplo, las cepas panaderas y cerveceras, aunque en linajes separados (Gonçalves *et al*., 2016), comparten su necesidad de fermentar eficientemente la maltosa, el principal azúcar de los cereales y, en consecuencia, muchos genes implicados en la utilización de este disacárido aparecen en mayor número de copias en las

poblaciones de estas levaduras (Gallone *et al*., 2016; Bigey *et al*., 2021). De hecho, el uso de aislados de *S. cerevisiae* de MM o de cepas comerciales de levaduras de panadería ha sido examinado previamente en mosto de cervecería (Marongiu *et al*., 2015; Mascia *et al*., 2015; Ripari *et al*., 2018; Cubillos *et al*., 2019) y es una práctica común en algunos estilos de cerveza populares en los países nórdicos y de Europa del Este, como Kvass (Loponen y Sibakov, 2013) y Sahti (Ekberg *et al*., 2015). En esta línea, se ha estudiado la posibilidad de utilizar levaduras non-*Saccharomyces* aisladas de MM para desarrollar cervezas con baja graduación alcohólica (Johansson *et al*., 2021). Esto pone de manifiesto el interés y la importancia del microbioma de la MM como fuente de una impresionante diversidad genética que puede ser explotada en diferentes procesos fermentativos, más allá de su uso tradicional en panificación.

Justificación y objetivos



"Si se quiere mejorar al pueblo, en vez de discursos contra los pecados, dénles mejores alimentos. El hombre es lo que come"

Ludwig Feuerbach

El interés por la producción artesanal de pan empleando métodos tradicionales viene marcado por cambios globales y el incremento en la incidencia de enfermedades relacionadas con las dietas modernas (diabetes de tipo II, obesidad y alergias alimentarias). En este sentido, cada vez son más las evidencias que respaldan el uso de la masa madre, no sólo para la producción tradicional de pan, sino también como la mejor alternativa para nuevos productos de panadería sin gluten o con bajo contenido en sal o en grasa, entre otros (Silow *et al*., 2018; Ramos *et al*., 2021). Esto refleja la presión que recibe el sector agroalimentario en términos de salud y seguridad, que además debe atender al constante crecimiento de la población. Todo ello, sin descuidar la satisfacción que se busca en el contexto sociocultural vinculado a la alimentación.

En este y otros muchos aspectos, es imprescindible poner a disposición de usuarios y consumidores una mayor diversidad genética de levaduras, que promueva la consolidación de una industria biotecnológica segura, controlada y flexible, capaz de afrontar los retos demográficos, junto con la necesidad de preservar el medio ambiente y que, además, permita diversificar la gama de productos con valor nutricional y organoléptico. Fiel a estas premisas, el presente trabajo de tesis doctoral, pretende ampliar nuestro catálogo de levaduras de masa madre, en el convencimiento de que cada una de ellas es única en cuanto a la diversidad de especies microbianas. Creemos también que es posible incrementar nuestro conocimiento sobre los mecanismos de respuesta a estrés en levaduras y explotar su diversidad en otros procesos biotecnológicos afines a la panificación, en los que la maltosa es el azúcar predominante, como el de cervecería. Por último, planteamos la hipótesis de que la estrategia de evolución adaptativa es adecuada para obtener levaduras mejoradas con mayor tolerancia a estrés y que mantengan su condición GRAS (Generally Recognised As Safe) para ser aptas en el proceso industrial al que van destinadas, sin sacrificar sus principales características fenotípicas y tecnológicas. Para ello, se ha seguido un

hilo conductor en torno a los objetivos generales y específicos que se enumeran a continuación.

1. Caracterizar, química y microbiológicamente, masas madre elaboradas a nivel industrial por la compañía Europastry.

1.1. Analizar los principales parámetros químicos de cada masa madre objeto de estudio.

1.2. Determinar las características fenotípicas y fermentativas de las levaduras aisladas de la masa madre.

2. Mejorar la tolerancia a ácido acético en aislados de masa madre de *S. cerevisiae* mediante estrategias de adaptación evolutiva.

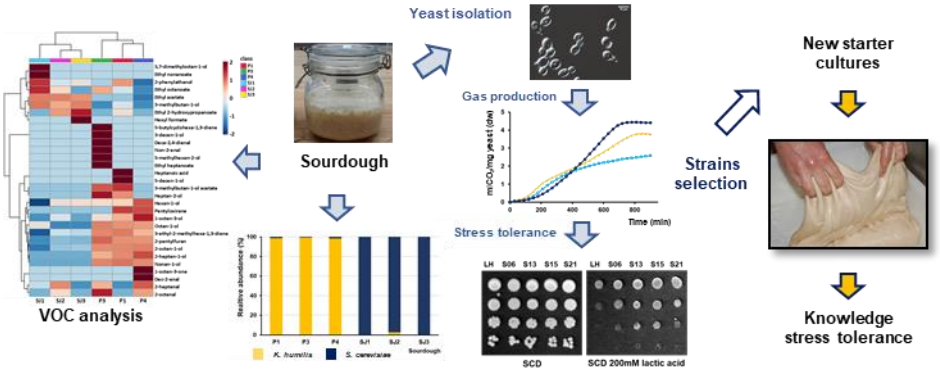
2.1 Obtener nuevas cepas de *S. cerevisiae* con mayor tolerancia a ácido acético, preservando su capacidad fermentativa en ausencia de estrés.

2.2. Caracterizar los cambios a nivel genómico de las cepas evolucionadas que determinan su adaptación a estrés.

3. Examinar el uso de aislados de masa madre de *S. cerevisiae* en la elaboración de cerveza.

3.1. Analizar la capacidad fermentativa de estas levaduras y su contribución al perfil aromático del mosto de malta.

Chapter 1: Technological and acid stress performance of yeast isolates from industrial sourdough



Keywords: *S. cerevisiae*, *K. humilis*, leavening capacity, volatile organic compounds, acid tolerance.

This chapter has been available with open access since June 4, 2023 and can be found online through the link included below.

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Summary

Yeast populations in sourdough have been poorly characterized from baking-relevant attributes as leavening activity in a stressful environment. In particular, the presence of extracellular acids represents an important challenge, which compromises the growth and fermentative metabolism of microbial starters. Here, we have analysed the fungal diversity of six industrial sourdoughs, identifying *Saccharomyces cerevisiae* and *Kazachstania humilis* as the predominant species. The volatile organic compounds (VOC) profile revealed differences between sourdough samples which can be attributed, at least in part, to their microbial composition and ingredients used in each one. In particular, *K. humilis* appeared to contribute to the presence of octan-1-ol, 2-hepten-1-ol, 2-octen-1-ol, nonan-1-ol and 2-pentylfuran in the sourdough. A total of 45 yeast isolates were then analysed in terms of CO_2 production and tolerance to NaCl, ethanol, oxidative-, cold- and heat-stress, which allowed the selection of 5 *S. cerevisiae* isolates and 2 from both *K. humilis* and *Torulaspora delbrueckii* for further characterization. Overall, *K. humilis* isolates showed a higher total CO_2 production and gassing rate than a commercial yeast strain used as a control. Hence, the isolated strains are potential candidates for the development of new sourdough starters and the basis for a further selection of variants with superior performance.

1. Introduction

Spontaneous fermentations have been traditionally used to endow cereal-based products with improved technological, nutritional and organoleptic properties. In artisanal bread production, sourdough, a spontaneous or starter-fermented mixture of flour and water, is used as a leavening agent (Brandt, 2019). Microbial activities also improve bread flavour and texture (Galle and Arendt, 2014; De Vuyst *et al*., 2023), and provide potential health benefits (Zannini and Gobbetti, 2019; Gabriele *et al*., 2023), including lower phytate and gluten levels, improved digestion and reduced glycemic index (Gobbetti *et al*., 2019; Rizzello *et al*., 2019). These properties account for the increased worldwide demand for sourdough bread, and the growing interest of the industry in sourdough-based baking technology.

Traditional sourdoughs are maintained by daily refreshments of an initial flour-water mixture that is spontaneously fermented by autochthonous microorganisms (Gänzle and Ripari, 2016; Landis *et al*., 2021; De Vuyst *et al*., 2023). Propagation of the initial dough over an extended period of time by backslopping cycles, allows the selection of the most adapted microorganisms, mainly lactic acid bacteria (LAB) and yeast, which define a mature sourdough that can be maintained for decades (Khlestkin *et al*., 2022). As an alternative, starter culture-initiated sourdough followed by backslopping can be used to speed up the process and to obtain a certain microbial composition with higher acidification capacity or flavour properties (Siragusa *et al*., 2009). However, this sourdough bread process is labor-intensive and time-consuming and requires tight control of the fermentation conditions and refreshment to ensure a stable microbiota and avoid undesirable contaminations (Venturi *et al*., 2012). This makes the production of traditional sourdough bread at the industrial level difficult or limited to a few products (Brandt, 2019).

Ready-to-use dried, pasty and liquid sourdough are commercialized and used as an ingredient in the dough formula in bakeries and industries. They are a source of acids and specific functional compounds targeted to improve flavour, taste and microbial self-life, and thus, they offer a clear added value (Quattrini *et al*., 2019; De Vuyst *et al*., 2023). Generally, they are metabolically inactive due to the high acidification and, consequently, the leavening activity of the bread dough relies on the use of commercial baker's yeast. However, this requirement must be taken into consideration when developing sourdough products in different countries. Some national regulations define sourdough as a leavening agent (Brandt, 2007), and they limit the use of commercial baker's yeast in the dough formula. Thus, natural starters for sourdough fermentation must display a high leavening capacity and be robust enough to ensure their viability and performance in different sourdough processes.

LAB and yeast, together with acetic acid bacteria (AAB), encompass the microbial ecosystem of natural sourdough. LAB are responsible for the acidification and protein degradation of the dough matrix, which has a positive influence on the final bread quality and shelf-life (Jin *et al*., 2021; Illueca *et al*., 2023). Heterofermentative LAB also contribute to dough rising, but leavening activity relies mostly on yeast metabolism. In general, mature nonstarter culture-initiated sourdoughs of different origins only contain one or two yeast species, among which *Saccharomyces cerevisiae* and *Kazachstania humilis* are the dominant species (De Vuyst *et al*., 2017; Comasio *et al*., 2020a; Landis *et al*., 2021). In addition, *Torulaspora delbrueckii*, *Kazachstania exigua*, *Wickerhamomyces anomalus*, *Issatchenkia orientalis* and *Candida glabrata*, among others, are commonly found in sourdough isolates at a given time, depending on the bakery environment, ingredients and process conditions (De Vuyst *et al*., 2017; Chiva *et al*., 2021). Microbial interactions also give shape to the sourdough community structure, as demonstrated by a worldwide survey of

microbial diversity in more than 500 sourdough starters (Landis *et al*., 2021). For example, maltose-negative species, like *K. humilis*, show positive interaction with the maltose-positive LAB species as *Fructilactobacillus sanfranciscensis* (Brandt *et al*., 2004; De Vuyst *et al*., 2016), which explains their co-occurrence in sourdough communities (Landis *et al*., 2021). Likewise, the presence, cell concentrations and leavening capacity of *S. cerevisiae*, the most powerful CO_2 producer among yeast (Xu *et al*., 2019), are affected positively or negatively by its associative growth with different heterofermentative LAB (Boudaoud *et al*., 2021; Oshiro *et al*., 2021).

The occurrence of *S. cerevisiae* in sourdough is strongly affected by the continuous acidification after each backslopping cycle. The metabolic cooperation between *K. humilis*, which releases fructose as a byproduct (Vogel *et al*., 2002), and *F. sanfranciscensis*, which uses this sugar as an electron acceptor, favors the production of acetic acid instead of ethanol by this late (Gänzle *et al*., 2007; Rogalski *et al*., 2021), which acts as a selective pressure on *S. cerevisiae*. This could explain the negative co-occurrence pattern for *S. cerevisiae* and *F. sanfranciscensis* as demonstrated by the amplicon sequence dataset and competition experiments (Landis *et al*., 2021), and the reduced counting of *K. humilis* as compared with LAB (Comasio *et al*., 2020a). The study by Landis and coworkers (2021) also pointed out a connection between microbial composition and dough rise, with special emphasis on AAB, a generally understudied sourdough bacteria component (De Vuyst *et al*., 2017; Comasio *et al*., 2020b). Indeed, the total relative abundance of AAB, including *Acetobacter* and *Gluconobacter* species, correlates negatively with leavening capacity, most likely by increased production of acetic acid and inhibition of yeasts (Landis *et al*., 2021).

The presence of extracellular acids represents an environmental challenge for many industrial bioproduction processes, including sourdough

baking applications. Acid toxicity in the cell is a combined function of its concentration and extracellular pH (Thomas *et al*., 2002; Graves *et al*., 2006), as not dissociated acids can diffuse through the cell membrane (Casal *et al*., 1996). This causes intracellular acidification, which compromises growth and metabolism. Under these conditions, *S. cerevisiae*, the best-known yeast at the physiological and molecular level, mounts a response that improves stress tolerance (Mira *et al*., 2010), but at high acid doses and low pHs, toxicity leads to loss of cell viability (for a review see Guaragnella and Bettiga, 2021). In this context, different strategies have been addressed to improve acid tolerance, mainly toward acetic acid, including overexpression of stress-tolerance genes, laboratory evolution, synthetic biology and metabolic engineering approaches (González-Ramos *et al*., 2016; Palma *et al*., 2018; Qin *et al*., 2020). However, the levels of tolerance reached by these approaches might be not enough, involve often recombinant DNA techniques and have been only tested in a laboratory or lignocellulosic ethanol-producing industrial strains (Guaragnella and Bettiga, 2021). Alternatively, the selection of naturally evolved strains, with relevant capacities to produce CO_2 even in high acidity and low pH conditions, appears as an interesting strategy.

Yeast populations in sourdough have been poorly characterized from baking-relevant attributes as leavening activity in a stressful environment. In general, *K. humilis* has been reported to be more sensitive to low pH than *S. cerevisiae*, while this late was less tolerant to acid concentrations. Nevertheless, the effects are strongly dependent on the strain analysed (Carbonetto *et al*., 2020). It is also clear that starter cultures used in sourdough processes have been selected based on a particular trait, like acid tolerance or flavour production (De Vuyst *et al*., 2017; Comasio *et al*., 2020a). In the present study, we have characterized the yeast population of a group of six industrial sourdoughs regarding their technological functionality under stress conditions. Our aim was

to isolate new yeast strains for the development of sourdough starter cultures with improved performance. The results demonstrate the importance of the sourdough microbial community as a source of yeast diversity and phenotypic traits of technological relevance. In addition, the novel strains could be the basis for a further selection of variants with superior performance and offer the opportunity to gain knowledge about the mechanisms that govern stress tolerance in the sourdough environment.

2. Materials and Methods

2.1. Sourdoughs

Six industrial sourdoughs used for the manufacture of bread elaborated and distributed by the baking company Europastry, S.A. (St. Cugat del Vallès, Barcelona, Spain) were studied. The sourdoughs are representative of two manufacturing plants located in Paterna, Valencia (indicated by the letter P) and Sant Joan Despí, Barcelona (indicated by the letters SJ). They ranged from semi-liquid to firm dough and were elaborated with wheat, wholemeal wheat or spelt flour. In all cases, a mature sourdough was obtained after a number of 6-7 backsloppings of the initial dough (mixture of flour and water fermented 24 h at 28ºC), these refreshments were carried out daily at 28ºC (8 h) in the proportion of 1:1:1 (flour:water:dough). Finally, the industrial sourdough was obtained after a single backslopping of the mature sourdough that was followed by a resting time of 24 h (4°C) before each baking-run. The ingredients and technological conditions for the industrial sourdough preparation are summarized in **Table 1**. As soon as received, 15 g of industrial sourdough was disaggregated in a bag mixer 400 lab blender (Interscience, St. Nom, France) in a final volume of 150 ml with 0.9 % NaCl solution. This initial homogenate was common to all chemical and microbiological assays, treating each aliquot in a specific way depending on the determination or test to be carried out, as described below.

Table 1. Industrial sourdoughs used in this study.

Sourdough	Flour	Dough Yield	Salt (%)	Backslopping	
				Time (h)	Temperature (ºC)
P1	T80	200	0	3	26
P3	Whole Wheat	200	0	3	30
P4	Spelt	200	0	3	30
SJ1	Wheat	200	0	4	27
SJ2	Wheat	153	1	4	25.5
SJ3	Wheat	155	0	4	25.5

2.2. Media and culture conditions

Previously described standard methods were followed for media preparation (Guthrie and Fink, 1991). Yeast cells were cultured at 25.5/30°C (see **Table 1**) in YPD (1 % yeast extract, 2 % peptone and 2 % glucose), or SCD (0.67 % yeast nitrogen base without amino acids, plus 2 % glucose). Counting and isolation of yeast cells from sourdough was carried out in SCD containing 100 µg/ml kanamycin (SCD-Kan). Biomass from yeast isolates was prepared by culturing cells in a liquid molasses medium according to an industrial recipe: 116.6 g of beet molasses (49 % sucrose), 1.5 g of ammonium sulfate, 0.15 g of orthophosphoric acid, and 20 µg of biotin per liter; adjusted to a final pH 5.0.

For gas measurement experiments, yeast cells were inoculated in a flour-free liquid-dough (LD) model system (Panadero *et al.*, 2005). Briefly, this consisted of 45 g l^{-1} maltose, 15 g l^{-1} glucose, 4.7 g l^{-1} $(NH_4)_2HPO_4$ and 2.5 g l^{-1} yeast extract, buffered with 14.14 g l^{-1} sodium citrate at pH 5.5 (adjusted with citric acid). The medium also contained 60 g l^{-1} sorbitol (0.33 M) and 13.5 g l^{-1} NaCl (0.23 M), which reduce water activity to bread dough values (aw ~ 0.97), and a mixture of essential nutrients, 2 g l^{-1} $MgSO_4 \cdot 7H_2O$, 0.8 g l^{-1} KCl, 40 mg l^{-1} nicotinic acid, 4.0 mg l^{-1} thiamine and 4.0 mg l^{-1} pyridoxine. Sour LD was prepared as above, except that lactic (200 or 300 mM) and/or acetic acid (20 mM) were included in the formula. All amino acids, sugars, antibiotics and

nutrient solutions were filter-sterilized and added to the autoclaved medium. Solid media contained 2 % agar.

For plate phenotype experiments, yeast cultures were diluted to OD_{600} = 0.8 and 10-fold serial dilutions spotted (3 µl) onto YPD- or SCD-agar solid media, lacking or containing 1 M NaCl, 9 % ethanol or 5 mM H_2O_2. Unless otherwise indicated, colony growth was inspected after 2–4 days at 30°C. For thermal stress, cells were incubated at low (12ºC) or high (39ºC) temperatures. Plates with maltose as a carbon source were also prepared.

2.3. Yeast isolation, counting and identification

To quantify microbiota naturally present in each sourdough, 10-fold serial dilutions of the initial homogenate (up to 10^{-5}) were made, and 100 µL of the last dilution was plated over the SCD-Kan medium. After incubation at 27°C for 48-72 h, 4-6 colonies from each plate were randomly selected, grown in a liquid medium, maintained as frozen (-80ºC) glycerol stocks and then rescued in YPD agar plates at 30ºC for 24 h before further analysis. These conditions preserved the characteristics of the isolates and ensured that yeast cells were growing under the same conditions. The number of microorganisms was expressed as a logarithm of colony-forming units (cfu) per g of sourdough analysed.

Yeast isolates were identified by the restriction fragments length polymorphism (RFLP) of the ITS-5.8S regions (Esteve-Zarzoso *et al*., 1999), and verified by DNA sequencing. DNA extraction from stationary phase-grown yeast cultures was performed according to Lõoke *et al*. (2011). The 5.8S-ITS internal transcribed region of the rDNA was amplified employing the primer pair ITS1-ITS4 (Toju *et al*., 2012) and the NZYTaq II DNA polymerase kit (NZYTech, Lisboa, Portugal), according to the manufacturer's instructions. PCR

products were digested without further purification with *Cfo*I, *Hinf*I and *Hae*III (FastDigest, Thermo Scientific), and the restriction fragments were separated by electrophoresis in 2 % agarose-Tris-acetate-EDTA buffer (pH 8.0), stained with RedSafe (iNtRON Biotechnology, Burlington, MA) and photographed under transilluminated UV light. Restriction patterns were compared with those reported in the database for rapid identification of yeast (www.yeast-id.org). In cases where the identification was doubtful, the PCR fragments were sequenced at the SCSIE Genomics Facility DNA sequencing services of the University of Valencia (Spain) and the sequences were compared by BLAST (Altschul *et al*., 1990).

2.4. Illumina MiSeq Sequencing and data processing

The analysis of microorganism communities associated with the sourdough samples under study was carried out by high-throughput Illumina MiSeq amplicon sequencing (Illumina, San Diego, CA) of the ITS1 region between the 18S and 5.8S rRNA genes (Toju *et al*., 2012) for yeast or the 16S rRNA for bacteria (Klindworth *et al*., 2013). Frozen portions (0.5 g) of sourdough were thawed on ice, and genomic DNA extraction was performed by using the DNeasy kit (Qiagen, Hilden, Germany).

Library preparation included two amplification steps. The first amplification was a nested PCR using primers ITS1-for/ITS4-rev and ITS5-for/ITS2-rev for yeast (Toju *et al*., 2012) or specific primers (**Table S1**), for variable V3 and V4 region of the 16S rDNA gene (Klindworth *et al*., 2013) for bacteria. Illumina overhang adapter sequences were added to ITS5-for and ITS2-rev primers to attach, as for the 16S gene, in a second PCR step indexes and Illumina sequencing adapters using the Nextera XT v2 Index kit (Illumina). Libraries were pooled and sequenced on the MiSeq System using a v3 reagent kit, 600 cycles (Illumina). To increase nucleotide base diversity for more

accurate base-calling, all libraries were spiked with 5 % PhiX Control v3 (Illumina).

Sequencing data have been demultiplexed using the Illumina bcl2fastq© program. Demultiplexed paired FASTQ sequences were imported into the QIIME2 v2022.2 for analysis. Quality control was carried out using the DADA2 pipeline incorporated into QIIME2 (Caporaso *et al*., 2012). The DADA2 program filtered out phiX reads removed chimeric sequences and assigned reads into Amplicon Sequence Variants (ASVs) (Callahan *et al*., 2016). 16S amplicon Taxonomic annotation was obtained using the SILVA v138 database for 16S amplicon and ITS unite v9 fungi database for ITS1 amplicon.

2.5. pH and titratable acidity (TTA)

The pH of sourdough samples was determined using a pH meter (HI99165, HANNA) equipped with an FC2423 penetrating electrode (HANNA Instruments, Padua, Italy). To TTA determination, 100 ml of the initial homogenate was clarified by centrifugation at 4,000 rpm for 15 min and 25 ml of the supernatant was titrated at 20ºC using the automatic potentiometric titrator Excellence T5 (Mettler Toledo, Columbus, OH). Results were expressed as the volume (ml) of 0.1 M NaOH standard solution (Merck, Darmstadt, Germany) needed for titrating 10 g of sourdough until a final pH of 8.5. Data represent the mean value and standard deviation (±sd) of at least three replicates.

2.6. Metabolite target analysis

Determination of lactic and acetic acids was carried out by Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC), using a chromatograph (Waters, Milford, MA) equipped with a variable wavelength (PDA) detector operating at 220 nm. Organic acids were extracted according to

Alfonzo *et al*. (2013). Extracts were filtered through 0.45 µm hydrophobic-PTFE filters and 20 µl were injected into a C18 3 µm column (4,6 x 150 mm; Atlantis T3, Waters). Chromatographic separation was conducted on isocratic conditions with a 1 ml/min flow rate of $HClO_4$ 0.1 mM at 35ºC. Run time, including separation and column washing, was 30 min.

2.7. Dry weight measurements

The cell density of saturated YPD-grown cultures was monitored by measuring the A_{600} after the appropriate dilution. Dry weights were determined gravimetrically after filtering and drying 50 units of OD_{600} at 55ºC for 24 h, and are expressed as mg of dry weight per OD_{600}.

2.8. Gas production

Molasses-grown cells were collected, washed, filtered and resuspended in distilled water (4ºC) containing 16 g l^{-1} of NaCl and the A_{600} of the resulting suspension was measured as previously described (Aguilera *et al*., 2010). The final yeast amount was adjusted to 160 OD units (uOD) and 15 ml was poured into a 250-ml screw cap bottle, placed in a 30°C water bath and gently shaken (80 rpm). After 15 min, 15 ml of 30°C-pre-warmed 2× LD model solution was added (Panadero *et al*., 2005) and the amount of CO_2 was recorded at 30 min intervals for 960 min in an AF-1101-10W Fermograph II apparatus (ATTO Corporation, Tokyo, Japan). Gas production measurements were also carried out by using a flour-basis dough system. For this, commercial wheat bread flour (Florencia from Harinas Polo; moisture <15 %; W 390-410 J; P/L, 0.9-1.0) was used. Bread dough was prepared by mixing 50 g of flour and 30 ml of 1.6 % NaCl containing 60 uOD_{600} of yeast suspension. The resulting dough was placed in a pre-warmed bottle and the CO_2 production was measured as above. In all cases, values are expressed as ml of CO_2 per mg of yeast cells (dry weight), and

represent the mean ± sd of at least three independent experiments. LD and sour bread doughs were prepared as above, with lactic (150, 200 or 300 mM) and/or acetic acid (15 or 20 mM) included in the formula.

2.9. Volatile organic compounds analysis

The volatile organic compounds (VOC) were analysed according to Wang *et al*. (2020) with some modifications. For each analysis, 0.5 g of sourdough was placed into a 20 ml headspace vial with 1 ml of milli-Q water and 5 µl of internal standard solution (2-heptanone, Sigma) at 42 µg/ml. A 95 µm Carboxen/Polydimethylsiloxane (CAR/PDMS) fiber (Agilent Technologies, Santa Clara, CA, USA) was used to extract VOC from sourdough. Using an MPS robotic autosampler (Gerstel, Germany), the vial, previously sealed with a silicon septum, was then heated in an oven at 50°C for 42 min and the SPME fiber was exposed during the last 30 min. Chromatographic separation of volatile compounds was carried out by an Agilent 7890B-5977B GC/MSD system equipped with an HP-5 capillary column (30 m × 0.25 mm × 0.25 µm) (Agilent Technologies). The fiber was desorbed in splitless mode at 250ºC for 3 min. The gas carrier was Helium with a flow rate of 0.9 ml/min. The temperature program was 35ºC for 3 min, increased to 160ºC at a rate of 4ºC/min and then increased to 240ºC at a rate of 10ºC/min, held for 2 min before post run at 250ºC for 2 min. The temperatures of the injection port, ion source and transfer line were 250, 230 and 260ºC, respectively. Mass spectra were acquired in the electronic impact of 70 eV and the scan mode in the m/z range of 29–400 amu (atomic mass unit). Peak identification was carried out by comparison of the mass spectral data obtained with those in the NIST (National Institute of Standards and Technology) library. Peak areas were expressed in arbitrary units based on detector output.

2.10. Statistical analysis

Normalization of VOC data, heatmap, and correlation matrix were performed using MetaboAnalyst (Xia *et al*., 2009- https://www.metaboanalyst.ca/).

3. Results and discussion

3.1. Biochemical profile and microbial counts

The main biochemical characteristics and the microbial accounts of the sourdoughs analysed are reported in **Table 2**. As it is shown, the six sourdoughs under study, three from Sant Joan Despí (SJ) and three from Paterna (P), were quite homogeneous concerning pH and TTA parameters, ranging from 3.7 to 3.9, and 7.1 to 15.2, respectively. Sourdough samples P1 and P3 showed the highest TTA values in consonance with their increased lactic and/or acetic content (**Table 2**). Finally, yeast colony counts on YPD varied from 5.1±0.3 to 6.29±0.05 cfu/g.

Table 2. Biochemical parameters and yeast counting.

Sourdough	pH	TTA[1]	Lactic acid[2]	Acetic acid[2]	CFU[3]
P1	3.91±0.15	15.2±0.2	11.7±0.9	0.9±0.2	6.07±0.09
P3	3.80±0.04	13.3±0.8	15.3±0.3	1.5±0.3	6.07±0.12
P4	3.70±0.08	7.6±0.8	8.7±0.8	0.6±0.1	5.1±0.3
SJ1	3.70±0.08	8.2±0.5	8.9±0.1	1.0±0.1	6.2±0.3
SJ2	3.90±0.11	9.9±0.6	9.5±0.9	1.6±0.4	6.29±0.05
SJ3	3.87±0.13	7.1±0.6	9.3±0.6	1.2±0.0	5.71±0.15

[1] expressed as the volume (ml) of 0.1 M NaOH standard solution needed for titrating 10 g of sourdough until a final pH of 8.5.
[2] expressed as mg acid/g of sourdough.
[3]expressed as log of colony forming unit (cfu)/g of sample.

3.2. Identification of yeast isolates

In total, 200 yeast isolates from the six sourdoughs analysed were arbitrarily recovered from kanamycin-containing YPD plates, propagated and stored at -80ºC for further identification. As expected, yeast diversity, as determined by RFLP-PCR analysis of the ITS-5.8S of each yeast isolate (Esteve-Zarzoso *et al*., 1999), was limited to a few species, *S. cerevisiae* (91 isolates), *K. humilis* (92 isolates), *T. delbrueckii* (9 isolates) and others (8 isolates). In addition, all sourdough analysed were characterized by a predominant one (**Table 3**). Samples from Sant Joan Despí contained mainly *S. cerevisiae* isolates, while *K. humilis* was the dominant specie in the sourdoughs elaborated in the Paterna's production plant. In addition, *T. delbrueckii* was found as minor specie in two of the three Paterna samples (P1 and P3), but not in sourdoughs prepared at the Sant Joan's facilities (**Table 3**). This suggests that the microbial environment of the bakery was the most important factor influencing the yeast composition of sourdoughs.

Table 3. Number of identified sourdough isolates.

SD[1]	No. of isolates	Sc[2]	Kh[3]	Td[4]	Other yeast
P1	37	7	21	5	4
P3	35	7	24	4	-
P4	32	5	27	-	-
SJ1	32	26	5	-	1
SJ2	34	22	10	-	2
SJ3	30	24	5	-	1

[1] SD: sourdough
[2] Sc: *S. cerevisiae*
[3] Kh: *K. humilis*
[4] Td: *T. delbrueckii*

3.3. Microbial diversity

The bacterial and fungal diversity of sourdoughs was explored by sequence-based analysis of the 16S rRNA gene and the ITS region, respectively. In all sourdoughs, the predominant bacterial phylum was Firmicutes (**Table S2**), with more than 97 % of the reads. At the specie level, *Fructilactobacillus sanfranciscensis* was the prevalent LAB in all the samples, independently of the location where the sourdoughs were elaborated. Unlike this, the fungal community was significantly different between sourdoughs (**Table S3**). The relative abundance (> 0.01 %) of ASVs (amplicon sequence variants) at the class assignment allowed for enlarging of the fungal diversity among the sourdoughs analysed (**Figure 4**).

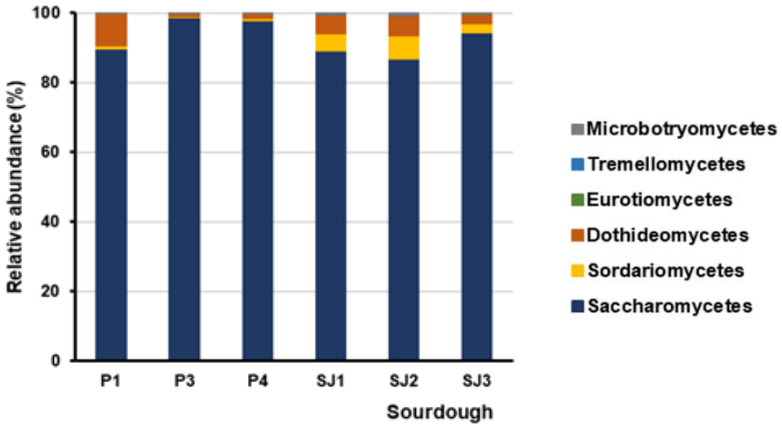


Figure 4. Relative abundance and distribution of major fungal classes from sourdoughs, elaborated in Sant Joan Despí (SJ1-SJ3) and Paterna (P1, P3 and P4).

As expected, the Saccharomycetes class was the most frequent (relative abundance) sourdough fungal group. Again, *S. cerevisiae* and *K. humilis* were the dominant yeasts in the SJ and P sourdoughs, with more than 97 % and 99 % of the reads, respectively. We also noted that *T. delbrueckii* represented less than 0.01 % of the reads, in contrast with the data obtained by identification of

randomly picked yeast colonies (**Table 3**), a circumstance that stresses the importance of both culture-dependent and -independent approaches in the study of sourdough microbial diversity. Finally, additional class-level groups, including Sordariomycetes, Dothideomycetes, Eurotiomycetes, Tremellomycetes and Microbotryomycetes, and species such as *Gibberella zeae*, *Monographella nivalis*, *Claviceps arundinis*, *Phoma herbarum*, *Stemphylium vesicarium*, *Mycosphaerella tassiana* and *Alternaria metachromatica* were detected in the sourdoughs sampled (**Figure 4**). Some of them are important fungal wheat pathogenic or endophytes species that are host by the flour employed in the sourdough elaboration (Aveskamp *et al*., 2008; Jayawardena *et a*l., 2019; Gagkaeva *et al*., 2020). Thus, the low number of non-fermenting species likely reflects the production during fermentation of acids, CO_2 and ethanol, and the reduction of aerobic conditions by O_2 consumption.

3.4. Impact of sourdough yeast composition on volatile compound profiles

Thirty-one volatile compounds (VOC), including higher alcohols, aldehydes or esters, were extracted, analysed and identified from SJ and P sourdoughs by using SPME-GC-MS. Among them, hexan-1-ol, 3-methylbutan-1-ol, ethyl octanoate, ethyl acetate, 1-octen-3-ol or 2-pentylfuran, were found in all sourdoughs analysed. **Figure 5** presents a heatmap of the relative abundance of major VOC in each sample. As can be seen, the different signal intensities determined the separation of the six sourdoughs into two clusters according to their production plant (SJ and P). In general, this arrangement was mainly based on the relative content of higher alcohols (ethanol was not determined) and esters that were more abundant in samples elaborated in Paterna and Sant Joan, respectively (**Figure 6**). As alcohols are described by green and herbaceous odor notes, meanwhile esters have a fruity and pleasant aroma (Lee and Noble, 2003;

Petel *et al*., 2017), our results suggest that sourdoughs prepared in each industrial location give rise to products with differential aromatic characteristics. This is also consistent with previous reports showing the profile of VOC detected in the fermentation of grape must and sourdough by *Kazachstania* spp. and *S. cerevisiae* (Ripari *et al*., 2016; Liu *et al*., 2020; Lin *et al*., 2022; Niçin *et al*., 2022).

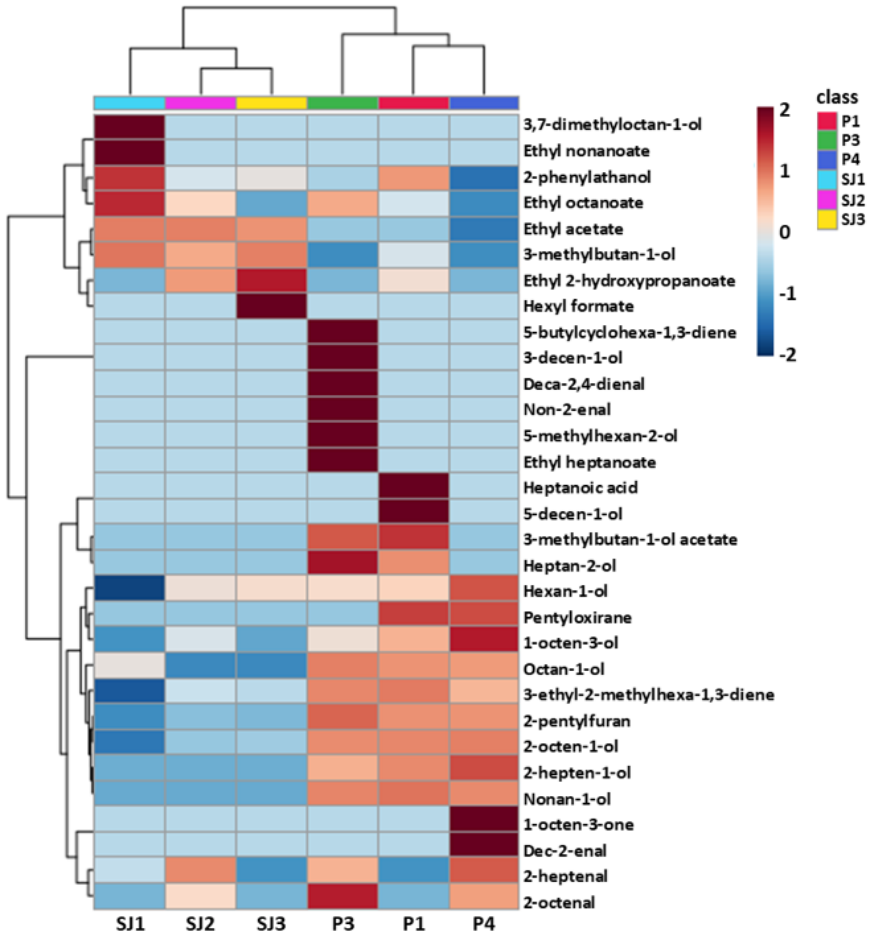


Figure 5. Heatmap of the relative abundance and distribution of VOC in the sourdoughs analysed. Columns represent the different sourdoughs analysed from Sant Joan Despí (SJ1-SJ3) and Paterna (P1, P3 and P4) production plants, and rows the 31 volatile compounds identified. Colors correspond with relative abundance values, where dark red indicates high levels, and dark blue indicates low levels (at least three biological replicates were analysed).

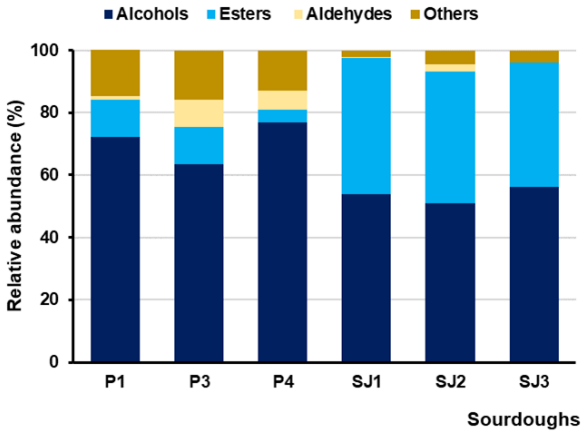


Figure 6. Chemical classes of volatile organic compounds detected from sourdough samples. Compounds belonging to each chemical class were grouped and their corresponding total amounts expressed as area percentages (%) related to the internal standard. Data represent the mean values of at least three independent replicates.

To further understand the correlation among the volatile fraction composition, the predominant yeast and the flour used in each sourdough, a Spearman's correlation index was calculated and the corresponding matrix was drawn (**Figure S1**). As expected, the VOC profile of sourdoughs was influenced by the type of wheat flour used. For example, a significantly strong correlation with deca-2,4-dienal, 5-buthylciclohexa-1,3-diene, 3-decen-1-ol, non-2-enal, 5-methyl-hexan-2-ol and ethyl heptanoate was found in P3 sourdough elaborated with wholegrain flour, while P1 and P4 samples, which were prepared with T80-wheat and spelt flour, correlate positively with 5-decen-1-ol and heptanoic acid, or 1-octe-3-one and dec-2-enal, respectively (**Figure S1**). In addition, 3-methylbutan-1-ol and ethyl acetate were specifically the major volatile compounds detected in all sourdoughs elaborated at the Sant Joan's plant (SJ1-SJ3), which have in common to be prepared with Florencia flour.

Regarding the predominant yeast, a significative positive correlation was found between *S. cerevisiae* and ethyl acetate or 3-methylbutan-1-ol, whereas in

sourdoughs with *K. humilis* as the predominant yeast, the correlation was established with nonan-1-ol, 2-hepten-1-ol, octan-1-ol, 2-octen-1-ol and 2-pentylfuran. In addition, these compounds showed a negative correlation with *S. cerevisiae*. In conclusion, our results highlight the importance of sourdough yeasts in modulating, together with flour type and elaboration parameters, flavour formation during fermentation. Consequently, it must be possible to fine-tune the desired aroma in bread by selecting the appropriate yeast starter.

3.5. Leavening ability and stress tolerance of yeast isolates

From the initial 200 identified yeast isolates, 45 strains (20 *S. cerevisiae*, 19 *K. humilis*, 3 *T. delbrueckii* and 3 others) displaying enough growth in molasses industrial medium, as required for their technological inspection, were selected for further characterization (**Table 4**). Biomass production, an important biotechnological parameter, expressed as dry cell weight, varied between the yeast isolates. In general, *S. cerevisiae* displayed the highest values (0.35±0.024 mg/OD_{600}), while the isolates from *K. humilis* and *T. delbrueckii*, with a mean value of 0.312±0.023 and 0.314±0.006 mg/OD_{600} respectively, showed a 12 % less of biomass production. Except for *Wickerhamiella pararugosa* (Wp01), all the strains tested exhibited leavening ability in a liquid dough model system, LD (**Table 4**), a synthetic medium that avoids the interference of the microflora naturally present in the flour and that mimics, at the physiological and molecular level, the nutritional and stressful environment of bread dough (Panadero *et al*., 2005). Nevertheless, there were strong differences in the fermentation capacity among isolates identified as the same specie (**Table 4**). For instance, gas production by *S. cerevisiae* strains was in the range between 3.12±0.13 and 1.06±0.06 ml CO_2/mg yeast dw; while for *K. humilis* strains this characteristic varied from 2.89±0.07 to 1.09±0.03 ml CO_2/mg yeast dw (**Table 4**).

Table 4. Gas production and stress behaviour of yeast isolates*.

Isolated	Sourdough	dw[1]	Gas[2]	NaCl	38ºC	12ºC	EtOH	H_2O_2
S01	P1	0.385	2.43±0.11	+	+++	++	+++	++
S02	P1	0.355	1.18±0.03	+	++	+	+++	+
S03	P1	0.362	2.834±0.015	++	+++	++	+++	-
S04	P1	0.327	2.41±0.03	-	-	++	++	-
S05	P3	0.368	2.44±0.12	-	-	++	++	-
S06	P3	0.333	2.94±0.02	+	+	+++	+++	++
S07	P3	0.303	3.12±0.09	-	-	++	++	-
S08	P3	0.392	1.695±0.013	++	+++	++	+++	++
S09	P4	0.388	1.46±0.03	++	++	+	+++	++
S10	P4	0.328	1.35±0.17	+	+	+	++	++
S11	SJ1	0.369	2.3±0.2	+	+	++	+++	++
S12	SJ1	0.336	1.42±0.06	+	+	+	++	+
S13	SJ1	0.337	2.88±0.05	+	+++	++	++	+
S15	SJ1	0.385	2.58±0.05	+	++	++	++	++
S16	SJ1	0.359	1.98±0.28	+	++	++	++	+
S20	SJ2	0.348	2.70±0.16	++	-	++	+++	+++
S21	SJ2	0.359	2.62±0.13	+	++	+++	++	++
S25	SJ3	0.335	1.06±0.08	+	+++	++	+++	+++
S27	SJ3	0.373	1.24±0.12	+	+++	++	+++	+++
S30	SJ3	0.341	2.41±0.06	++	+++	++	++	++
K02	P1	0.304	1.34±0.06	++	-	+	+	++
K03	P1	0.345	1.92±0.04	++	-	++	++	++
K04	P1	0.316	1.92±0.06	++	-	++	++	++
K06	P1	0.351	1.13±0.02	++	-	++	+++	+++
K07	P1	0.308	1.16±0.08	++	-	++	++	+++
K08	P1	0.290	2.61±0.05	++	-	++	+++	+++
K09	P3	0.294	2.70±0.03	+	-	+	++	+++
K10	P3	0.328	2.6±0.2	+	-	+	+	+++
K11	P3	0.314	2.89±0.07	++	-	+++	+++	+++
K12	P3	0.329	1.77±0.08	+	-	++	++	+++
K13	P3	0.282	2.842±0.016	+	-	++	++	+++
K14	P3	0.328	1.53±0.04	+	-	++	++	+++
K15	P4	0.310	1.60±0.08	+	-	++	++	+++
K17	P4	0.333	1.63±0.02	+	-	++	++	+++
K18	P4	0.335	2.02±0.09	+	-	++	++	+++
K19	SJ1	0.289	1.31±0.02	++	-	+	++	++
K21	SJ2	0.276	1.163±0.014	+	-	++	++	++
K24	SJ2	0.286	1.45±0.02	+	-	+	++	++
K31	SJ3	0.320	1.09±0.03	+	-	+	+	++
T01	P1	0.320	1.76±0.09	++	-	++	-	+++
T02	P1	0.313	1.33±0.03	++	-	++	++	+++
T03	P3	0.308	1.67±0.08	+++	-	++	+	+++
W02	P1	0.421	1.32±0.02	+++	+	+++	+++	+++
C01	P1	0.412	0.642±0.016	+++	-	+++	-	++
Wp01	SJ2	0.560	np	++	++	+	-	+

[1] dw= dry weight expressed as mg per OD_{600}. **[2]** expressed as ml of CO_2 per mg of cells (dw) at 480 min fermentation time. Values are means ± sd of three replicates. *****For phenotype experiments, yeast cultures were diluted to OD_{600} = 0.5 and 10-fold serial dilutions (10^{-1}-10^{-3}) spotted (3 µl) onto YPD-agar solid media lacking (12ºC, 38ºC) or containing 1 M NaCl, 9% EtOH or 5mM H_2O_2. Growth was inspected after 2-5 days at 30ºC or at the indicated temperature. – indicates no growth and +, ++ and +++ visible growth at the 10^{-1}, 10^{-2} and 10^{-3} dilution, respectively. np (no gas production).

The tolerance of yeast isolates was tested under stress conditions of biotechnological relevance. In general, all strains analysed grew well at low temperature (12ºC; **Table 4**), indicating an adaptation to the cold resting period employed in all sourdoughs analysed in this study (see the Materials and Methods section). Nevertheless, other stress conditions had a differential impact on growth. Salt stress provided by 1 M NaCl reduced notably, and even avoided, e.g. S04, S05 and S07, the proliferation of *S. cerevisiae* strains, but *K. humilis* and *T. delbrueckii* still maintained noteworthy growth levels under this condition (**Table 4**), in good consonance with previous characterization of isolates hosted by traditional sourdoughs from Portugal (Hernandez-Lopez *et al*., 2003, 2006; Fernandes *et al*., 2021). A high NaCl tolerance among species of the *Kazachstania* group isolated from sourdough has been also reported (Korcari *et al*., 2021). Likewise, most of the *K. humilis* and *T. delbrueckii* strains displayed higher tolerance to oxidative stress than those of the *S.* cerevisiae group. On the contrary, neither *K. humilis* nor *T. delbrueckii* strains were able to grow under heat conditions (38ºC), while *S. cerevisiae* strains were characterized by a moderate or high tolerance, e.g. S01, S03 or S13, among others (**Table 4**). This is an important biotechnological property since microorganisms for dry sourdough products and starters are often exposed to temperatures above the ambient (Brandt, 2019). Finally, the presence of high concentrations of ethanol (9 %) in the culture medium caused a strong inhibition of all strains except those grouped as *S. cerevisiae* or three *K. humilis* strains (K06, K08 y K11) that still displayed moderate growth (**Table 4**).

3.6. Effects of lactic acid on gassing rate

We were interested to analyse more in deep the technological performance of the sourdough yeast isolates in the presence of lactic acid. Based on the leavening performance and stress tolerance survey shown above (**Table**

4), five *S. cerevisiae isolates* (S03, S06, S13, S15 and S21) and 2 from both *K. humilis* (K08 and K13) and *T. delbrueckii* (T01 and T03) were selected for further characterization of their gassing rate. **Figure 7** shows the results of CO_2 production for each strain measured for 600 min in an LD medium lacking or containing 300 mM of lactic acid, which reduces the pH of the liquid dough from 5.5 to 4.2. L'Hirondelle (LH) were also tested. Concretely, all the isolates of *S.*

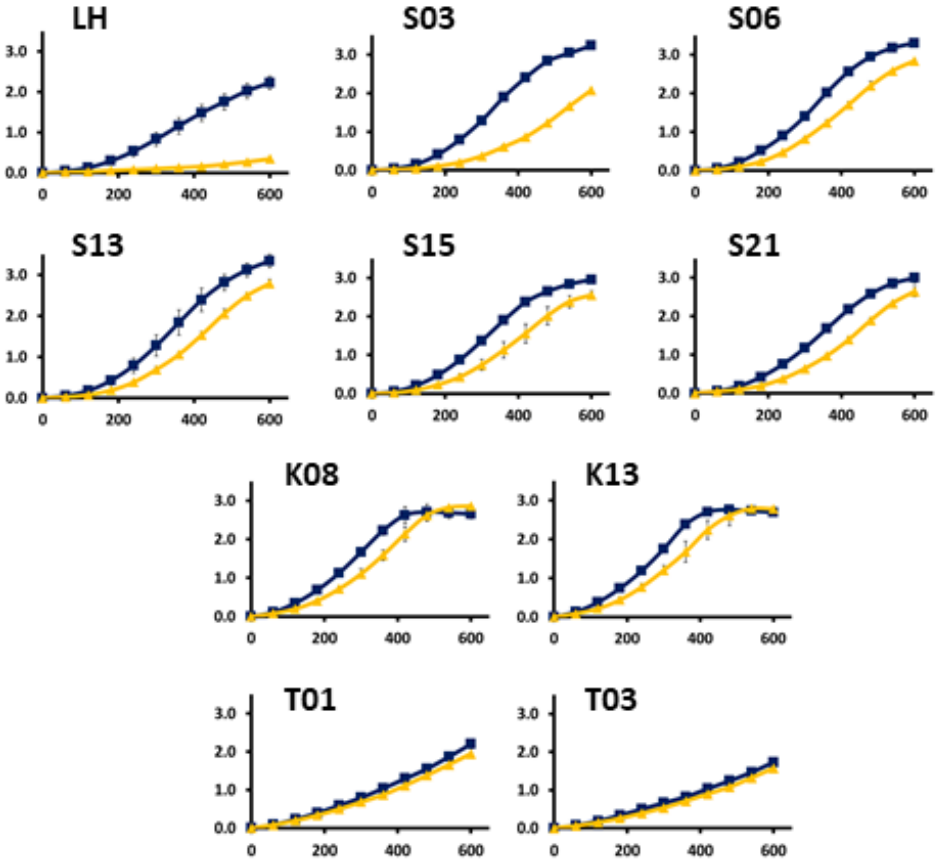


Figure 7. Gassing rate of selected yeast isolates of sourdoughs. Yeast suspensions of the indicated *S. cerevisiae* (S03, S06, S13, S15 and S21), *K. humilis* (K08 and K13) or *T. delbrueckii* (T01 and T03) isolates were mixed with a 30°C-pre-warmed LD model solution lacking (blue square) or containing (yellow triangle) 300 mM of lactic acid and the amount of CO_2 evolved was recorded at 30 min intervals for 600 min in a Fermograph II apparatus. The commercial bakers' yeast strain L'Hirondelle (LH) was used as a control. Values are expressed as ml of CO_2 per mg of yeast cells (dw) and represent the mean±sd of at least three independent experiments.

cerevisiae and *K. humilis* assayed displayed higher fermentative capacity than the commercial strain under control conditions (**Figure 7**). L'Hirondelle strain produced 1.16±0.20 ml of CO_2 per mg of cells (dw) after 6 h, while the mean value among the *S. cerevisiae* isolates was 1.97±0.19. In turn, the two selected isolates of *K. humilis* exhibited a high performance along the CO_2 production curve and the *Torulaspora* strains were the lowest (**Figure 7**). As expected, the differences in gassing rate were more pronounced in LD samples supplemented with 300 mM lactic acid. Thus, the commercial strain displayed a loss of around 89 % of CO_2 production at 6 h (**Figure 7**). For the sourdough isolates of *S. cerevisiae*, the drop ranked from 39 (S06) to 68 % (S03), while leavening after 6 h decreased by around 29 % and 15 % among the *K. humilis* and *T. delbrueckii* isolates, respectively (**Figure 7**).

Then, the impact of lactic acid on the CO_2 production ability of the sourdough isolates was investigated in flour-based dough samples lacking or containing 300 mM lactic acid or a mix with 150 mM lactic and 15 mM acetic acid. Compared with the LD, flour-basis dough contains lower amounts of readily fermentable sugars such as glucose, fructose and maltose, this late resulting from starch hydrolysis. In addition, *K. humilis* is maltose-negative, which should reduce its fermentation rate in flour-basis dough, in particular, if *F. sanfranciscensis* is not present. Indeed, *K. humilis* and *F. sanfranciscensis* have been reported to show a trophic relationship of cross-feeding (Brandt *et al*., 2004; De Vuyst *et al*., 2016; Landis *et al*., 2021), by which the LAB supplies glucose from maltose to *K. humilis* allowing its prevalence and co-occurrence in sourdoughs (Carbonetto *et al*., 2018, 2020; De Vuyst *et al*., 2023). Nevertheless, the *K. humilis* isolates showed the highest gassing power in the flour-basis dough system (**Table 5**). Their CO_2 production ability in monoculture was in general 54 % over that provided by the S. cerevisiae sourdough isolates (**Table 5**). In addition, their fermentation rate in the presence of lactic and acetic acid was

superior, ranking from 95 to 100 % of the gas production in control conditions. The commercial strain used showed a lower acid tolerance in comparison to any other sourdough isolates (**Table 5**).

Table 5. Gassing performance of selected sourdough isolates in flour-basis dough.

Isolated/Strain	Yeast species	Gas at 240 min fermentation (ml CO_2/mg of yeast dw)		
		Control	150mM lactic 15 mM acetic	300 mM lactic
LH	Commercial *S. cerevisiae*	2.84±0.13	2.08±0.02	1.14±0.14
S03	*S. cerevisiae*	3.32±0.19	2.37±0.05	2.49±0.03
S06	*S. cerevisiae*	3.10±0.08	2.61±0.015	2.05±0.05
S13	*S. cerevisiae*	3.28±0.02	2.80±0.03	2.40±0.03
S15	*S. cerevisiae*	2.26±0.03	1.93±0.05	1.80±0.13
S21	*S. cerevisiae*	3.24±0.07	3.06±0.14	2.75±0.12
K08	*K. humilis*	5.20±0.19	5.15±0.06	3.80±0.09
K11	*K. humilis*	4.63±0.19	4.67±0.2	3.55±0.03
K13	*K. humilis*	5.02±0.14	4.9±0.2	4.62±0.4
K18	*K. humilis*	3.84±0.02	3.66±0.03	2.98±0.2
T01	*T. delbrueckii*	1.08±0.02	1.57±0.09	1.26±0.13
T03	*T. delbrueckii*	0.56±0.011	0.81±0.05	0.62±0.17

3.7. The presence of acetic acid impairs drastically the CO_2 production of *S. cerevisiae* strains but has no major effect on *K. humilis* gas performance

Previous work by Carbonetto *et al*. (2020) showed that tolerance to low pH and acetate is the main difference between *K. humilis* and *S. cerevisiae* sourdough strains. Accordingly, we were interested to analyse if these differences affect the ability of our yeast isolates to leaven sourdoughs. Gassing rate measurements in LD samples containing 300 mM lactic acid, 200 mM lactic acid plus 20 mM acetic acid, or without acids were recorded for the sourdough isolates K08 and S13, the best CO_2 producers among the *K. humilis* and *S. cerevisiae* isolates (**Table 5**), using again the commercial baker's yeast strain L'Hirondelle (LH) as a control. As shown in **Figure S2**, the kinetics of CO_2 production at pH 4.1 were similar to that recorded in control LD at pH 5.5 for

the three strains analysed. This confirms that the inhibitory effect of 300 mM lactate on CO_2 production (**Figure 7** and **Figure S2**) was just due to the acid concentration and not by pH sensitivity, an effect that was exacerbated by the combined addition of 200 mM lactic and 20 mM acetic acid in the LD. Indeed, under these conditions, the gassing rate by both *S. cerevisiae* strains, LH and S13, was almost negligible, while the *Kazachstania* strain was insensitive to the presence of acetic acid and the CO_2 production remains as high as in control LD (**Figure S2**). Thus, the *K. humilis* isolates provide a high fermentation potential in sourdough systems lacking or containing lactic and/or acetic acid when used as a monoculture.

4. Conclusions

Our results highlight the potential of *K. humilis* as a leavening agent in lean dough, exceeding the capacity of commercial baker's yeast of *S. cerevisiae*. The gassing capacity of the K08 isolate was remarkable in sourdough-like environments containing high amounts of lactic and acetic acid, making it highly applicable as a sourdough starter. In addition, our results suggest that *K. humilis* provides a characteristic aroma profile that together with that produced by *S. cerevisiae* isolates, could contribute to broadening the bread flavour complexity. Lastly, the stress tolerance properties of *K. humilis*, such as oxidative and salt stress tolerance make this yeast adequate for its industrial production.

Supplementary material

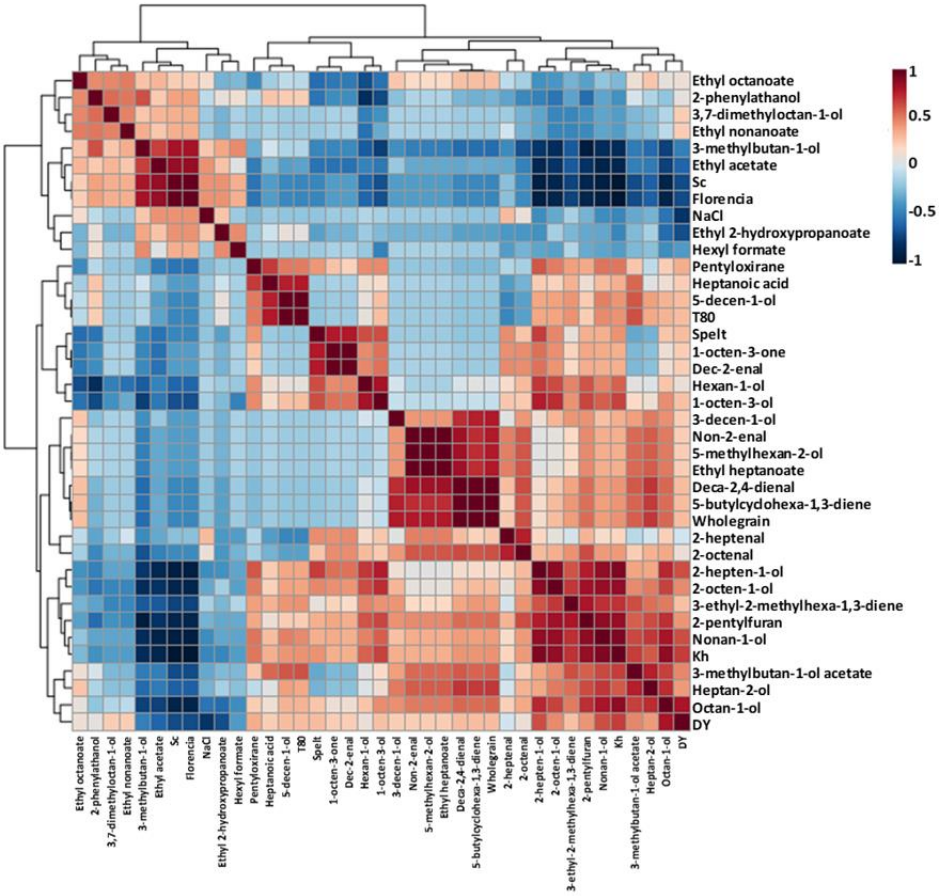


Figure S1. Correlation plot of volatile organic compounds, isolated yeast species, flours and other parameters used to elaborate sourdoughs. The Spearman correlation index was indicated at the scale bar, denoting the nature of the correlation with 1 for perfect positive correlation (dark red) and -1 for perfect negative correlation (dark blue). DY: Dough yield, Sc: *Saccharomyces cerevisiae* and Kh: *Kazachstania humilis*.

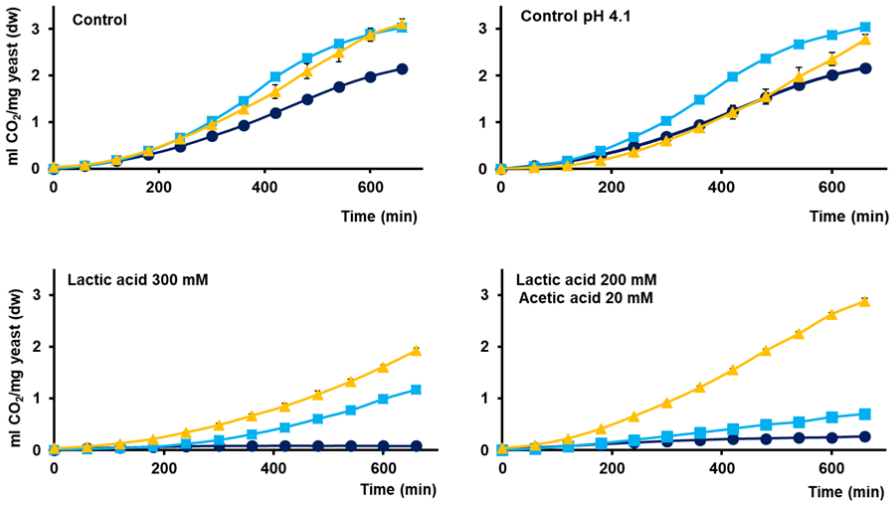


Figure S2. Effect of pH, lactic and acetic acid on CO_2 production by sourdough yeast isolates K08 and S13. Yeast suspensions of the *K. humilis* K08 (yellow triangle) and *S. cerevisiae* S13 (light blue square) isolates were mixed with a 30°C pre-warmed LD model solution lacking (control; pH 5.5), containing 300 mM lactic acid, 200 mM lactic and 20 mM acetic acid, or adjusted to pH 4.1, and the amount of CO_2 evolved was recorded at 30 min intervals for 600 min in a Fermograph II apparatus. The commercial bakers' yeast strain L'Hirondelle, LH (dark blue circle), was used as a control. Values are expressed as ml of CO_2 per mg of yeast cells dry weight (dw) and represent the mean ± sd of at least three independent experiments.

Table S1. Oligonucleotides used in this study.

Name	Sequence	Used for	Reference
PCR1_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG	Bacteria library	Klindworth et al. (2013)
PCR1_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC	Bacteria library	Klindworth et al. (2013)
ITS1-f	CTTGGTCATTTAGAGGAAGTAA	Yeast library	White et al. (1990)
ITS4-r	TCCTCCGCTTATTGATATGC	Yeast library	White et al. (1990)
ITS5-f	GGAAGTAAAAGTCGTAACAAGG	Yeast library	White et al. (1990)
ITS2-r	GCTGCGTTCTTCATCGATGC	Yeast library	White et al. (1990)

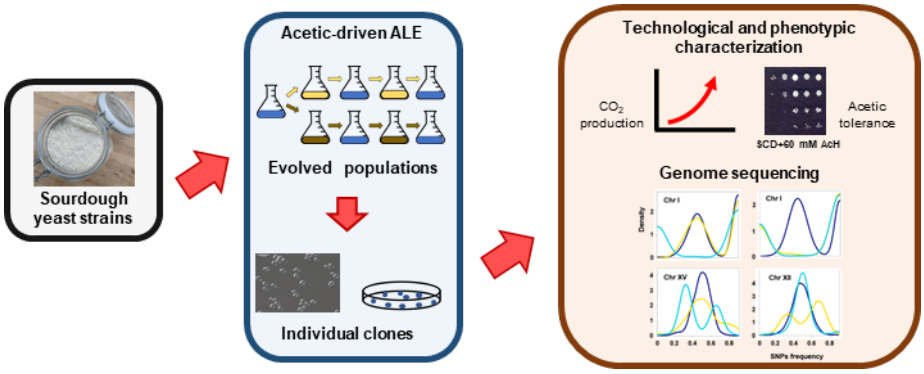
Table S2. Number of reads assigned to bacteria species level in each sourdough.

Kingdom	Phylum	Class	Order	Familly	Genus	Species	P1	P3	P4	SJ1	SJ2	SJ3
Bacteria	Actinobacteriota	Actinobacteria	Micrococcales	*Micrococcaceae*	*Kocuria*	*Rothia kristinae*	0	24	0	0	0	0
	Firmicutes	Bacilli	Lactobacillales	*Lactobacillaceae*	*Limosilactobacillus*	*Limosilactobacillus pontis*	0	0	0	0	10	0
						Limosilactobacillus mucosae	0	0	0	15	0	0
					Fructilactobacillus	*Fructilactobacillus sanfranciscensis*	54687	95722	93470	79826	116762	76168
						Fructilactobacillus vespulae	0	5	0	0	10	0
					Pediococcus	*Pediococcus* sp.	107	1891	43	0	0	0
	Proteobacteria	Gammaproteobacteria	Enterobacteriales	*Erwiniaceae*	*Pantoea*	*Pantoea* sp.	94	233	77	417	161	103
			Pseudomonadales	*Pseudomonadaceae*	*Pseudomonas*	*Pseudomonas viridiflava*	14	19	20	95	35	27
						Total assigned to species level	54902	97894	93610	80353	116978	76298
						unassigned	54	201	15	258	84	89

Table S3. Number of reads assigned to fungi species level in each sourdough.

Kingdom	Phylum	Class	Order	Familly	Genus	Species	P1	P3	P4	SJ1	SJ2	SJ3
Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	*Saccharomycetaceae*	*Kazachstania*	*Kazachstania humilis*	35309	63054	44028	284	18437	1819
					Saccharomyces	*Saccharomyces cerevisiae*	355	290	614	397004	602928	612595
				Incertae sedis	*Candida*	*Candida tropicalis*	0	0	0	34	192	92
		Sordariomycetes	Hypocreales	*Nectriaceae*	*Fusarium*	*Fusarium oxysporum*	11	2	0	21	107	31
					Gibberella	*Gibberella zeae*	216	184	139	18607	42611	13652
				Clavicipitaceae	*Claviceps*	*Claviceps arundinis*	7	1	18	1558	803	466
				Incertae sedis	*Sarocladium*	*Sarocladium strictum*	3	1	3	139	255	56
			Trichosphaeriales	*Trichosphaeriaceae*	*Nograspora*	*Nigrospora oryzae*	18	0	54	127	315	135
			Xylariales	*Hyponectriaceae*	*Monographella*	*Monographella nivalis*	14	36	10	1794	3049	927
		Dothideomycetes	Pleosporales	*Pleosporaceae*	*Alternaria*	*Alternaria metachromatica*	2876	356	474	12640	23386	8195
					Stemphylium	*Stemphylium vesicarium*	302	13	3	579	3347	1134
				Didymellaceae	*Didymella*	*Didymella fabae*	0	1	0	0	13	2663
					Neoascochyta	*Neoascochyta paspali*	2	0	8	163	527	102
					Phoma	*Phoma herbarum*	164	44	32	2210	5572	2536
			Capnodiales	*Mycosphaerellaceae*	*Mycosphaerella*	*Mycosphaerella tassiana*	334	60	98	8566	9434	3816
			Dothideales	*Aureobasidiaceae*	*Aureobasidium*	*Aureobasidium pullulans*	10	0	7	53	153	69
		Eurotiomycetes	Eurotiales	*Aspergillaceae*	*Penicillium*	*Penicillium polonicum*	0	0	2	20	116	20
					Aspergillus	*Aspergillus penicilloides*	4	1	0	25	179	49
						Aspergillus flavus	1	0	1	50	117	44
						Aspergillus ruber	16	1	5	147	255	201
	Basidiomycota	Tremellomycetes	Filobasidiales	*Filobasidiaceae*	*Filobasidium*	*Filobasidium wieringae*	5	1	12	21	105	30
			Cystofilobasidiales	*Cystofilobasidiaceae*	*Cystofilobasidium*	*Cystofilobasidium macerans*	1	2	0	89	378	79
			Tremellales	*Bulleribasidiaceae*	*Vishniacozyma*	*Vishniacozyma victoriae*	6	2	7	160	492	194
		Microbotryomycetes	Sporidiobolales	*Sporidiobolaceae*	*Sporobolomyces*	*Sporobolomyces roseus*	14	2	29	647	2379	715
						Total assigned to species level	39668	64051	45544	444938	715150	649620
						unassigned	3498	389	184	4331	16694	6286

Chapter 2：Adaptive laboratory evolution for acetic acid-tolerance matches sourdough challenges with yeast phenotypes



Keywords: adaptive laboratory evolution, evolved sourdough yeast, acetic acid stress tolerance, aneuploidy, copy number variation

This chapter has been available with open access since September 12, 2023 and can be found online through the link included below.

Sánchez-Adriá IE, Sanmartín G, Prieto JA, Estruch F, Fortis E, Randez-Gil F. Adaptive laboratory evolution for acetic acid-tolerance matches sourdough challenges with yeast phenotypes. *Microbiol. Res.* 2023 277:127487. https://doi:10.1016/j.micres.2023.127487

Summary

Acetic acid tolerance of *Saccharomyces cerevisiae* is an important trait in sourdough fermentation processes, where the accumulation of acid by the growth of lactic acid bacteria reduces the yeast metabolic activity. In this work, we have carried out adaptive laboratory evolution (ALE) experiments in two sourdough isolates of *S. cerevisiae* exposed to acetic acid, or alternatively to acetic acid and myriocin, an inhibitor of sphingolipid biosynthesis that sped-up the evolutionary adaptation. Evolution approaches resulted in acetic tolerance, and surprisingly, increased lactic susceptibility. Four evolved clones, one from each parental strain and evolutionary scheme, were selected on the basis of their potential for CO_2 production in sourdough conditions. Among them, two showed phenotypic instability characterized by strong lactic sensitivity after several rounds of growth under unstressed conditions, while two others, displayed increased constitutive acetic tolerance with no loss of growth in lactic medium. Genome sequencing and ploidy level analysis of all strains revealed aneuploidies, which could account for phenotypic heterogeneity. In addition, copy number variations (CNVs), affecting specially to genes involved in ion transport or flocculation, and single nucleotide polymorphisms (SNPs) were identified. Mutations in several genes, *ARG82*, *KEX1*, *CTK1*, *SPT20*, *IRA2*, *ASG1* or *GIS4*, were confirmed as involved in acetic and/or lactic tolerance, and new determinants of these phenotypes, *MSN5* and *PSP2*, identified.

1. Introduction

Sourdough-baking technology is experiencing an increased demand because it provides products with improved bread flavour and texture (De Vuyst *et al*., 2023), as well as reduced phytate, gluten and glycemic index (Gobbetti *et al*., 2019; Ribet *et al*., 2023). These properties rely mostly on the sourdough microbiota composed by stable associations of lactic acid bacteria (LAB) and yeasts.

Up to 40 different yeast species have been identified in sourdoughs collected worldwide (De Vuyst *et al*., 2017), although a given sourdough usually contains one prevailing yeast (Comasio *et al*., 2020a), quite often *Kazachstania humilis* or *Saccharomyces cerevisiae*. It is assumed that the yeast species diversity is influenced by its interaction with LAB, based on mutual relationships. A well-known example is the nutritional mutualism of *Fructilactobacillus sanfranciscensis* and *K. humilis* (Sieuwerts *et al*., 2018). Nevertheless, the nature of these interactions is influenced by a variety of factors, among them, the stress intrinsic tolerance of each species (De Vuyst *et al*., 2017). The weak acids produced by LAB, but also by acetic acid bacterias (AAB), reduce dough pH and generate toxicity promoting the exclusion in the mature sourdough of less-adapted community members (Carbonetto *et al*., 2020). For example, *K. humilis* strains appear to differ from *S. cerevisiae* strains by displaying higher sensitivity to low pH, but higher tolerance to acetate (Carbonetto *et al*., 2020). Indeed, CO_2 production rate measurements in flour-basis dough model systems, indicate that the presence of acetic acid impairs drastically the leavening activity of *S. cerevisiae*, but has no major effect on *K. humilis* gas performance (Sánchez-Adriá *et al*., 2023a). Hence, acetic-tolerance of baker's yeast strains of *S. cerevisiae* is a trait that needs to be improved in

order to ensure a high counting of this species in mature sourdoughs and their leavening activity in the baking process.

It is generally accepted that the toxicity of different weak acids relies on their undissociated form, which diffuses across the plasma membrane due to their high solubility in the phospholipids (PLs) fraction (Lindgren and Dobrogosz, 1990). This explains why reducing plasma membrane permeability, by increasing lipid saturation, provides tolerance to weak acids (Lindberg *et al*., 2013). In full agreement, the complex sphingolipids (SLs) content in the acetic-tolerant *Zygosaccharomyces bailii* is higher than in *S. cerevisiae* (Lindberg *et al*., 2013). In addition, the synthesis of these lipids increases in acetic acid exposed cells of both species (Guerreiro *et al*., 2016; Lindahl *et al*., 2016). Likewise, several sterol biosynthesis pathway genes have been identified, as acetic acid-tolerance determinants and/or with increased transcript levels in response to acetic (Mira *et al*., 2010). Hence, strong evidence supports a causal relationship between lipid saturation and acid tolerance (Lindberg *et al*., 2013).

Here, we have induced genomic changes in two sourdough isolates of *S. cerevisiae* (S06 and S13) by adaptive laboratory evolution (ALE). The ALE approach allows developing highly specialized yeasts in technologically important properties, on the basis of the natural selection of beneficial mutations under specified growth conditions. In addition, the technique is ease to implement, does not require any prior knowledge and provides basic information on molecular aspects of evolution and genotype–phenotype interconnections (Mavrommati *et al*., 2022; Fernandes *et al*., 2023). In our study, ALE was based on successive culture of yeast cells in medium with or without increased concentrations of acetic acid, a strategy that induces constitutive tolerance to different stresses (González-Ramos *et al*., 2016). In parallel, evolved clones were obtained by alternating cultivation cycles in the presence of acetic acid or

myriocin, an effective ALE-driver to obtain evolved clones with increased SLs abundance (Randez-Gil *et al*., 2020). Our results demonstrate the powerful of our ALE strategies to induce acetic tolerance and to generate novel strains with higher performance in the sourdough environment. The evolved yeast populations have been characterised in deep and the results are discussed in terms of the mechanisms that govern the yeast response to acetic acid.

2. Materials and methods

2.1. Strains, media and culture conditions

Two *S. cerevisiae* strains, S06 and S13, isolated from two industrial sourdoughs of the baking company Europastry, S.A. (St. Cugat del Vallès, Barcelona, Spain) and characterized by their high leavening performance and stress tolerance (Sánchez-Adriá *et al*., 2023a) were used as parental strains. In some experiments, the commercial *S. cerevisiae* strain L'Hirondelle (LH), produced by the Lesaffre Group (Lille, France), was used as control.

Previously described standard methods were followed for media preparation (Guthrie and Fink, 1991). Yeast cells were cultured at 30°C and 200 rpm in YPD or SCD supplemented with the appropriate amino acid drop out (ForMedium). Myriocin at 1.5-3.0 µM (final concentration) was added from a stock solution of 2 mM (ethanol:DMSO; 80:20, v:v). Media containing increased concentrations of acetic acid were prepared by mixing the appropriate volume of filter-sterilized SCD and SCD containing 120 mM acetic acid, both adjusted to pH 4.0. Solid media contained 2 % agar.

For plate phenotype experiments, cells were grown to the mid-exponential phase at 30ºC (OD_{600} ~ 0.5). Then, 10-fold serial dilutions were spotted (3 µl) onto SCD agar plates lacking or containing lactic, acetic or a

combination of both as indicated. Myriocin tolerance was checked in YPD plates. Colony growth was inspected after 2-4 days of incubation at 30ºC.

2.2. Adaptive laboratory evolution

Evolution experiments were conducted using batch culture techniques in 250 ml Erlenmeyer flasks as previously described (Aguilera *et al*., 2010). SCD-grown pre-cultures of the two parental strains under study were used to inoculate 50 ml of liquid SCD medium containing 60 mM acetic acid (pH 4) at initial OD_{600} ~ 0.05. Cultivations were carried out at 30ºC and 200 rpm for 24 h, after which, cells were washed with water (González-Ramos *et al*., 2016), transferred to the same medium lacking acetic acid at OD_{600} of 0.05, and grown for 24 h under the same conditions. This scheme of alternating cultivation cycles in the presence and absence of acetic acid was followed until the end of the evolution experiment. After a few cycles, as specify in **Figure 8**, the acid concentration was increased, first from 60 to 90 mM and then to 120 mM, when a consistent final OD_{600} of 4-5 (~ 5-6 generations) was obtained. When growth was delayed, at the highest acid concentrations, cultures were extended for 48 h in order to obtain the mentioned increase in cell density. Acetic acid/Myriocin-driven ALE (A/M) was conducted similarly, except that cells were grown by alternating cultivation in SCD and YPD containing acetic acid and myriocin, respectively. Myriocin concentration was 1.5 µM (final concentration) at the beginning and was further increased to 3.0 µM and maintained over the course of the experiment (**Figure 8**). Evolution experiments involved a total of at least 34-37 cultivations in acid-containing media, which allow attaining 200 generations evolved populations when the experiment was ended. Cells from 50, 100 and 150 generations evolved cultures were also sampled in order to characterize the gradual acquisition of stress tolerance and to select the best phenotypes. The number of generations in each cultivation was inferred from the equation

n = 3.3 x log (final OD_{600} / initial OD_{600}), and the total number in evolved populations was calculated by summing generations in acid media. Samples from the evolved populations were taken, maintained as frozen (-80°C) glycerol stocks and then rescued in YPD agar plates at 30°C for 24 h before further analysis.

2.3. Selection of evolved clones

Cell populations of each yeast background and evolutionary line attained after 100, 150 and 200 generations were compared for acid tolerance and gassing rate under sourdough conditions with their parental counterparts, and those showing the best performance, $S06A_{100}$, $S06A/M_{100}$, $S13A_{150}$ and $S13A/M_{100}$ were chosen for screening individual clones. For this, cells of each population were cultivated in solid YPD, SCD-75 mM acetic acid or SCD-175 mM lactic acid plus 30 mM acetic acid, and 5 colonies from each medium, marked with subscripts 01-05 (YPD), 06-10 (acetic acid) and 11-15 (lactic plus acetic), were randomly picked. Cells from these colonies (60 in total; S06A01-15; S06A/M01-15; S13A01-15 and S13A/M01-15) were grown overnight in YPD and then inoculated into two replicates of 4.5 ml of SCD-4 % glucose with 175 mM lactic and 30 mM acetic acid (pH 4.0). The initial weight of each tube was recorded and cultivation was carried out at 80 rpm and 30ºC for 24 h after which the tubes were open and the final weight was measured. The weight difference was used as a gross estimation of the gassing production of each isolate under this cultivation conditions. From this assay, the best two evolved clones from each set were selected for further characterization of gas production (see below), and finally, the top clone of each parental strain and evolution line was analysed for genomic changes.

2.4. Gas production

Fermentative capacity was determined by measuring gas production in both liquid dough (LD) (Panadero *et al*., 2005), lacking or containing lactic and/or acetic acid (sour LD), or flour-based dough (FBD). Molasses-grown cells (Hernandez-Lopez *et al*., 2003) were collected, washed, resuspended in distilled water (4ºC) containing 16 g l^{-1} of NaCl, and 15 ml of the yeast suspension [30 mg (dry weight) per ml] was diluted with the same volume of a 30ºC-prewarmed LD solution (Panadero *et al*., 2005). Then, the suspension was incubated at 30ºC with low shaking (80 rpm), and the amount of CO_2 evolved recorded at 20 min intervals in a Fermograph equipment (ATTO Corporation, Tokyo, Japan). In all cases, values are expressed as ml of CO_2 per mg of yeast cells (dry weight), and represent the mean ± sd of at least three independent experiments.

2.5. Sequencing and bioinformatics analysis

Whole-genome sequencing and bioinformatics analysis were performed at the Genomics service of the Valencia University (Valencia, Spain). Briefly, Illumina sequencing libraries from S06, $S06A_{15}$, $S06_{A/M13}$, S13, $S13_{A14}$ and $S13_{A/M14}$ strains were constructed using the Truseq nano DNA library preparation kit (Illumina Inc., San Diego, CA). The number of raw pair-end 251 bp reads collected were in average 2,795,074±689,772. Raw reads were quality trimming and filtering using AfterQC (Chen *et al*., 2017), with filter of minimum phred-quality score 15 and minimum read size as 50. Raw and processed reads quality control was made with FastQC v0.11.8 (http://www.bioinformatics.babraham.ac.uk) and AfterQC tools (**Table S4**). Resulting reads were aligned to the *S. cerevisiae* R64-1-1 reference strain (S288C), using the bowtie2 mapping tool (Langmead and Salzberg, 2012). Samtools 1.19 and Picard 2.18 (Li *et al*., 2009) were used for mapping post-

processing and remove duplicates. Only proper paired reads with a mapping quality score above 30 were retained from the alignment. Indel realignment and depth of coverage calculation was performed with GATK-3.6.

Alignment data files were quality check with Qualimap v2.2.1 (García-Alcalde *et al*., 2012; Okonechnikov *et al*., 2016). For SNP-calling (SNPs and indels detection) and filtering we use VarScan (v2.3.9; min-avg-qual 20 –min-var-frequation 0.3 –min-coverage 24 –min-reads 25 –min-freq-for-hom 0.70 –p-value 0.05). MiModD 0.1.9 tool (http://doi.org/10.5281/zenodo.2582000) was used for variant post-processing, including genotype filtering and annotation with SnpEff v4.3t (Cingolani *et al*., 2012) settled on approach that required >10 % base-call supporting a SNP in the evolved genomes and < 2 % base-call supporting the same base in the parental genome data and genotype quality above 20. The variant calling files (.vcf) were used to compare changes between parental strains and their corresponding evolved clones and to select those that appear specifically during evolution. Indel, missense variants or frame shift in coding gene regions were kept after manually confirmed by visual inspection in the JBrowse (Diesh *et al*., 2023) and position of early STOP codon.

To detect chromosome copy-number changes we use the nQuire tool (Weiß *et al*., 2018) with mapping quality and minimum coverage filters set to 10 and applying lrdmodel to assess ploidy level.

2.6. Statistical analysis

Sample averages were compared by a student's t-test with the Excel software (Microsoft). The samples denoted with * were significantly different ($p<0.05$). The kernel density graphic was performed with the platform www.bioinformatics.com.cn/en.

3. Results and discussion

3.1. Myriocin-driven evolution speed-up the acquisition of acetic acid-tolerance

We used two *S. cerevisiae* strains isolated from industrial sourdoughs, S06 and S13 (Sánchez-Adriá *et al*., 2023a), as parental strains to carry out evolution experiments addressed, to endow them with increased acetic acid tolerance and performance under sourdough conditions. Two evolutionary lines, referred as AcH and A/M, were conducted. In the first, yeast cells were cultivated in the presence or absence of acetic acid, which promotes the acquisition of constitutive rather adaptive tolerance (González-Ramos *et al*., 2016). In the second strategy (A/M), the acetic acid-stressed SCD cultures were alternated with refreshments in YPD containing myriocin, a drug that affects the lipid profile (Randez-Gil *et al*., 2020).

Figure 8 shows the growth of the yeast strains S06 and S13 after 24h of culture in the successive acetic-stressed cycles of the two evolutionary schemes. In general, the two strains exhibited improved acetic tolerance after around 100 generations of exposure to increased concentrations of acid (pH 4.0). By comparing, the presence of myriocin in alternation with acetic resulted in a more rapid acquisition of growth capacity in the presence of acid, a result that reinforces the idea of a connexion between lipid saturation and acid tolerance (Lindberg *et al*., 2013). Nevertheless, at concentrations of 120 mM acetic acid, no apparent further improvement was obtained. Neither the AcH-evolutionary experiments rendered cells adapted to this acid concentration (**Figure 8**). This observation likely reflects the existence of drawbacks and genetic constraints that preclude additional improvements (Meijnen *et al*., 2016).

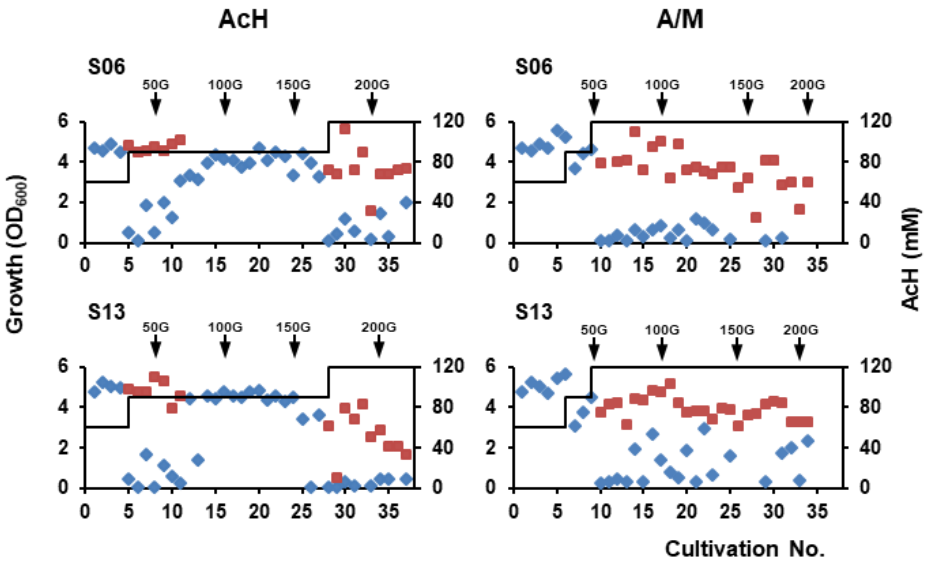


Figure 8. Characterization of evolved populations of sourdough strains in acetic acid-containing media. The graphs show the growth, measured as OD_{600}, of the yeast strains S06 and S13 after 24 (blue diamonds) and/or 48 h (red squares) of culture in the successive acetic-stressed cycles of the two evolutionary lines, referred to as AcH and A/M. Starting from 60 mM acetic acid and initial OD_{600} of 0.05, cultivations took 24 h in order to obtain a significant increase in cell density, but when the selective pressure increased and growth was impaired, they were extended to 48 h. Growth in media lacking acetic acid is not shown. The black line indicates the concentration of acetic acid (AcH) and the black arrows the sampling of 50-, 100-, 150- and 200-generations evolved populations. In the A/M evolution line, the myriocin concentration increased from the initial 1.5 to 3.0 µM after 8 cycles of cultivation and was longer maintained until the experiment was ended.

3.2. Phenotypic characterization of evolved populations

We investigated more in deep how the evolutionary experiments affected the growth of S06 and S13 under different conditions. **Figure 9** shows the growth on solid media of parental, 50- and 100-generation evolved populations under non-stressed and acid-stressed conditions. As expected, the evolved populations showed a gradual increase in acetic acid tolerance that was first observed in cells of both strains evolved in the presence of myriocin (**Figure 9**, SCD+60 mM AcH; compare e.g. S06, A_{50} and A/M_{50}). However, no additional

phenotypic change could be observed for the yeast populations evolved from 100- to 200-generations (data not shown), in good correspondence with the OD_{600} data displayed in **Figure 8**. A/M-evolved populations exhibited a high resistance to myriocin as compared with their parental (**Figure S3**), although a slight tolerance was also evident in AcH-evolved populations that were not previously exposed to the drug (**Figure S3**; S06 and S13, A_{50} and A_{100}).

Then, we examined the growth of the evolved cultures on a medium containing lactic acid, such as is an important trait to consider as this is the major weak acid in sourdough. To our surprise, the acquisition of acetic acid tolerance caused a loss of growth in lactic acid-containing medium that was especially pronounced in the presence of myriocin, likely due to their effects on the evolutionary process, and in the S06 strain (**Figure 9**; SCD+200 mM LA). To our knowledge, this is the first time that such a negative effect on lactic tolerance has been reported when yeast cells are adapted to acetic acid. The microbial inhibition by hydrophilic weak organic acids, such as acetic and lactic, has been traditionally related with their common ability to cause acidification and reduction of intracellular pH (Ullah *et al*., 2012; Stratford *et al*., 2013; Palma *et al*., 2018; Peetermans *et al*., 2021). However, it seems that these *S. cerevisiae* strains activated on the course of their evolution response mechanisms unrelated with low pH, or that these acids may not act in the same manner on the yeast cell. In this line, different effects on glucose uptake and ethanol production by yeast cells have also been reported in response to acetic and lactic acid stress (Narendranath *et al*., 2001a; Thomas *et al*., 2002). Earlier studies also reported a strong induction by acetic acid stress of the plasma membrane H+-ATPase Pma1 (Piper *et al*., 2001). In addition, *PMA1* overexpression confers acetic tolerance (Lee *et al*., 2017). Unlike this, a decrease instead of an increase in Pma1 activity has been reported in response to lactic acid stress (Narendranath *et al*., 2001a). Neither *PMA1* has been found to be upregulated in evolved *S. cerevisiae* strains

with enhanced tolerance to lactic acid (Fletcher *et al*., 2017). Hence, the evolutionary changes that were useful for improving acetic acid tolerance may have overridden the intrinsic high resistance to lactic of the strains analysed.

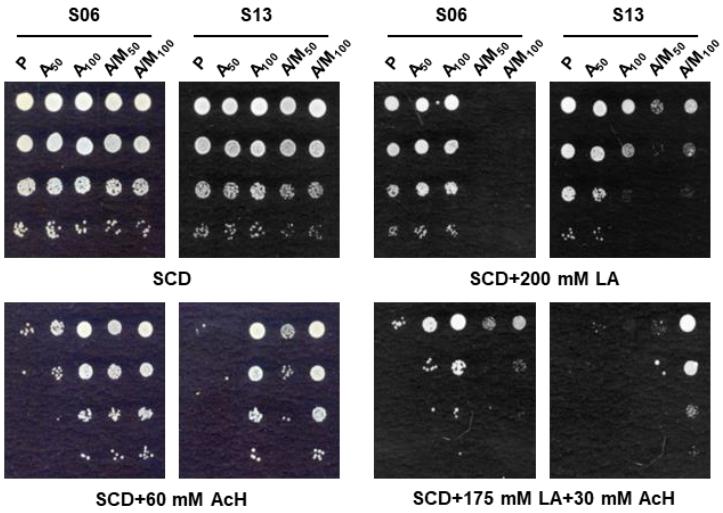


Figure 9. Tolerance of evolved populations to acetic acid and/or lactic acid. Cell samples of the parental (P) S06 and S13 strains and their corresponding 50- and 100-generations evolved populations in each evolutionary line, AcH (A_{50}, A_{100}) and A/M (A/M_{50}, A/M_{100}) were examined for acid tolerance in SCD solid medium lacking or containing acetic acid (AcH) and/or lactic acid (LA) at the indicated concentrations. Colony growth was inspected after 48 h or 4-5 days of incubation at 30ºC for control (SCD) or acid-containing medium, respectively. For more details, see the Materials and Methods section.

The phenotypic interaction between acetic and lactic acid was also examined (**Figure 9**; SCD+175 mM LA+30 mM AcH). Strong synergistic toxicity between lactic and acetic acid have been described resulting in a much stronger growth inhibition and reduction in metabolic activities (Narendranath *et al*., 2001b). Nevertheless, A_{100} and A/M_{100} evolved populations of S06 and S13, respectively, were still able to show increased tolerance in a medium containing both lactic and acetic acid (**Figure 9**). Our results reveal the strong background dependency of acid stress tolerance in natural strains and how the particular evolutionary conditions determine the phenotype of the evolved populations.

Finally, we checked if the acquisition of acetic tolerance in our experimental populations was achieved at the expense of their fermentative capacity. CO_2 production measurements were carried out in liquid dough (LD) model system lacking (control) or containing 175 mM lactic plus 30 mM acetic acid. Yeast populations of S06 and S13 evolved for 100, 150 and 200-generations through the two evolutionary lines were tested. As shown in **Figure S4**, evolved cells of both strains showed a slight reduction in their fermentative activity under control conditions. This result likely reflects the redistribution of energy resources toward acid resistance activities (Zakrzewska *et al*., 2011). However, this energy investment had no major cost in terms of gassing power in cells exposed to acid stress (**Figure S4**).

3.3. Isolation, fermentative and phenotypic characterization of individual clones

Individual clones were isolated from the evolved populations $S06A_{100}$, $S06A/M_{100}$, $S13A_{150}$ and $S13A/M_{100}$, which showed the best fermentation performance in fermentation trials within each evolutionary scheme and parental strain (**Figure S4**). The screening included 15 individual clones from each experimental population, 5 grown in YPD (subscript 1-5), 5 in acetic-SCD (subscript 6-10) and 5 in lactic plus acetic-SCD (subscript 11-15; see the Materials and Methods section). Thus, a total of 60 colonies were randomly selected and tested subsequently for weight losses during fermentation, which allow for a gross evaluation of the CO_2 released by the fermentative activity of yeasts. As an example, **Figure S5** shows the results of the initial characterisation of clones 1-15 from the A/M_{100} population of the S13 strain. Based on these results, the two top performing clones from each population: $S06A_{12}$ - $S06A_{15}$; $S06A/M_6$ - $S06A/M_{13}$; $S13A_{13}$ - $S13A_{14}$; and $S13A/M_{11}$ - $S13A/M_{14}$, were chosen for a more detailed characterization of gassing production in LD medium and

LD containing lactic plus acetic acid (**Figure 10**). The analysis included the evolved heterogeneous populations from which the clones were isolated and the strain L'Hirondelle as example of commercial baker's yeast. In general, the evolved clones showed a similar behaviour than their corresponding ancestor in control LD, and increased gassing power in acid-containing medium (sour LD), a result that was especially pronounced when compared with the commercial strain (**Figure 10**).

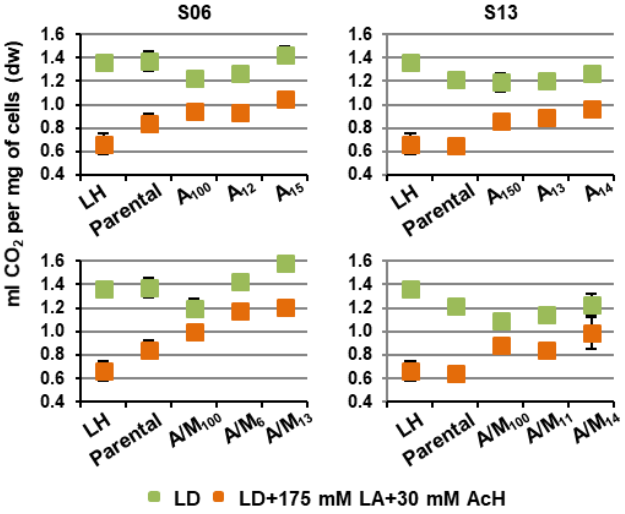


Figure 10. Technological traits of evolved clones. The parental strains S06 and S13, the evolved populations selected from each evolutionary line, AcH ($S06A_{100}$ and $S13A_{150}$) and A/M ($S06A/M_{100}$ and $S13A/M_{100}$), and the two individual clones isolated from each of them, A_{12} - A_{15}, A/M_6 - A/M_{13}, A_{13} - A_{14} and A/M_{11} – A/M_{14}, respectively, were assayed for CO_2 production in liquid dough model system (LD) and LD containing lactic (LA) plus acetic (AcH) acid, at the indicated concentration. The analysis also included the strain L'Hirondelle (LH) as example of commercial baker's yeast. In all cases, values are expressed as ml of total CO_2 released per mg of yeast cells (dry weight) after 480 min of fermentation, and represent the mean ± sd of at least three independent experiments.

Then, we examined the phenotypic stability of four clones, $S06A_{15}$, $S06A/M_{13}$, $S13A_{14}$ and $S13A/M_{14}$, one from each background and evolutionary line, selected as the best performers in the fermentation trials (**Figure 10**). Cells were grown for several generations in YPD and then a drop test was conducted

in the presence of acetic and/or lactic acid. As shown in **Figure 11**, clones $S06A/M_{13}$ and $S13A_{14}$ exhibited lactic sensitivity, a phenotype that was already observed in some evolved heterogeneous populations (**Figure 9**). In addition, the two clones hardly differed from their parental in acetic tolerance, suggesting genome alterations (see later). On the contrary, clones $S06A_{15}$ and $S13A/M_{14}$ still exhibited strong acetic tolerance as compared with their ancestors combined with no loss of growth in lactic medium (**Figure 11**). The results highlight the important heterogeneity in the response of genetically homogeneous *S. cerevisiae* cultures to acetic acid stress as it has been previously reported (Swinnen *et al*., 2014; Franco-Duarte *et al*., 2015; Palma *et al*., 2018).

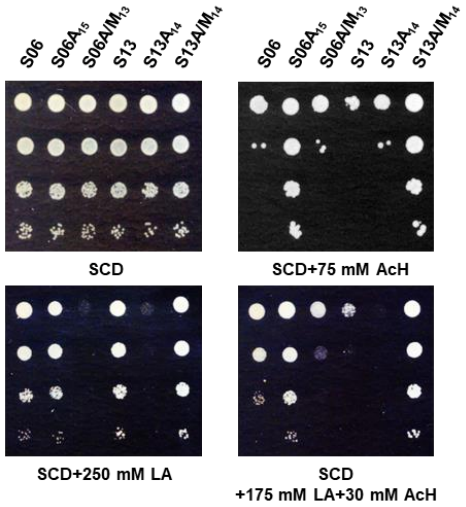


Figure 11. Phenotypic traits of selected individual clones. The acid-phenotypic stability of four clones, $S06A_{15}$, $S06A/M_{13}$, $S13A_{14}$ and $S13A/M_{14}$, one from each background and evolutionary line, selected as the best performers in the fermentation trials (**Figure 10**), was examined after several rounds of culture in the absence of acetic acid (YPD medium). A drop test assay was used to evaluate the tolerance of yeast cells to acetic (AcH) and/or lactic (LA) acid at the indicated concentration. Colony growth was inspected after 2-4 days of incubation at 30ºC. For more details, see the Materials and Methods section.

3.4. Genome sequencing and ploidy level

We sought to identify genomic alterations and casual mutations in the evolved clones than could explain their phenotypic changes. The four clones, $S06A_{15}$, $S06A/M_{13}$, $S13A_{14}$ and $S13A/M_{14}$, and their parental counterparts were sequenced and compared to the laboratory *S. cerevisiae* reference strain S288C. First, we carried out DNA content analysis by flow cytometry. As many yeast isolates from natural environments (Bigey *et al*., 2021), the parental strains showed near two copies of their genomes, 1.88±0.12 and 1.87±0.14 for S06 and S13 respectively. Comparing with this, the evolved clones showed values in a similar range, 1.80±0.30 and 2.01±0.05 for $S06A_{15}$ and $S06A/M_{13}$, respectively, and 2.40±0.30 for $S13A/M_{14}$. Only the clone $S13A_{14}$ displayed a noticeable increase in its ploidy level, 3.30±0.30, suggesting a stress-driven large-scale genomic amplification.

We also examined the changes in heterozygous SNPs frequency compared to the overall genome frequency distribution (**Figure S6**) and the read-depth between chromosomes (**Figure 12A**), to estimate ploidy levels (Morard *et al*., 2019). Both methods confirmed the 2n ploidy level in both unevolved parental strains. However, aneuploidies were evident in some evolved clones (**Figure 12A and 12B**). Strains $S06A/M_{13}$, $S13A_{14}$ and $S13A/M_{14}$ showed monosomy of chromosome I. Aneuploidies in this chromosome have been reported frequently in *S. cerevisiae* strains, a change that could be associated to its small size, and consequently weak effect on strain robustness (Peter *et al*., 2018; Morard *et al*., 2019). Remarkably, additional changes were found in the lowest lactic-tolerant strains. Strain $S06A/M_{13}$ showed an extra copy of chromosome XV (**Figure 12A and 12B**), while strain $S13A_{14}$ appeared to display an extra copy of chromosome XII (**Figure 12B**). Nevertheless, we observe a decrease of read depth on this chromosome (**Figure 12A**). Given the

ploidy level of this strain (3.30±0.30), the results suggest a gross change in the chromosome dosage of this strain, including likely overall duplication of chromosomes, after dropping one copy of chromosome I, and a later loss of a copy of chromosome XII. Weather these changes are responsible of the phenotypic variations of these clones remains unclear. Distinct chromosomal instability patterns have been reported in cells exposed to different stress conditions (Mendes *et al*., 2017; Shen *et al*., 2020). In addition, ploidy changes and aneuploidies causes gene copy number imbalances, which results in non-genetic phenotypic variability (Beach *et al*., 2017).

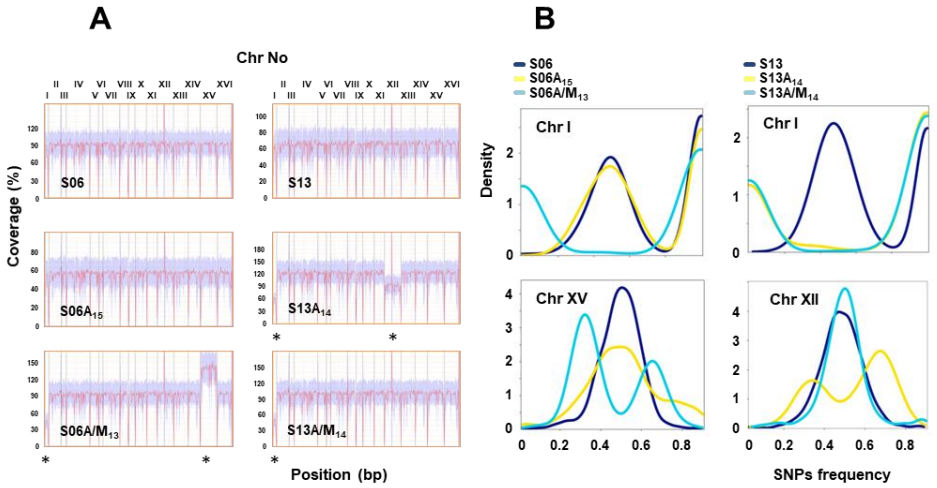


Figure 12. Genome composition of sourdough strains and evolved clones. (A) Sequencing coverage across the reference *S. cerevisiae* S288C, genome of parental sourdough strains S06 and S13 and their evolved clones $S06A_{15}$, $S06A/M_{13}$, $S13A_{14}$ and $S13A/M_{14}$. Deviations from the average of the genome are labelled with *. **(B)** Frequency SNPs density distribution in chromosomes of evolved clones showing aneuploidies (I, XII and XV). Each graph shows the SNPs distribution for the parental strain and the corresponding evolved clones. The graphics were created with the programme http://bioinformatics.com.cn/en

3.5. Copy number variations

Small local variations in the genome, referred as copy number variations (CNVs), including deletions and amplifications, were also detected across all sequenced sourdough strains (**Figure 13**). As the genome from other industrial strains, S06 and S13 strains displayed significantly less transposable element and the telomeres regions were underrepresented in comparison to the reference strain S288C (**Figure 13**). These features have been considered as a sign of selection in a man-made environment (Argueso *et al.* 2009; Babrzadeh *et al.* 2012; Gallone *et al*., 2016) like sourdough, and are related with important phenotypic characteristics that determine the technological potential of yeast strains (Franco-Duarte *et al*., 2016, 2022). Comparison of the CNVs resulted in the identification of 143 genes, mostly underrepresented, that showed altered copy number in the two backgrounds (**Table S5**), a result that is consistent with the common origin of the parental strains. The results agree well with previous reports that point out genes involved in ion transport or flocculation, including *FLO1*, *FLO9*, *FLO5*, *FLO11* or *FLO10* as the most heavily influenced by CNVs (Dunn *et al*., 2012; Bergström *et al*., 2014; Gallone *et al*., 2016). The list of underrepresented CNVs also contained respiration-, redox-, and ion balance-encoding genes found to provide protection against acetic acid stress (Henriques *et al*., 2017), like *PAU* genes, which encodes yeast cell wall mannoproteins (Marguet *et al*., 1988; Rachidi *et al*., 2000), the YRF/uncharacterized module of yeast helicases and the *CUP* gene family (Kang *et al*., 2019). Finally, some CNVs that have been reported to be niche-linked (Gallone *et al*., 2016), as *IMA5*, *MAL31* or *MAL33*, involved in maltose metabolism (Bigey *et al*., 2021), were found to be amplified (**Table S5**). Overall, the results are consistent with the high intrinsic acid tolerance of the parental S06 and S13 strains and the presence of maltose as main carbon source in sourdough.

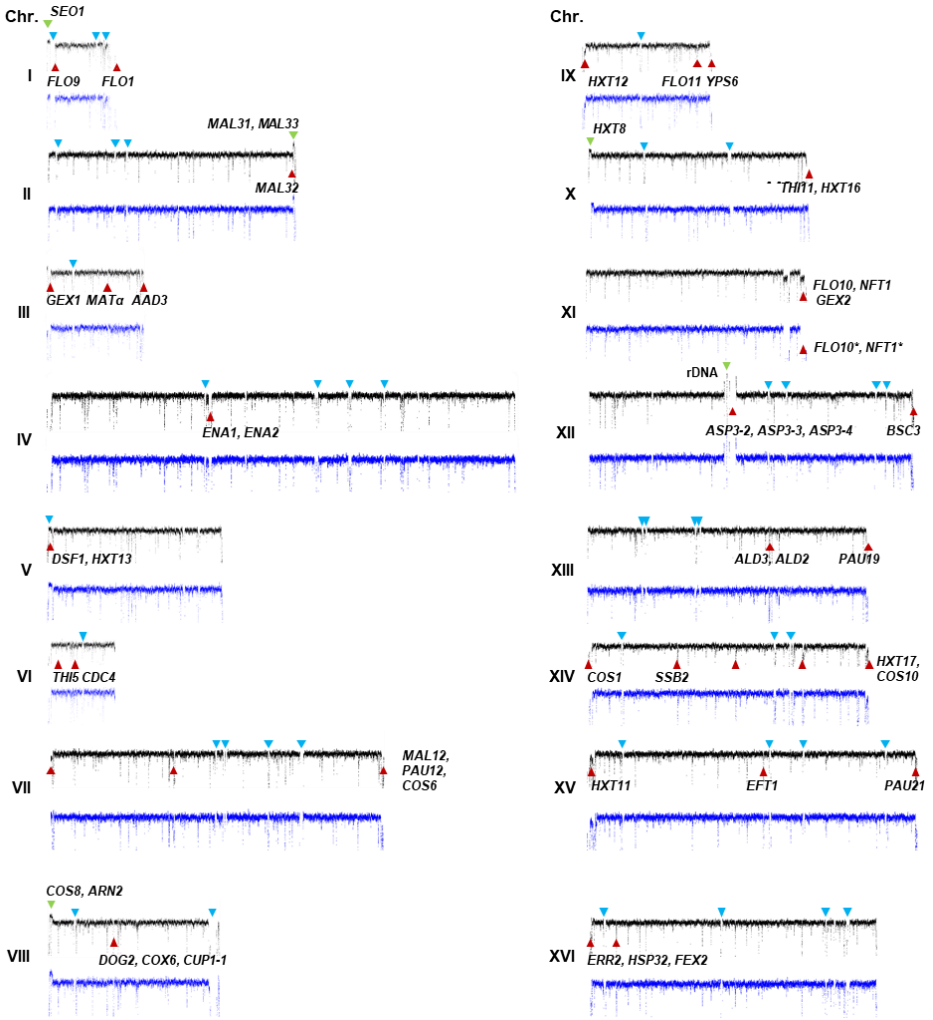


Figure 13. Sourdough strains show a large amount of copy number variations (CNVs) events. Relative gene dosage plots through mapping S06 (black lines) and S13 (blue lines) sequencing reads onto S288C chromosome sequences. Genomic regions overrepresented and underrepresented are indicated with green and red triangles, respectively. Representative *S. cerevisiae* genes in these regions are named. Blue triangles denote Ty transposons or δ elements.

3.6. Single nucleotide polymorphisms

Single nucleotide polymorphism (SNP) analysis identified a total of 148,857 variants, between the reference strain S288C and the parental and

evolved strains under study. In general, SNPs were present in similar frequency in both homozygosity and heterozygosity, and uniformly present along the genome. We wonder if this accumulation of SNPs could be driven by adaptation to the acid-environment of the sourdough. SNPs are the most abundant form of sequence variation (Bergström *et al*., 2014). Thus, we first analysed the accumulation of SNPs in candidate genes that have been previously associated to acetic acid tolerance in different studies. Only genes identified by different approaches or phenotypically confirmed were considered. **Table S6** shows, as a heatmap, the number of SNPs found in each selected gene and strain analysed. As can be seen, most of the examined genes accumulated a big number of homozygous or heterozygous variants in both the parental and the evolved clones of the two strains analysed. This suggests that the continuous selective pressure due to the acid conditions during the sourdough fermentation, conformed a genetically separated yeast population. On the other hand, only eight genes, four in the S06 (*SRB6*, *COS9*, *CWP1*, *ISO1*), two in the S13 (*TPO2*, *YPT7*) and one in both strains (*ADY2*), were found to be unaltered as compared with the reference S288C strain (**Table S6**). The absence of SNPs in *ADY2* is remarkable as this gene encodes an acetate transporter, which deletion has been reported to improve growth and fermentation under acetic acid stress in the laboratory BY4741 strain (Zhang *et al*., 2017). Interestingly, *ADY2* alleles harbouring different SNPs have been reported to swap their function from acetate to lactate transporters in *S. cerevisiae* (de Kok *et al*., 2012). Hence, the lack of SNPs in *ADY2* could be the result of a balance between acetic and lactic acid tolerance in sourdough conditions.

Then, in a second approach, the SNPs list was filtered (see the Materials and Methods section) in order to search for nucleotide variants and indels that introduce a non-conserved or a missense change into the coding region, which rendered a catalogue of 75 genes (**Table S7**). From them, we first paid attention

to genes, 43 in total, with SNPs that arose prior to the start of the laboratory evolution experiments. As it is shown, most of the mutations were strain-dependent, present in homozygous and did not vary in response to the environmental conditions imposed by the ALE (**Table S7** and **Figure 14A**; upper Venn diagram). Among the mutated genes, only *ARG82*, *PSP2* and *JJJ2* were shared by the two parental backgrounds. In addition, 11 genes showed loss of homozygous or heterozygous SNPs (**Figure 14A**; lower Venn diagram) and 8 included new stop codons or changes in the frame shift. We evaluated the effect on acid tolerance of the deletion of all these genes in the BY4741 laboratory strain. Only the knock-out of 8 genes altered the growth of the wild-type strain in the presence of acetic and/or lactic acid (**Figure 14B).** Deletion of *JJJ2* and *SGF73* had a weak effect, but absence of Arg82, the inositol polyphosphate multikinase 2 (Ipk2; Saiardi *et al*., 1999; Odom *et al*., 2000), Crz1, a calcineurin-dependent transcription factor (Stathopoulos and Cyert, 1997) and the cell-death protease Kex1 (Carmona-Gutierrez *et al*., 2013), resulted in a strong growth defect in lactic-containing medium, indicating that their function contributes to lactic tolerance. In addition, *crz1* and *kex1* mutants showed acetic-sensitivity (**Figure 14B**). Knock-out of *SPT20*, a subunit of the SAGA transcriptional regulatory complex (Grant *et al*., 1998) and *CTK1*, cyclin-dependent protein kinase (Lee and Greenleaf, 1991), decreased acid tolerance, but these mutants already showed a growth defect under unstressed control conditions (**Figure 14B**). Previous results reported the implication of Arg82, Kex1, Ctk1 and Spt20 in low-pH tolerance, which could explain the phenotype of these mutants (Kawahata *et al*., 2006). Crz1 and calcineurin have been also reported to play a role in the inorganic acid stress adaptation (de Lucena *et al*., 2012). We also found new determinants of acetic tolerance (**Figure 14B**). In particular, deletion of *PSP2*, which encodes a protein with RGG motifs that regulates autophagy (Yin *et al*., 2019), conferred protection against acetic acid stress (**Figure 14B**), a phenotype not previously reported (Mira *et al*., 2010). Although the mutation

found in *PSP2* (deletion of a N in a N-rich zone; **Table S7**) is expected to have no major impact in the Psp2 function, the finding that it was shared between all the strains analysed, suggests it might contribute together with other genetic changes to the phenotype of the sourdough strains.

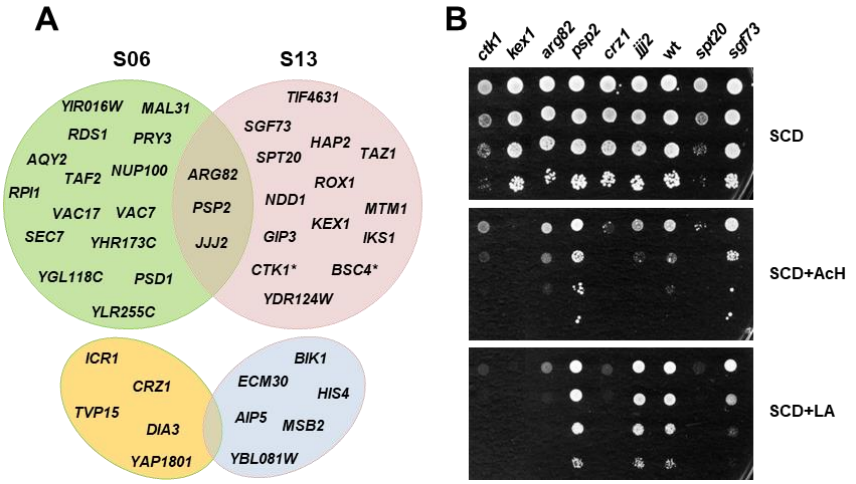


Figure 14. Identification of genes involved in acetic acid tolerance. (A) Venn diagrams showing genes containing filtered SNPs in the parental strains S06 and S13, relative to the reference S288C strain, and their adaptation pattern in the evolved clones analysed. The upper circles illustrate genes containing homozygotic variants in the parental strains that did not vary in the two evolved clones. (*) denotes genes containing heterozygotic SNPs that change to homozygotic in at least one evolved clone. The intersection illustrates the set of genes shared in the two backgrounds. Lower circles display genes containing homozygotic variants that change to heterozygotic in at least one evolved clone. SNPs have been detailed in Table S7 **(B)** BY4741 mutants carrying deletions in genes shown in panel A were analysed for growth under acid-stress conditions. Only those showing a differential phenotype relative to the wild-type strain are shown. Cells were grown to the mid-exponential phase at 30ºC (OD_{600} ~ 0.5). Then, 10-fold serial dilutions were spotted (3 µl) onto SCD agar plates lacking or containing lactic (LA) or acetic acid (AcH). Colony growth was inspected after 2-4 days of incubation at 30ºC. For more details, see the Materials and Methods section.

Finally, we focused on those genes containing SNPs that arose during the evolution experiment (**Figure 15**). As can be seen, the identified genes were strain- and evolutionary line-dependent. Remarkably, most of the genes, 22 out

GENE	S6Ac	S6Ac/M	PHENOTYPE		BIOLOGICAL PROCESS
			Acetic acid 50 mM	Lactic acid 200 mM	
HSP30			Ø	Ø	Regulation of ATP-dependent activity
MRX14			±	Ø	Mitochondrial translation
MSN5			+	-	Protein export from nucleus
DNF1			Ø	Ø	Stablishment or maintenance of cell polarity
LAM4			Ø	Ø	Sterol transport
GAR1			inviable		snRNA pseudouridine synthesis
MLP2			±	Ø	Spindle pole body organization
BBC1			Ø	±	Actin cytoskeleton organization
APL2			Ø	+	Golgi to vacuole transport
MIF2			inviable		Kinetochore assembly
YEF3			inviable		Translation elongation
GIM5			++	±	Regulation of transcription elongation
YNL319w			++	±	Hexose transmembrane transport
GAS4			Ø	Ø	Ascospore wall assembly
KIN4			±	Ø	Spindle pole body organization
MEC1			inviable		Signal transduction in response to DNA damage
OSW7			Ø	Ø	Ascospore wall assembly
GIS4			Ø	Ø	Intracellular signal transduction
IRA2			-	Ø	Regulation of Ras protein signal transduction
MDM20			±	±	Actin cytoskeleton organization
RAD53			inviable		DNA repair
TFC1			inviable		Transcription by RNA polymerase III
YKR011c			Ø	Ø	Unknown

GENE	S13Ac	S13Ac/M	PHENOTYPE		BIOLOGICAL PROCESS
			Acetic acid 50 mM	Lactic acid 200 mM	
GIS4			Ø	Ø	Intracellular signal transduction
FCP1			inviable		Transcription by RNA polymerase II
APC1			inviable		Protein ubiquitination
YBL081w			+	+	Unknown
YER152c			Ø	Ø	Unknown
OSW7			Ø	Ø	Ascospore wall assembly
ASG1			++	+	Unknown
BBC1			Ø	±	Actin cytoskeleton organization
SMC5			inviable		Mitotic chromosome condensation
SAM4			Ø	Ø	Homocysteine metabolic process
CAM1			Ø	Ø	Transcription by RNA polymerase II

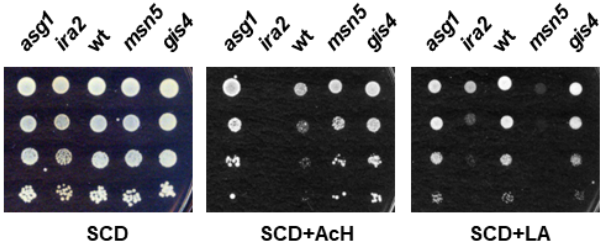


Figure 15. Identification of genes showing specific SNPs variants in evolved clones and their phenotypic characterization in BY4741 deletion mutants. The tables display those genes with SNPs present exclusively in any of the evolved clones (marked in green). The sequence variation appears in heterozygosis in all cases, except *MDM20* in $S06A_{15}$ with both alleles switched. Neutral (Ø), positive (+) and negative (-) phenotypes relative to the wild type BY4741 strain are shown. Inviable mutants and the biological process terms for each gene are also indicated. A drop test assay in SCD medium lacking or containing 45 mM acetic acid (SCD+AcH) or 200 mM lactic acid (SCD+LA) for BY4741 wild-type (wt) and their corresponding deletion mutants, *asg1*, *ira2*, *msn5* and *gis4* is shown. Cells were grown and cultures were diluted, spotted and incubated as described in **Figure 14B**.

23, harbouring filtered SNPs acquired through the evolutionary procedures in the S06 background were found in the acetic-tolerant $S06A_{15}$ strain. The S13 strains presented a lower number of mutated genes, 11 in total, with only three, *GIS4*, *OSW7* and *BBC1*, shared between the acetic-tolerant strains, $S06_{A15}$ and $S13A/M_{14}$ (**Figure 15**). As above, mutants of all these genes from the EUROSCARF haploid mutant collection were screened for susceptibility phenotypes to acetic and lactic acids. From them, knock-out of *GIS4*, *ASG1* and *MSN5* improved the acetic acid tolerance, while absence of Ira2 caused increased susceptibility to both acetic and lactic (**Figure 15**). *IRA2*, which encodes a GTPase-activating protein (Tanaka *et al*., 1990), has been previously connected with acetic acid tolerance (Stojiljkovic *et al*., 2020). Previous reports following laboratory evolution approaches have also identified mutations in a group of four genes, between them *GIS4* and *ASG1*, as causal for the acquisition of constitutive acetic acid tolerance (González-Ramos *et al*., 2016). *ASG1* encodes a zinc cluster protein that regulates the utilization of fatty acids and accumulation of lipids in response to stress (Jansuriyakul *et al*., 2016), while Gis4, has been proposed to play a role in ion homeostasis (Ye *et al*., 2006) and glucose regulation (La Rue *et al*., 2005). Remarkably, mutation of *GIS4*, which results in an early stop codon, was exclusively found in the two acetic tolerant strains, $S06A_{15}$ and $S13A/M_{14}$ (**Table S7**). On the other hand, the enhanced acetic-tolerance of the *msn5* mutant is intriguing, as this phenotype was accompanied by a loss of protection against lactic acid. The exportin Msn5 is involved in nuclear export of Haa1, the transcription factor in charge of the transcriptional induction of most acetic- and lactic acid-tolerance genes (Abbot *et al*., 2008; Mira *et al*., 2010). Disruption of *MSN5* leads to the accumulation of Haa1 in the nucleus (Sugiyama *et al*., 2014). This unusual constitutive nuclear localization of Haa1 could affect its turnover and activity. Absence of Msn5 could also destabilize the nuclear export of other factors, as the exportin has been implicated in a variety of signalling systems (Alepuz *et al*., 1999). For example, *msn5* mutant cells block

the nuclear export of Msn2, which reduces its protein levels and causes delayed growth under chronic stress conditions (Durchschlag *et al*., 2004). More work is required to shed light to the underlying mechanisms operating in the phenotypic response of *msn5* cells to acid conditions.

4. Conclusions

This work has evidenced the powerful of our targeted ALE strategies to induce acetic tolerance in *S. cerevisiae* and to generate novel strains with higher performance in the sourdough environment. Phenotypic characterization of evolved populations demonstrated that acetic acid tolerance acquisition was quite often accompanied by a loss of protection against lactic acid, indicating that these weak acids may not act in the same manner on the yeast cell. In addition, we have confirmed the involvement of several genes as *ARG82*, *KEX1*, *CTK1*, *SPT20*, *IRA2*, *ASG*1 or *GIS4*, and identified new determinants, *MSN5* and *PSP2*, in acetic and/or lactic tolerance. Several of these genes presented weak impact mutations suggesting a combination of many genetic changes as causative of the tolerance phenotype. Nevertheless, the observation that SNPs in *GIS4* were shared by the superior evolved strains, underline the importance of this mutation for acetic acid tolerance.

Supplementary material

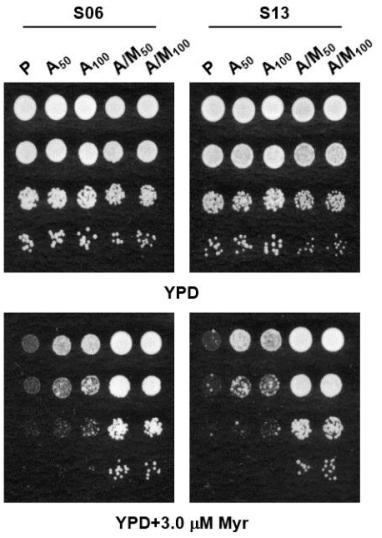


Figure S3. Acetic-driven evolved populations show increased myriocin tolerance. Parental strains (P) S06 and S13 and their corresponding 50- and 100-generation evolved populations from both, acetic (A_{50} and A_{100}) and acetic/myriocin (A/M_{50} and A/M_{100}), evolutionary lines were tested for myriocin tolerance (Myr). Cells were grown in liquid YPD to the mid-exponential phase at 30ºC (OD_{600} ~ 0.5). Then, 10-fold serial dilutions were spotted (3 µl) onto YPD plates lacking or containing 3 µM myriocin. Colony growth was inspected after 2-4 days of incubation at 30ºC. For more details, see the Materials and Methods section.

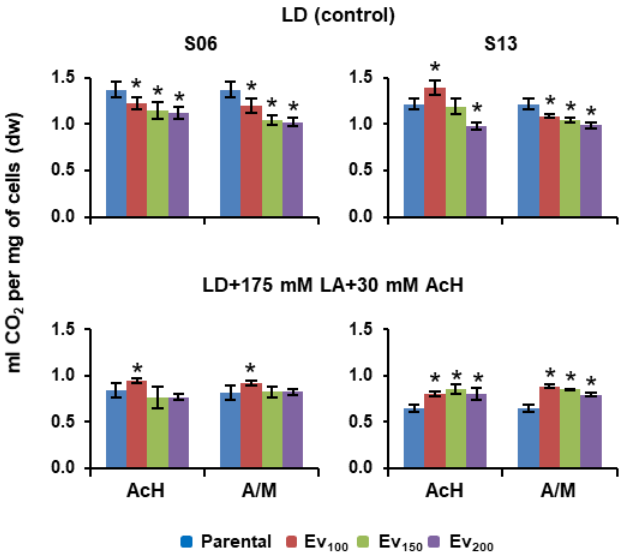


Figure S4. Fermentative power of evolved populations. Yeast cells of *S. cerevisiae* sourdough strains (S06 and S13) and their corresponding 100- (Ev_{100}), 150- (Ev_{150}) and 200-generation (Ev_{200}) evolved populations from both, acetic (AcH) and acetic/myriocin (A/M) evolutionary lines, were tested for total CO_2 production after 240 min of fermentation in liquid dough (LD) lacking or containing lactic and acetic acid at the indicated concentrations. In all cases, values are expressed as ml of CO_2 per mg of yeast cells (dry weight), and represent the mean ± sd of at least three independent experiments. Bars labelled with * indicated significant differences ($p < 0.05$) with their parental counterpart in the condition assayed.

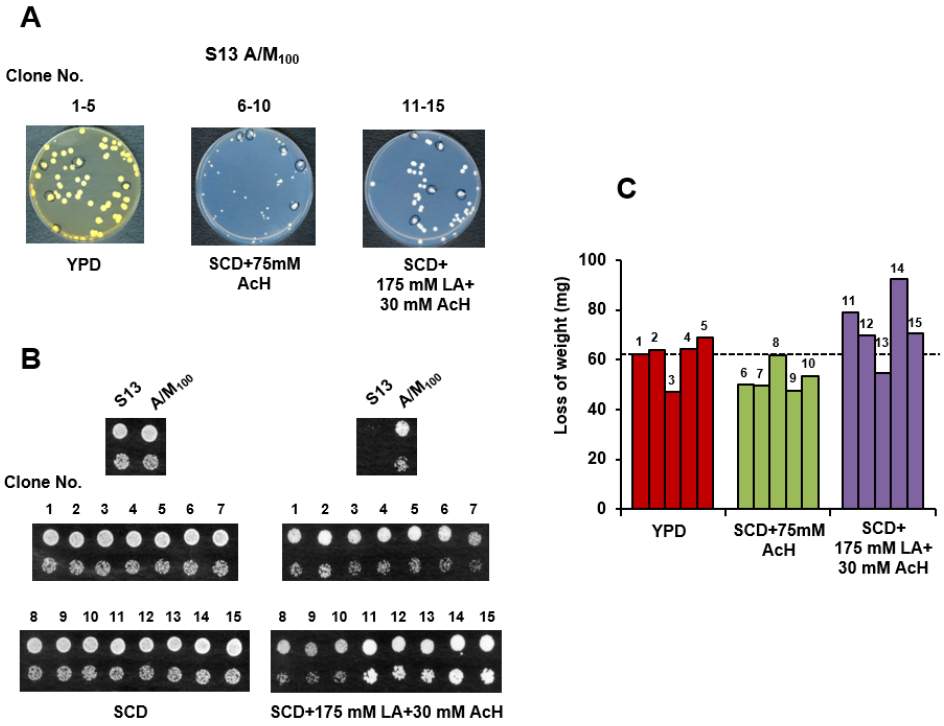


Figure S5. Isolation and selection of evolved clones from heterogeneous evolved populations. (A) An SCD-grown saturated culture of cells from the evolved population S13A/M_{100} was diluted and plated in three media YPD, SCD-containing 75 mM acetic or SCD containing 175 mM lactic plus 30 mM acetic acid. After 1-3 days of incubation at 30ºC, five colonies of each culture medium were randomly picked. **(B)** SCD-grown cultures of the 15 isolated colonies (1-5 from YPD; 6-10 from SCD+75mM AcH and 11-15 from SCD+175mM LA + 30mM AcH), were diluted and spotted onto solid SCD control medium or the same medium containing a mix of acids as indicated. Growth was inspected after 2-3 days of incubation at 30°C. **(C)** The gas production of each evolved isolate was estimated by the loss of weight in an SCD media containing 4% glucose and 175mM lactic plus 30mM acetic acid after 24 h at 30 ºC. Values represent the mean of two biological replicates.

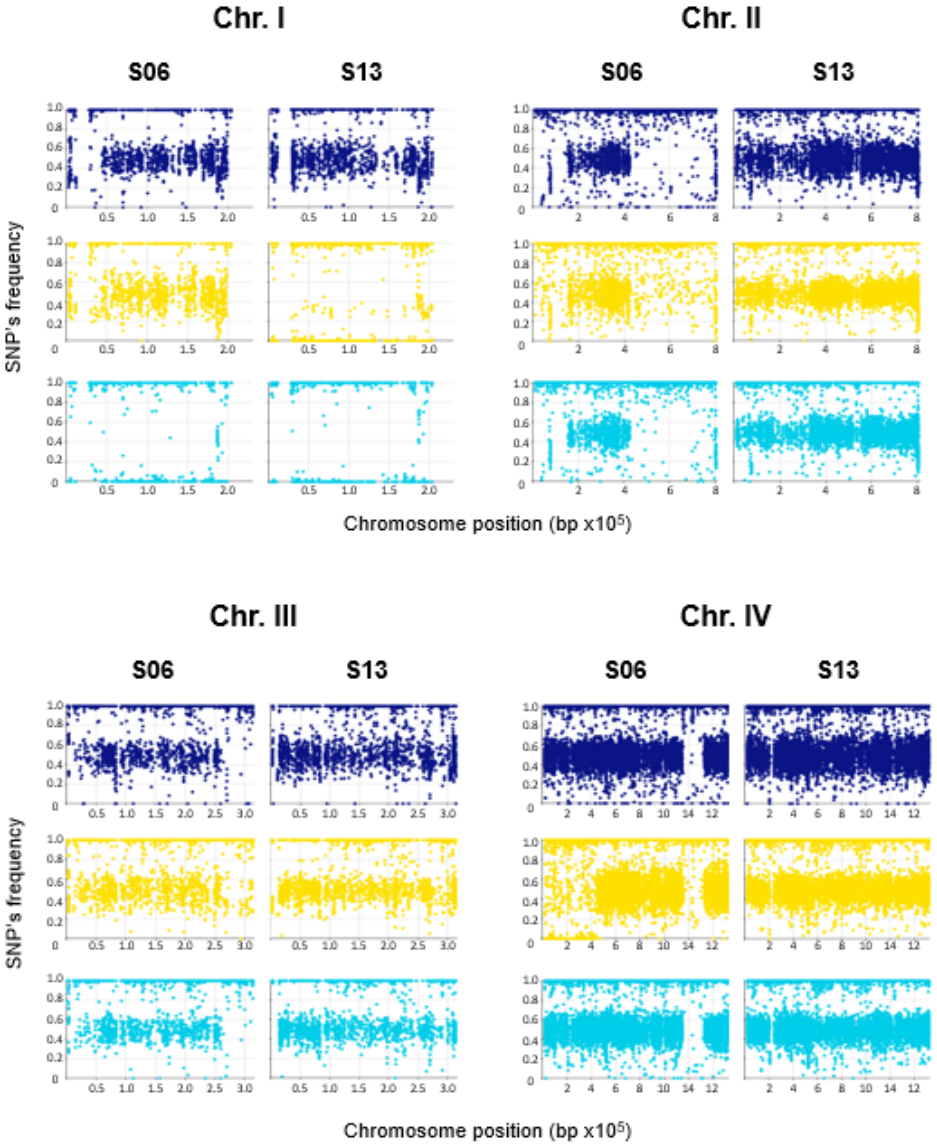


Figure S6. SNPs frequency representation. The graphics show the SNPs frequency along each chromosome of the parental (dark blue) S06 and S13 strains and their corresponding evolved clones isolated in the two evolutionary lines, acetic acid (yellow) or acetic plus myriocin (light blue), compared to the S288C strain.

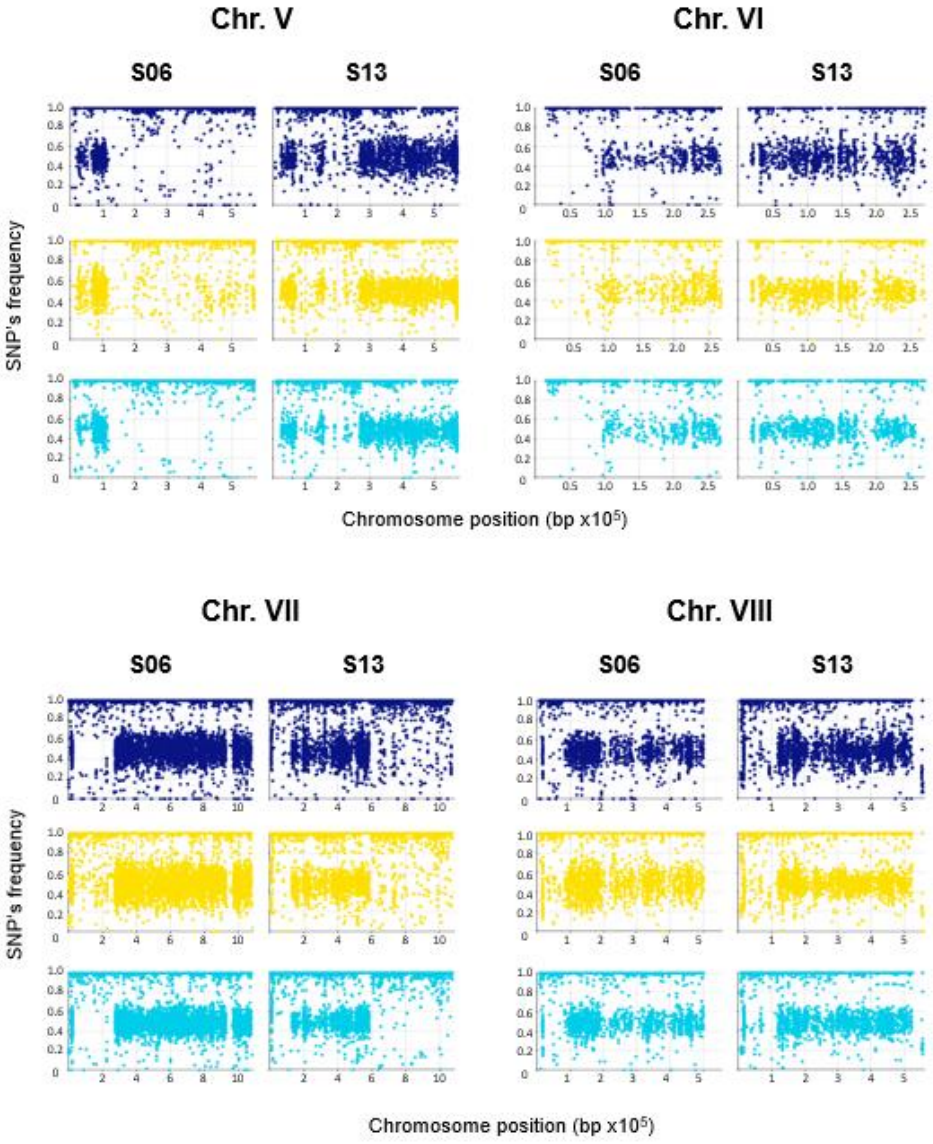


Figure S6 (Continuation).

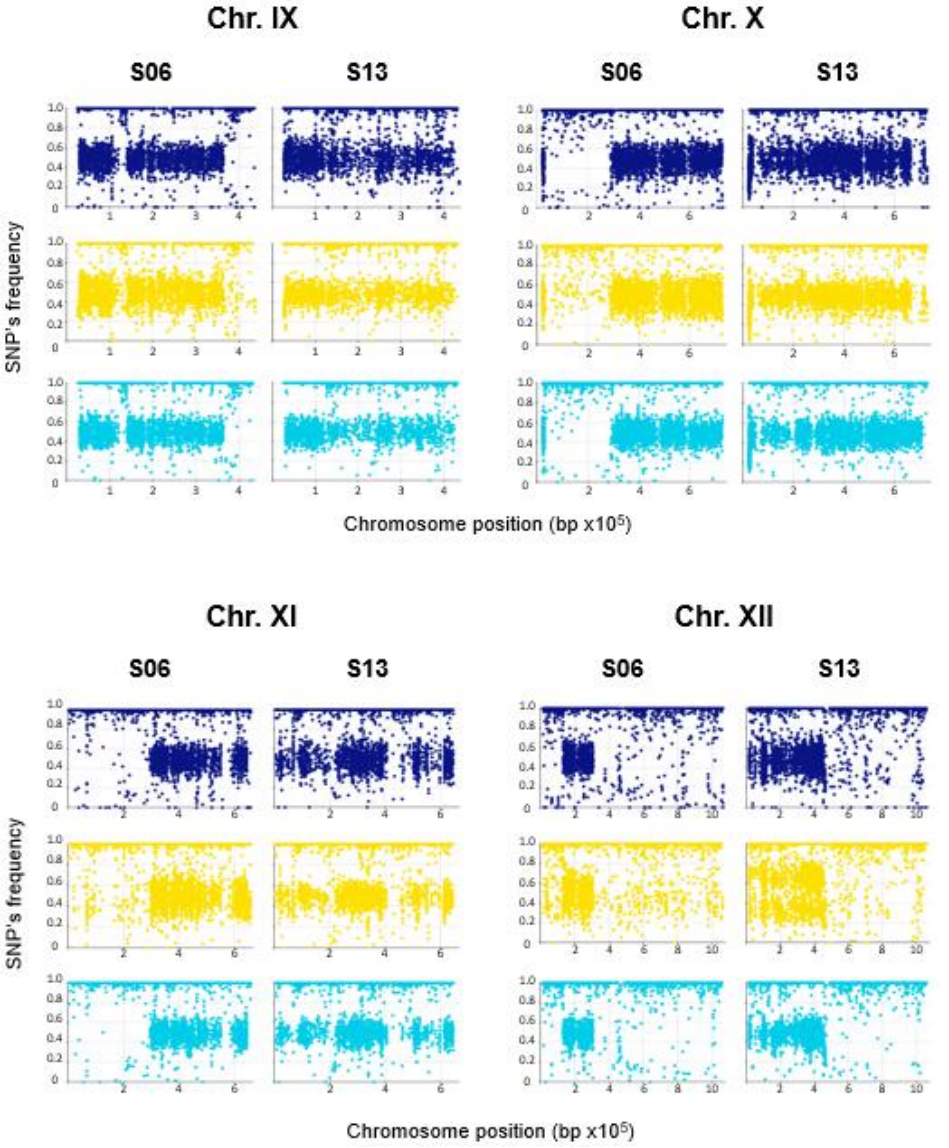


Figure S6 (Continuation).

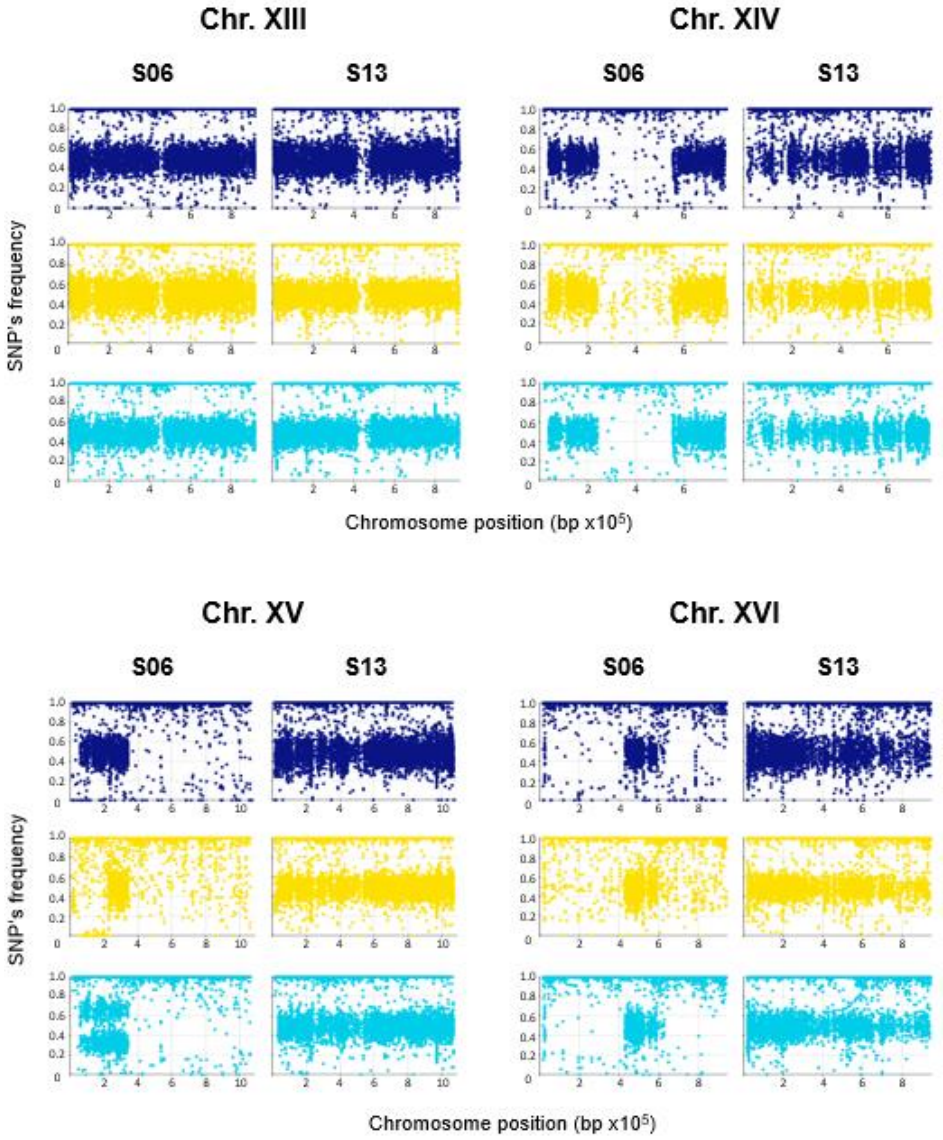


Figure S6 (Continuation).

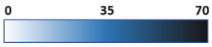
Table S4. Sequencing and bioinformatic data analysis.

Sample	Total raw reads	Total clean reads	Clean reads (%)	Total read bases	Average read-depth (%)	bases covered above 10 reads (%)
S06	2937335	2890730	98.4	1070445823	88.05	92.9
S06A$_{15}$	1714654	1684261	98.2	632795117	52.05	92.5
S06A/M$_{13}$	3058749	3008640	98.4	1133112563	93.21	92.9
S13	2103182	2041904	97.1	771714714	63.48	92.5
S13A$_{14}$	3782757	3711515	98.1	1380121368	113.52	92.8
S13A/M$_{14}$	3173769	3119692	98.3	1156689002	95.15	92.8

Table S5. Copy number variations detected across all sourdough strains under study.

Chr.	Genes	
	underrepresented	overrepresented
I	*FLO9, SSA1, FLO1, YAR061w, YAR064w, PHO11*	*SEO1*
II	Nt-*NHP6-B, TIM12, PHO3, MAL32*	*MAL31, MAL33*
III	*GEX1,VBA3, HMLALPHA1, HMLALPHA2, MATALPHA LEU2, NFS1, DCC1, YCR102c, AAD3*	
IV	*MPH2, SOR2, HXT15, ENA1, ENA2*	
V	*DSF1, HXT13*	
VI	Ct-*COS4, DDI2, SNO3, SNZ3, THI5, CDC4*	
VII	*MAL12, PAU12, YRF1-3*	
VIII	*YHL050c, YHL049c, DOG2, CUP1-1, YHR054c, CUP1-2 FLO5, PHO12*	*COS8, ARN2, PAU13, YHL045w, YHL044w ECM34, YHL042w*
IX	*VTH1, IMA3, HXT12, CAB2, FLO11, YPS6, PAU15*	
X	*PAU1, VTH2, IMA4, YJR154w, AAD10, THI11, HXT16 SOR1, MPH3, COS5*	*REE1, IMA5, HXT8*
XI	*FLO10*, NFT1*, YKR104w, VBA5, GEX2,*	
XII	*ASP3-2, ASP3-3, ASP3-4, YLR162w, BSC3, YRF1-4, YRF1-5*	*RDN25, RDN37, RDN58 RDN18, RDN5, MAS1 SHH4, PUS5, Ct-SEC10*
XIII	*DDR48, ALD3, ALD2, YMR321c, SNO4, ERR3, PAU19 YRF1-6, COS1, DDI3, SNO2, SNZ2, SSB2,* Nt-*YNL035c*	
XIV	*YNL034w, YNL033w YNL018c, YNL019c, YNRO65c, HXT17 MAN2, AIF1, COS10, PAU6 AAD15, BDS1, YOL163w, YOL162w, IMA2, HXT11, EFT1*	
XV	Ct-*PRH1, YOR387c, FDH1, YOR389w, FEX1, HSP33, ERR1 PAU21*	
XVI	*YRF1-7, PAU22, ERR2, HSP32, FEX2, YPL278c, YPR202w YPR203w, YPR204w*	

Table S6. Heat map showing the occurrence and number of SNPs in genes reported to be linked to acetic acid tolerance*.



		S06		$S06A_{15}$		$S06A/M_{13}$		S13		$S13A_{14}$		$S13A/M_{14}$		
Chr	**Gene**	**Hm**	**Ht**	**Hm**	**Ht**	**Hm**	**Ht**	**Hm**	**Ht**	**Hm**	**Ht**	**Hm**	**Ht**	**Biological Process and reference**
I	***CCR4***													mRNA decay [1]; [2]
II	***PDR3***													Cellular response to drug [1]
II	***QDR3***													Transmembrane transport [3]
II	***YRO2***													Unknown [4]
II	***IRA1***													Regulation of Ras protein [5]
II	***SRB6***													Transcription regulation [1]
III	***PDI1***													Protein folding [2]
III	***HIS4***													Histidine biosynthesis [6]
III	***ADY2***													Acetate transport [2]; [7]
III	***SRB8***													Transcription regulation [1]
IV	***NUP145***													Protein import into nucleus [2]; [8]
IV	***KNH1***													1,6-β-D-glucan biosynthesis [1]
IV	***STE5***													Invasive growth [9]
IV	***SAC6***													Actin filament organization [2]
IV	***VHS1***													Regualtion of protein localization [2]
IV	***MSN5***													Protein export from nucleus [8]
IV	***PDR15***													Response to xenobiotic stimulus [7]
IV	***EUG1***													Protein folding [2]
V	***GIP2***													Glycogen metabolism [2]
VII	***KEX1***													Apoptosis [10]
VII	***PMR1***													Calcium ion transport [9]
VII	***PDR1***													Cellular response to drug [1]; [11]
VII	***PMA1***													Proton transmembrane transport [1]; [7]; [12]
VII	***ADH6***													Alcohol metabolism [9]
VII	***ESP1***													Apoptosis [9]
VII	***TPO2***													Spermine transport [1]
VIII	***STE12***													Invasive growth [2]
IX	***MLP2***													Protein import into nucleus [8]
IX	***ASG1***													Unknown [2]
IX	***QDR1***													Transmembrane transport [1]
XI	***COS9***													Protein transport to vacuole [2]
XI	***JEN1***													Plasma membrane lactate transport [7]; [13]
XI	***STE6***													Peptide pheromone export [2]
XI	***CWP1***													Cell wall organization [1]
XI	***CTS1***													Chitin catabolism [11]
XII	***FPS1***													Acetate transport [1]
XII	***ACE2***													Transcription regulation [8]; [9]
XII	***FRE1***													Iron ion transport [9]

Table S6 (Continuation)

		S06		S06A$_{15}$		S06A/M$_{13}$		S13		S13A$_{14}$		S13A/M$_{14}$		
Chr	**Gene**	**Hm**	**Ht**	**Hm**	**Ht**	**Hm**	**Ht**	**Hm**	**Ht**	**Hm**	**Ht**	**Hm**	**Ht**	**Biological Process and reference**
XIII	***ERG13***													Ergosterol biosynthesis [9]
XIII	***WAR1***													Transcription regulation [1]; [8]
XIII	***GSF2***													Protein folding [15]
XIII	***GIS4***													Intracellular signal transduction [2]
XIII	***YPT7***													Endocytosis [12]
XIII	***ERG5***													Ergosterol biosynthesis [9]
XIII	***ADH3***													Ehrlich pathway [2]
XIII	***ASC1***													Translation regulation [16]
XIII	***UBP15***													Protein deubiquitination [9]
XIII	***SCW10***													Conjugation with cellular fusion [9]
XIII	***GAS1***													Cell wall organization [9]
XIII	***GLC8***													Glycogen biosynthesis [9]
XIII	***ELP6***													Transcription regulation [9]
XIII	***PRE5***													Proteasomal protein catabolism [9]
XIII	***DIA1***													Invasive growth [9]
XIV	***PDR16***													Response to xenobiotic stimulus [1]
XIV	***RAS2***													Regulation of pseudohiphal growth [1]; [13]
XIV	***AQR1***													Monocarboxylic acid transport [1]
XIV	***PDR18***													Response to xenobiotic stimulus [1]; [17]
XV	***GAS4***													Ascospore wall assembly [9]
XV	***SHR5***													Protein palmitoylation [1]
XV	***MSH2***													DNA insertio or deletion binding [5]
XV	***IRA2***													Regulation of Ras protein [5]
XV	***MET22***													Hiperosmotic stress [6]
XV	***RAS1***													Regulation of adenylate cyclase [2]
XV	***AZF1***													Regulation of cell wall organization [8]
XV	***ADH2***													Ehrlich pathway [9]
XV	***BAG7***													Regulation of Rho protein [1]
XV	***PDR5***													Response to xenobiotic stimulus [7]
XV	***GAC1***													Glycogen metabolism [9]
XV	***HRK1***													Monoatomic ion homeostasis [1]; [18]
XVI	***ISU1***													Iron-sulfur cluster assembly [9]
XVI	***PDR12***													Organic acid transport [8]
XVI	***VPS16***													Protein targeting to vacuole [2]
XVI	***SKS1***													Transcription regulation [2]
XVI	***HAA1***													Transcription regulation [1]; [12]
XVI	***TPO3***													Spermine transport [1]; [15]
XVI	***TDA6***													Unknown [15]

***** homozygous (Hm) and heterozygous (Ht) SNPs are shown. [1] Mira *et al.*, (2010); [2] Gonzalez-Ramos *et al.*, (2016); [3] Ma *et al.*, (2015); [4] Mira *et al.*, (2011); [5] Stojiljkovic et *al.*, (2020); [6] Fernandez-Niño *et al.*, (2018); [7] Guaragnella and Bettiga (2021); [8] Pereira *et al.*, (2019); [9] Fletcher *et al.*, (2017); [10] Houptmann and Lehle (2008); [11] Marad *et al.*, (2018); [12] Meijnen *et al.*, (2016); [13] Salas-Navarrete *et al.*, (2022); [15] Baek *et al.*, (2016); [16] Lee *et al.*, (2015); [17] Godinho *et al.*, (2018); [18] Guerreiro *et al.*, (2017).

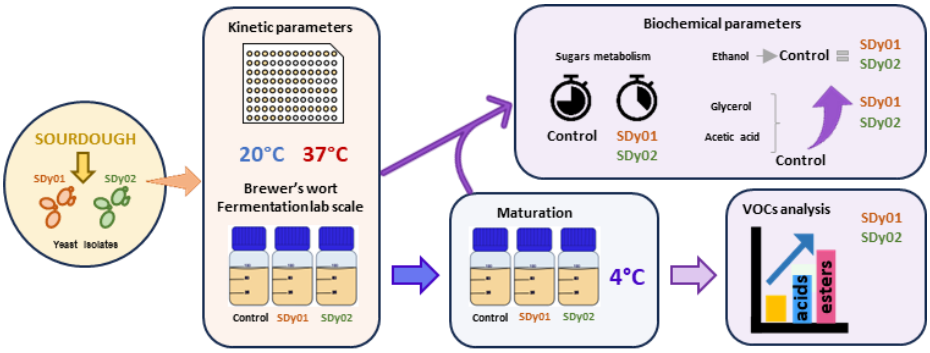
Table S7. List of filtered SNPs found in the parental and selected evolved strains under study.

Chr	Gene[1]	Frequency[2]	S06 SNPs[3]			S13 SNPs			
		P/A15/A/M13	P	A15	A/M13	Gene	P/A14/A/M14	P	A14	A/M14
II	*MAL31*	82/90/86		T802656C (N to S)						
II	*MEC1*	0/40/0		C508086A (L to I)						
		0/66/0		G508764A (V to M)						
II						*YBL081w*	9/54/50		A72374G (H to R)	
							32/2/8	A72371G (N to S)		
II	*TFC1*	11/59/16		GT485927G (FS[4])						
III						*BIK1*	100/36/39	A68852G (L to S)	A68852G (L to S)	
III						*HIS4*	100/49/40	T67820C (K to E)	T67820C (K to E)	
III	*HSP30*	0/48/0		C156783A (G to V)						
III	*RDS1*	83/95/95		GAAT312257G (EYI to DI)						
III	*TAF2*	82/100/89		CCCCT204422C (FS: STOP)						
III	*FIG2*	9/33/12		A270074G (T to A)						
III	*VAC17*	86/79/80		T18468TCTGAAC (in[5]: LN)						
IV	*ARG82*	78/86/90		C810744CATCATT (in: ND)		*ARG82*	79/90/94	C810744CATCATT (in: ND)		
IV	*DIA3*	38/0/57	G408812A (A to V)		G408812A (A to V)					
		50/0/46	T409788C (R to G)		T409788C (R to G)					
IV	*TVP15*	31/15/12	C655350T (S to F)							
IV	*MRX14*	0/48/0		T682482C (L to S)						
IV	*MSN5*	0/44/0		C1141423A (L to M)						
IV	*SEC7*	86/74/79		del[6]801915T (-8 aa)						
IV						*YDR124w*	82/87/91	del701136A (-5 aa)		
V	*DNF1*	0/48/0		A514046G (T to A)						
V						*YER152c*	14/59/47		G472853C (T to S)	
VI						*AIP5*	90/69/68	del179312T (-5 aa)	del179312T (-5 aa)	
VI	*OSW7*	0/50/0		T233004C (N to E)		*OSW7*	10/46/37		del232990A (del and change D to M)	
							12/51/41		TCA233006T (E to V and FS)	
VII						*MSB2*	31/6/11	T518111C (I to T)		
VII						*HAP2*	84/92/90	T53152TGCC (in: R)		
VII						*KEX1*	76/93/100	C112960T (D to N)		
VII						*MTM1*	84/91/89	TG1006274T (FS: STOP)		
VII						*SGF73*	82/88/85	TCAGCAG379162T (del: QQ)		
VII						*TIF4631*	88/80/72	A824582ATTCTAC (in: SY)		
VII	*YGL118c*	82/85/90		GAT288375G (FS: STOP)						
VIII	*YAP1801*	85/58/67	del420550C (-4 aa)		del420550C (-4 aa)					
		95/71/75		T420572G (Q to P)						
VIII	*LAM4*	0/53/0		A263057C (I to R)						
VIII	*GAR1*	0/54/0		C283250T (G to S)						
VIII	*YHR173c*	81/89/88		TA451138T (FS: STOP)						
IX						*ASG1*	13/32/37		A105420G (N to S)	
IX	*YIR016w*	89/76/84		A382914ATG (FS: STOP)						
IX	*ICR1*	86/59/55	A394835in		A394835in (ncRNA of *FLO11*)					
IX	*MLP2*	0/54/0		G63327A (P to S)						
IX	*RPI1*	83/94/89		ATTT137704A (in: K)						

X	***BBC1***	0/47/0			**AG**401793**A** (FS: **STOP**)					***BBC1***	17/48/50			**T**399292**C** (**Q** to **R**)								
X										***IKS1***	83/92/97	**C**329323**T** (**V** to **I**)											
X	***JJJ2***	85/93/94	**T**114765**TTTC** (in: **E**)							***JJJ2***	81/88/93	**T**114765**TTTC** (in: **E**)											
X	***PRY3***	82/92/89	**G**293478**GGAC** (in: **S**)																				
XI	***APL2***	0/40/0			**C**188143**A** (**K** to **N**)																		
XI										***CTK1***	40/88/76	**A**184503**in** (**FS**)			**A**184503**in** (**FS**)							
XI	***MIF2***	4/44/6			**CA**274411**C** (FS: **STOP**)																		
XI	***NUP100***	87/80/72	**T**310563**TTAATAACAA** (in: **NNN**)																				
XI	***YKR011c***	10/46/15			**AT**461693**A** (FS: **STOP** loss)																		
XII	***AQY2***	88/77/77	**G**36038**GAAACACCAGCA** (in: **FAGV** and **FS**)																				
XII										***ECM30***	97/62/71	**del**1011122**C** (-10 aa)			**del**1011122**C** (-10 aa)							
XII	***YLR255c***	88/78/75	**T**645754**TTCTCTC** (in: **ER**)																				
XII	***YEF3***	0/46/0			**C**637762**A** (**A** to **D**)																		
XIII										***FCP1***	0/0/54										**G**820884**A** (**R** to **K**)		
XIII	***GIM5***	0/50/0			**A**82642**G** (**K** to **R**)																		
XIII	***GIS4***	0/40/0			**G**257327**GT** (FS: **STOP**)					***GIS4***	0/0/56										**G**257327**GT** (FS: **STOP**)		
XIII	***PSP2***	80/86/90	**GATA**238330**G** (del: **N**)							***PSP2***	89/83/80	**GATA**238330**G** (del: **N**)											
XIV										***APC1***	0/0/48										**C**315764**T** (**A** to **V**)		
XIV										***BSC4***	67/92/93	**GA**138052**G** (FS: **STOP**)			**GA**138052**G** (FS: **STOP**)								
XIV	***CRZ1***	83/69/76	**A**579948**in** (in: **QQ**)		**A**579948**in** (in: **QQ**)			**A**579948**in** (in: **QQ**)		***CRZ1***	86/84/73	**A**579948**ACAACAG** (in: **QQ**)											
XIV	***PSD1***	80/97/95	**A**317360**ATCT** (in: **E**)																				
XIV	***VAC7***	84/92/89	**CCAG**527489**C** (del: **Q**)																				
XIV	***YNL319w***	1/48/3			**GA**38658**G** (**FS**)																		
XV	***GAS4***	0/43/4			**G**72083**GT** (**FS**)																		
XV	***IRA2***	0/41/0			**G**173313**GA** (FS:**STOP**)																		
XV	***KIN4***	0/62/0			**T**776714**C** (**L** to **P**)																		
XV	***MDM20***	0/100/0			**C**187309**A** (**H** to **N**)																		
XV										***NDD1***	89/79/77	**CTGT**1036136**C** (del: **Q**)											
XV	***KIN4***	0/62/0			**T**776714**C** (**L** to **P**)																		
XV										***SMC5***	15/20/40										**T**261362**A** (**D** to **E**)		
XV										***SPT20***	95/82/80	**AATT**45897**A** (del: **N**)											
XVI										***SAM4***	15/51/30				**A**25297**C** (**N** to **H**)								
XVI										***CAM1***	12/37/37				**A**465116**G** (**K** to **E**)								
XVI										***GIP3***	72/92/88	**G**1296526**A** (**S** to **F**)											
XVI	***RAD53***	0/0/44						**G**292867**T** (**F** to **L**)															
XVI										***ROX1***	80/90/96	**G**680007**GCAA** (in: **Q**)											
XVI										***TAZ1***	89/74/85	**A**814896**AGAAAGTAAG** (in: **KVR**)											

***** Frequency of reads, positions of the SNPs in the ORFs as well as their impact in the amino acid sequence are indicated. [1] Genes showing a frame shift or stop codon are shown in red. [2] Variants whose frequenty was >70 were considered homozygotic and they were considered heterozygotic when the frequency was ≥30 and ≤70. [3] Colors represent the change of SNPs frequency between the parental and the evolved clones. yelow: homozygotic mutations found in all the strains of both S06 and S13 backgrounds; grey: homozygotic mutations found in all strains of at least one background; salmon, reduction in SNPs frequency in at least an evolved clone of either S06 or S13; blue, gain in SNPs frequency in at least one evolved clone of either S06 or S13. [4] FS: Frame Shift. [5] IN: Insertion. [6] DEL: Deletion. *SEC7* (DEL: GDEDEDED). *YAP1801* (DEL: QQPQ). *YDR124w* (DEL: EYFPR). *AIP5* (DEL: EEEEE). *ECM30* (DEL: RTSSPSPSLS).

Chapter 3: Sourdough yeast strains: the forgotten starters for beer fermentation



Keywords: sordough; yeast; *Saccharomyces cerevisiae*; brewing; thermal tolerance; fermentation rate; flavour

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Sánchez-Adriá IE, Sanmartín G, Prieto JA, Estruch F, Randez-Gil F. Sourdough yeasts strains: the forgotten starters for beer fermentation.

Summary

The increasing popularity of home brewing and the fast evolution of craft beer companies have fuelled the interest in novel yeasts as the main actors to diversify the beer portfolio. Here, using small lab-scale fermentation, we have characterized the thermal tolerance and brewing-related features of two sourdough isolates of *Saccharomyces cerevisiae*, SDy01 and SDy02, at different temperatures, 20 and 37ºC, comparing them with the commercial brew strains, BRY-97 and VOSS. The sourdough strains exhibited increased growth rates and lower lag phases than the reference beer strains at both temperatures. Consistent with this, SDy01 and SDy02 displayed a higher fermentative activity than the control commercial strains in terms of sugar rate depletion and release of metabolic by-products. As expected, the sourdough strains consumed maltose more quickly independently of the temperature tested, but they were unable to reduce maltotriose content. Nevertheless, alcohol yield did not vary significantly, while glycerol production was superior in wort fermented by SDy01 and SDy02 at 20 or 37ºC. Comparing with the reference BRY-97 strain, SDy01 and SDy02 brewing at 20°C increase total amount of volatile compounds (VOCs), in particular of esters and carboxyl compounds, which provide fruitiness and acidity. In contrast, fermentation at 37ºC resulted in a drastic reduction in the total volatile content in wort fermented with sourdough yeast, especially in the level of esters. As a result, the wort fermented with SDy01 and SDy02 showed a similar profile of VOCs than VOSS at 37ºC, with alcohols as the prominent chemical group. In conclusion, our results stress the high fermentative performance of sourdough strains in beer's wort and their ability to provide a complex and specific aromatic profile in a wide range of temperatures.

1. Introduction

The microbial ecosystem of natural sourdoughs encompasses a mix of lactic acid bacteria (LAB) and yeast able to face a stressful environment characterized by a high content of weak acids in a microaerophilic matrix where maltose predominates as a carbon source. These specific conditions limit microbial diversity, which often is reduced to one or two species of LAB and yeast, being *Kazachstania humillis* and *Saccharomyces cerevisiae* the most frequently found baking yeasts (Sánchez-Adriá *et al*., 2023a). Nevertheless, sourdough of different origins exhibits large strain diversity as microbial composition depends on the bakery environment, ingredients and process conditions (Comasio *et al*. 2020a; De Vuyst *et al*., 2023). In addition, microbial heterogeneity results from continuous backslopping (Landis *et al*., 2021), which offers the opportunity to make up a genotypic composition and fixe the best-adapted evolved variants in the mature sourdough.

Strain diversity of sourdough baker's yeast might be used to improve traditional fermentations closely related to baking. Indeed, most baker's yeast strains share a traditional common use and domestication trajectory with beer strains that have been evidenced by large-scale phenotyping and genotyping. Analysis of hundreds of industrial strains of *S. cerevisiae* has demonstrated that most baker's yeast strains are grouped in a mixed clade shared with some beer strains (Gonçalves *et al*., 2016). This is somewhat expected given that human-driven selection toward features suited to distinct niches is the main source of genetic and phenotypic variation amongst *S. cerevisiae* lineages (Gallone *et al*., 2016). For example, many genes involved in maltose metabolism, the main carbon source of cereal-based fermentations, are amplified in beer, sake and bread yeast populations (Gallone *et al*., 2016; Bigey *et al*., 2021). Like this,

osmotolerance genes are overrepresented in both baking and brewing yeasts (Gonçalves *et al*., 2016).

Instead of brewing yeast, baker's yeast is frequently used to brew common Eastern European beers like kvass (Loponen and Sibakov, 2013) and Finland's sahti (Ekberg *et al*., 2015; Catallo *et al*., 2020). Previous studies have also demonstrated that sourdough isolates of *S. cerevisiae* can be employed to ferment brewer's wort (Mascia *et al*., 2015; Rossi *et al*., 2018; Johansson *et al*., 2021). Beers obtained with these strains are characterized by their aromatic differences. Although this is an important property, criteria such as fermentation rate, lag phase or broad temperature tolerance were secondary or not considered. However, there is a growing interest in brewing at temperatures of 35-40ºC, well above those typically used for commercial ales (Garshol, 2021). Warmer fermentations result in shorter lag phases and fermentation times, which reduces the risk of contaminations, increases the economy of the process and saves cooling costs, particularly in geographical locations such as Southern Europe. Moreover, brewing at high temperatures appears to have no undesirable effects on sensory properties or even reduce off-flavours. For example, wort pitching with traditional Norwegian kveik ale thermotolerant yeasts produces clean and fresh-tasting beers (Garshol, 2021; Kits and Garshol, 2021; Foster *et al*., 2022).

The increasing demand for novel beer styles, the emergence of the craft brewing community and the limited number of beer strains used in industrial production (Lengeler *et al*., 2020) have stimulated the interest in exploring novel sources of yeast strains with potential use in brewing (Gibson *et al*., 2017; Hittinger *et al*., 2018; Cubillos *et al*., 2019). Here, we have examined the brewing potential of two sourdough isolates of *S. cerevisiae* by comparing them with commercial brewing strains typically used for conventional and warm pitching. In our hands, the use of these baker's strains is a promising alternative to improve

technological parameters and endow the final beer with a specific and complex aromatic character.

2. Materials and methods

2.1. Strains, media and culture conditions

Two *S. cerevisiae* strains, SDy01 and SDy02, isolated from sourdoughs in our laboratory, and two commercial brewing yeast, LalBrew Voss™, a traditional kveik Norwegian farmhouse yeast, and Lalbrew BRY-97, an American West Coast Ale Yeast, both from Lallemand Brewing (Montreal, Canada), were used throughout this work.

Previously described standard methods were followed for media preparation (Guthrie and Fink, 1991). Yeast cells were propagated from cryo-cultures in solid YPD at 30°C. Ale malt wort with an extract content of 10º Plato (10ºP) was prepared by dissolving 131.8 g of hop-less Pilsen Light liquid malt extract, LME (Briess Malt & Ingredients Co., Chilton, WI) in distilled water to a total volume of 1 L. The suspension was centrifuged at 3,000 rpm for 10 min/4ºC, and the clarified wort was sterilized by autoclaving at 110ºC for 10 min (Thesseling *et al*., 2019). Finally, the wort obtained was chilled and stored at 4ºC until use.

2.2. Growth kinetics

Yeast strains were grown overnight in wort medium at 25-30ºC and 180 rpm in biological triplicates. Then, aliquots of cultures were inoculated into a final volume of 200 µl in 96-well microplates (initial OD_{600} ~ 0.1). At least 4 technical replicates of each culture were analysed. Specific wells were run without inoculation for background correction. Plates were then sealed and incubated at 20 or 37ºC under static conditions using a SPECTROstar analyser

(OMEGA) inside an incubator (EQUITEC) with control of temperature and humidity (70%). Growth was automatically recorded by measuring OD_{600} at 2 h-intervals during a 48 h period with vigorous shaking before each read. The observed blank in each run was subtracted from the measured OD_{600} values, and the resulting data (observed OD_{600}; OD_{obs}) were then converted to corrected OD_{600} (OD_{cor}) according to the formula $OD_{cor} = OD_{obs} + 0.449(OD_{obs})^2 + 0.191(OD_{obs})^3$ (Warringer and Blomberg, 2003). Relevant growth parameters, the specific growth rate constant μ_{max} (the slope of the linear portion), the generation time g (equals to 0.301/slope), the division rate ν (equals to $1/g$) and the lag time λ (the interception between the slope and a straight line corresponding to the initial OD_{cor}) were calculated from the curve of the logarithmic transformed OD_{cor} data represented as a function of time. The maximal OD_{600} (OD_{max}) was calculated as the mean of the OD_{cor} measurements corresponding to the six last time points (Warringer and Blomberg, 2003). The data presented represent the average ± standard deviation (sd) of at least three curves obtained from independent cultures.

2.3. Small lab-scale fermentations

Yeast strains were inoculated from YPD slants in wort and cultivated overnight in an orbital shaker at 180 rpm agitation and 25 or 30ºC for low (20ºC) and high (37ºC) temperature fermentations, respectively. Cultures were carried out in 100-ml bottles containing 60 ml wort inoculated with 0.3 units of OD_{600} (~3 x 10^6 cells/ml), without shaking at 20ºC or 37ºC. Polypropylene screw caps with gas permeable 0.2 μm PTFE membranes (Fisher Scientific) were used to ensure aeration and pressure equalisation. Fermentation progress was tracked by monitoring weight loss using a balance and was considered to be finished after 72 and 96 h for fermentation temperatures of 37 and 20ºC, respectively. After the indicated periods, the experimental beers were maturated at 4ºC for 3 days.

Sterile wort controls and samples taken throughout and at the end of the experiments were stored at -80ºC for further characterization. At least three biological replicates were conducted for each strain and condition.

2.4. Metabolite target analysis

Determination of main fermentation and wort metabolites, maltotriose, maltose, glucose, fructose, glycerol, ethanol and acetic acid was carried out by High Performance Liquid Chromatography (HPLC), using a chromatograph (Waters, Milford, MA) equipped with a quaternary pump, micro vacuum degasser, and refraction index and variable wavelength (PDA) detectors. Samples were filtered through 0.45 µm hydrophilic-PTFE filters and 20 µl were injected into an Ion Exclusion 6 µm column (8 x 300 mm; SH-1011, Shodex) with a guard column SH-G. Chromatographic separation was conducted on isocratic conditions with a 0.5 ml/min flow rate of 1.5 mM H_2SO_4 at 50ºC. Run time, including separation and column washing, was 40 min. Triplicate samples were analysed.

2.5. Volatile organic compounds analysis

The volatile organic compounds (VOC) were analysed according to Wang *et al*. (2020) with some modifications. For each analysis, 1 ml of maturated beer, previously clarified by centrifugation and filtration, was placed into a 20 ml headspace vial with 15 µl of internal standard solution (2-heptanone, Sigma) at 42 µg/ml. A 95 µm Carboxen/Polydimethylsiloxane (CAR/PDMS) fiber (Agilent Technologies, Santa Clara, CA, USA) was used to extract VOCs from wort. Chromatographic separation, compound detection and identification were carried out as described in Sánchez-Adriá *et al*. (2023a). Quantification was performed using external standards representative of different chemical families, hexanol, phenylethyl alcohol and 2,3-pentanodione. Calibration curves were

constructed with at least six points ($R^2 \geq 0.999$). In all cases, values are expressed as mg/L of wort and represent the mean ± sd of at least three independent experiments.

2.6. Statistical analysis

Sample averages were compared by a student's t-test with the Excel software (Microsoft). The samples denoted with * or # were significantly different ($p<0.05$). The comparison of more than two sets of samples was analysed by One-way ANOVA in XlStat 2019 and values with different letter in the same row indicate that there are significant differences between samples ($p<0.05$) according to Tukey's test. Normalization of VOC data, heatmap, correlation matrix and principal component analysis were performed using MetaboAnalyst (Xia *et al*., 2009) (https://www.metaboanalyst.ca/).

3. Results and Discussion

3.1. Growth parameters

One of the key features that determine the selection of yeast strains for brewing is their ability to proliferate under the array of stress conditions imposed during the fermentation process. In particular, growth kinetics are relevant as small growth delays after inoculation or reduced growth rate can compromise a healthy and efficient fermentation, the flavour and quality of the final product and the economy of the process. Therefore, we first examined the growth and thermal tolerance of two *S. cerevisiae* strains, SDy01 and SDy02, isolated from sourdough in our laboratory. Cells were grown at 20 and 37ºC in microplates containing ale malt wort with 10º Plato (10ºP) prepared by diluting a hop-less Pilsen-style malt barley extract. As control, we used two commercial brewing yeast strains typically used for conventional and warm pitching, BRY-97 and

Voss™. The optimal temperature range for BRY-97 is 15-22ºC, while maximum growth for Voss varies between 35-40ºC.

Table 6 shows the main kinetic parameters of the strains under study. As can be seen, the two sourdough strains showed similar behaviour in worth at 20 or 37ºC. At low temperature, both displayed the highest growth rate and the lowest lag phase as compared with the commercial BRY-97 strain. The cell mass yield, estimated by the OD_{max}, was also significantly higher for SDy01 and SDy02 (**Table 6**).

Table 6. Kinetic parameters of yeast strains cultured in brewing wort*.

	Temperature					
	20°C			37°C		
Parameter[1]	BRY-97	SDy01	SDy02	VOSS	SDy01	SDy02
λ (h)	5.93±0.57^a	2.86±1.20^b	1.04±0.81^b	3.65±0.51^a	2.63±0.09^b	2.31±0.19^b
μ_{max} (h^{-1})	0.068±0.004^a	0.082±0.004^b	0.080±0.005^b	0.261±0.019^a	0.262±0.013^a	0.266±0.014^a
g (h)	4.56±0.34^a	3.70±0.20^b	3.79±0.24^b	1.18±0.08^a	1.16±0.06^a	1.14±0.06^a
OD_{max}	4.89±0.10^a	5.38±0.16^b	5.47±0.20^b	4.44±0.54^a	5.09±0.24^a	5.94±0.50^b

[1] Relevant growth parameters, the specific growth rate constant (μ_{max}), the generation time (g), the lag time (λ) and the maximal OD_{600} (OD_{max}) were calculated for the sourdough yeast strains SDy01 and SDy02, and the commercial brewing yeast BRY-97 and VOSS, as described in the Materials and Methods section.
***** values are means ± sd of at least three replicates (n = 3). Values within a row and temperature with different superscript letters are significantly different ($p< 0.05$).

Regarding the growth at 37ºC, the sourdough strains behave like the control yeast VOSS, except that again, they exhibited a significantly shorter lag phase (**Table 6**). Increased specific growth rates and relatively lower lag phases than representative beer strains have been previously reported for some baker's yeast in fermentations carried out in hopped wort mashed at 20ºC (Marongiu *et al*., 2015). We conclude that the use of sourdough strains in beer production has the potential to provide reduced fermentation times and wider thermal tolerance.

3.2. Fermentative activity

We investigated the fermentative activity of the strains analysed and their capacity to transform wort into a final product with the main beer features. Fermentations at laboratory scale were carried out according to Thesseling *et al*. (2019) and tracked by monitoring weight loss, which allows for a gross evaluation of the CO_2 released by yeast. **Figure 16** shows the profile of weight loss accumulation for wort fermented at 37 and 20ºC for 72 and 96 h, respectively. The final weight loss at the end of the storage period at 4ºC was also recorded.

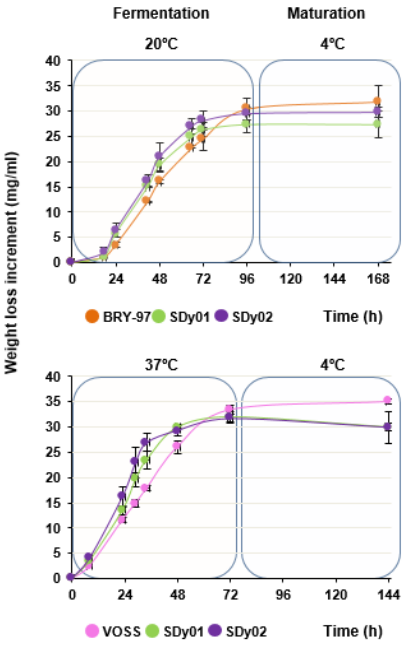


Figure 16. Estimation of the fermentative activity on wort of sourdough yeast strains. The graphs show the kinetics of weight loss accumulation for wort fermented at 37 and 20ºC for 72 and 96 h, respectively, by the sourdough strains SDy01 and SDy02. Results for the commercial strains VOSS and BRY-97, which were used as reference of high and low temperature fermentations, as well as the final weight loss at the end of the storage period at 4ºC for 96 h, are also shown. Data represent the average ± standard deviation (sd) of at least three curves obtained from independent cultures.

As can be seen, the sourdough strains, and in particular SDy02, displayed the fastest weight loss rate as compared with the control commercial strains (**Figure 16**). Differences were especially pronounced at 37ºC, with slope values at the straight-line portion of the curve of 0.579±0.034, 0.641±0.015 and 0.788±0.071* mg/ml·h for VOSS, SDy01 and SDy02 (* p<0.05 respect to the

control). At 20ºC, SDy02 showed again the highest slope 0.580±0.011* mg/ml·h (* $p<0.05$ concerning the control) followed by SDy01 and BRY-97, with 0.562±0.028 and 0.516±0.022, respectively. Finally, there were no major differences in weight loss accumulation at the end of de storage period at 4ºC. The results are consistent with the kinetic parameters shown above, in which the sourdough strains displayed increased μmax and/or shorter λ (**Table 6**).

3.3. Biochemical profile

Then, we examined whether the above results were caused by differences in the rate of sugar consumption through the fermentation pathway. For this, the concentration in wort of glucose, fructose, maltose, maltotriose, ethanol, glycerol and acetic acid was analysed before the end of the fermentation (**Figure 17**). Wort samples at 48 and 72 h after the onset of the fermentation at 37 and 20ºC were taken and analysed by HPLC. Likewise, aliquots of stored samples at 4ºC were assayed as representative of the final product (**Figure 17**). As expected, glucose and fructose were not detected at the time of the analysis in any of the strains under study. The hexoses, which together represented around 15% of total sugars in the wort utilized, are the preferred substrate of *S. cerevisiae* (Gancedo, 1998). In addition, the two sourdough strains achieved the full consumption of the initial maltose content regardless of the fermentation temperature, while 32% and 23% of residual maltose were still present in wort fermented by BRY-97 or VOSS at 20 and 37ºC, respectively (**Figure 17**). The commercial strains also consumed maltotriose, although the extent of its utilization varied among them, being higher for BRY-97 at 20ºC than for VOSS at 37ºC (**Figure 17**), a result that could be related to the presence of specific transmembrane transporters and the strong temperature dependence of sugar transport (Magalhães *et al*., 2016). Unlike this, SDy01 and SDy02 exhibited a low ability to utilize the trisaccharide, with no more than 8% of the initial content

(**Figure 17**). Maltotriose utilization has been considered a trait shared between bakery and brewing strains acquired during interaction with humans (Gallone *et al*., 2018), although their uptake varies largely between strains (Catallo *et al*., 2020). Traditionally, the phenotype maltotriose positive has been considered beneficial as its residual concentration in beer correlates inversely with alcohol yield (Catallo *et al*., 2020; Nikulin *et al*., 2020; Hutzler *et al*., 2021). Nevertheless, the consumer demand for low-alcohol and non-alcohol beers is constantly increasing (Research and markets, 2023). In addition, maltotriose-negative strains produce beer with lower attenuation leaving behind residual sweetness, body, and mouthfeel (Dillon and Sanderson, 2021).

The activity of the fermentation pathway was further evaluated by inspecting the by-product content of wort. At both, 20 and 37ºC the sourdough strains produced a slightly higher amount of ethanol than their corresponding reference strains at 72 and 48 h, respectively, but differences were not statistically significant (**Figure 17**). Interestingly, wort fermented with SDy01 and SDy02 also contained 66 and 76% higher glycerol concentration than BRY-97 ($p<0.05$) at 20ºC, and 52 and 46% concerning VOSS ($p<0.05$) at 37ºC (**Figure 17**), confirming thus the highest fermentative activity of the sourdough strains in consonance with their fastest consumption of maltose and CO_2 release. Glycerol adds body fullness and enhances the intensity of flavour (Zhao *et al*., 2015). Like the polyol, acetic acid content was again higher in wort fermented with sourdough strains at 20ºC, 0.6-0,7 g/L ($p<0.05$) as compared with that prepared with BRY-97, around 0.2 g/L. However, values were similar, 0.5-0.7 g/L in samples elaborated at 37ºC with VOSS or sourdough strains (**Figure 17**).

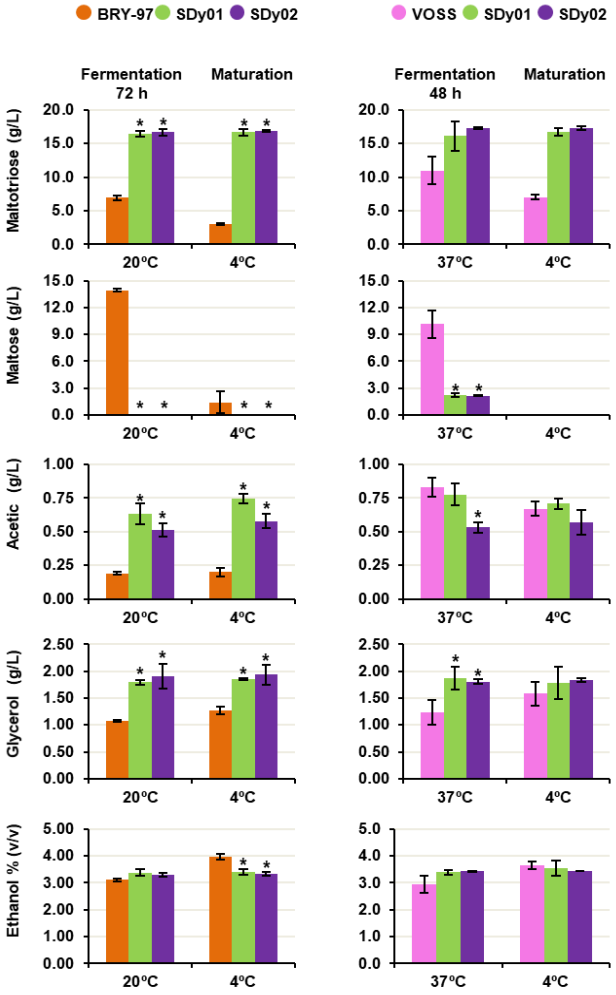


Figure 17. Comparison of main fermentation and wort metabolites between brewering and sourdough yeast. The content of maltotriose, maltose, glucose, fructose, glycerol, ethanol and acetic acid of wort after 48 h (37ºC) or 72 h (20ºC) of fermentation by the sourdough strains SDy01 and SDy02, and the commercial strains VOSS and BRY-97, which were used as reference of high and low temperature fermentations is shown. Results after the 96 h-maturation period at 4ºC are also shown. The concentration of each analyte was carried out by HPLC using standard calibration curves. Data represent the average ± standard deviation (sd) of at least three biological samples conducted for each strain and condition. SDy01 and SDy02 samples denoted with * were significantly different (p<0.05) to their corresponding reference strain at 20 or 37ºC.

Finally, we examined the biochemical composition of wort after the storage at 4ºC. During this step, the commercial strains depleted maltose and

reduced the content of maltotriose, with a corresponding increase in the level of glycerol and ethanol, while there was hardly change in the metabolite profile of wort fermented with SDy01 and SDy02 (**Figure 17**). As a result, the final content of ethanol and other by-products in wort fermented at 37ºC was similar between VOSS and the sourdough strains. However, at 20ºC, the alcohol level achieved by BRY-97 was a little higher, while remaining lower the acetic and glycerol content (**Figure 17**). Again, the results stress the potential of the sourdough strains to reducing fermentation time at both temperatures tested, without affecting or even improving the main beer's biochemical features.

3.4. VOCs

Samples of maturated wort driven by the strains under study were analysed by GC-MS to determine the influence of fermentation temperature and strain in the volatile profile. In total, 30 compounds, 13 esters, 7 alcohols, 4 carboxyls, 2 carbonyls and 4 belonging to other families were identified and quantified (**Table 7**). As is shown, the use of sourdough strains had a great influence on the aromatic profile of wort fermented at 20ºC. Remarkably, SDy01 and SDy02 increased the total amount of VOCs by 1.7 and 1.8 times as compared with the reference BRY-97 strain, respectively. The increase was extended to all functional groups, but the change was not significant for alcohols and carbonyls in SDy01 samples. As a result, esters, which confer a fruity-flowery aroma to beer (Branyik *et al*., 2008), were the dominant chemical group in wort fermented with both sourdough strains, ranging 50-44% of the total (**Table 7**).

Interestingly enough, these VOCs are highly flavour-active compounds in beer (Olaniran *et al*., 2017) and variations in their concentration and proportion contribute to its specific 'bouquet' (Vera *et al*., 2011). Differences were notable for the most abundant molecular species, acetate esters like 2-phenyl ethyl acetate (roses, honey) and 1-butanol-3-methyl acetate (banana and

pear), and fatty acid esters, between them, ethyl octanoate (sour apple), ethyl decanoate (brandy, grape, pear) and ethyl 9-decenoate (fruity, fatty). Unlike this, alcohols predominated, as could be expected, in the BRY-97 wort, 69% (**Table 7**). Higher alcohols account for the highest absolute concentrations in wort fermented with most brewing yeast, ale or lager (Olaniran *et al*., 2017), and enhance the flavour of beer by adding alcoholic perception and warm mouthfeel (He *et al*., 2014). Phenyl ethanol (rose, floral) and 3-methyl-1-butanol (fruity, sweet) were the most abundant alcohol species in all samples and their content didn´t differ too much between strains. These are commonly found in beers and involved in the aroma profile of those fermented by *Saccharomyces* strains (Larroque *et al*., 2021). In contrast, minor higher alcohols, like 1-heptanol, were produced by BRY-97, while 1-octanol or 2-decen-1-ol were significantly more abundant in samples elaborated with SD strains (**Table 7**).

The higher level of esters in wort elaborated with sourdough yeast suggests that the synthesis of medium-chain fatty acids, which are the precursors and the limit factor for fatty acid esters formation (Saerens *et al*., 2008), is much higher in SDy01 and SDy02 than in the brewing yeast BRY-97 under these conditions. Consistent with this, the total amount of medium-chain fatty acids, including octanoic, 9-decenoic and decanoic acid, increased 9- and 12-times in SDy01 and SDy02, concerning the reference strain, respectively (**Table 7**). Regarding other compounds, the two SD strains displayed the "phenolic-off-flavour"-positive (POF+) status (Thurston and Tubb, 1981). Indeed, increased levels of styrene and 4-vinylguaiacol were found in wort samples fermented at 20ºC with SDy01 and SDy02 as compared with those elaborated with BRY-97, although the POF productivity was low (**Table 7**). These compounds are produced mainly by enzymatic decarboxylation during fermentation from the phenolic acids, cinnamic acid and ferulic acid, respectively (Gramatica *et al*., 1981; McMurrough *et al*., 1996; Schwarz *et al*., 2012). Their presence in

excessive concentrations, e.g. >4 mg/L, generate an undesirable clove-like aroma (Vanbeneden *et al*., 2008). Nonetheless, they are essential contributors to the aromatic profile of Belgian white beers, German Weizen beers and Rauch beers, among others, and at low concentrations they also play a role in the flavour perception of many other blond and dark ale-style beers (Vanbeneden *et al*., 2008).

The wort fermented with VOSS at 37ºC (**Table 7**) showed a similar profile of VOCs than BRY-97 (at 20ºC), with alcohols as the prominent chemical group followed by esters, but with a more complex composition characterized by the presence of lower content of ethyl hexanoate and higher levels of carboxyls and alkanes as tetradecane (**Table 7**). On the contrary, the total volatile content was drastically reduced (more than 50%) in wort fermented with SD strains at high temperatures (**Table 7**). The results were in some way unexpected, as an increase in temperature for lager- (12-15ºC) or ale-type (18-25ºC) fermentations, had been reported to increase the concentration of volatile compounds (Webersinke *et al*., 2018; Lasanta *et al*., 2021; Castro *et al*., 2022), and in particular of esters (Pires *et al*., 2014). Nevertheless, Aasen (2020) found a strong decrease in esters formation when beer brewed with Kveik yeasts was tested in a range of temperatures from 20 to37ºC. In line with this, fermentation at 37ºC also reduced the content of 4-vinylguaiacol to levels of the commercial VOSS strain, with values that were 9 and 23% of those observed at 20ºC for SDy01 and SDy02, respectively (**Table 7**). The result suggests that the regulation and/or activity of the phenylacrylic acid decarboxylase (Pad1) of sourdough yeast strains, which transform ferulic acid in 4-vinylguaiacol (Clausen *et al*., 1994) could be temperature-sensitive. Altogether, our results stress the ability of sourdough strains to provide aroma diversity in a wide range of temperatures.

Table 7. Concentration of VOCs in maturated worts prepared with the selected strains*.

	Temperature					
	20°C			37°C		
Compound (mg/L)	**BRY-97**	**SDy01**	**SDy02**	**VOSS**	**SDy01**	**SDy02**
Ethyl acetate	4.9±0.2^{a}	11.1±1.3^{b}	4.9±0.6^{a}	10.0±1.5^{a}	6.2±1.4$^{b\#}$	6.52±1.08^{b}
3-Methylbutyl acetate	14.9±0.3^{a}	52±6^{b}	38±2^{c}	12.9±1.7^{a}	15±2$^{a\#}$	15±3$^{a\#}$
2-Methylbutyl acetate	1.57±0.12^{a}	8.3±1.6^{b}	8.6±0.6^{b}	1.9±0.4^{a}	3.1±0.4$^{ab\#}$	4.34±0.97$^{b\#}$
2-Phenylethyl acetate	7.2±1.7^{a}	47±7^{b}	43±8^{b}	7±2^{a}	13±3$^{b\#}$	16.7±1.8$^{b\#}$
Octyl acetate	0±0^{a}	1.0±0.3^{b}	0.27±0.03^{a}	0±0^{a}	0±0$^{a\#}$	0±0$^{a\#}$
TOTAL acetate esters	28.6±1.9^{a}	119±4^{b}	94±8^{c}	32±4^{a}	36.9±1.7$^{a\#}$	43±7$^{a\#}$
Ethyl butanoate	0.33±0.04^{a}	0±0^{b}	0±0^{b}	0±0^{a}	0±0^{a}	0±0^{a}
Ethyl hexanoate	31±3^{a}	37.8±1.3^{a}	33±5^{a}	15±2^{a}	4.36±0.09$^{b\#}$	11±3$^{a\#}$
Ethyl octanoate	27±5^{a}	60±7^{b}	64.3±1.6^{b}	24±5^{a}	9±2$^{b\#}$	21±4$^{a\#}$
Ethyl 9-decenoate	1.3±0.2^{a}	22±2^{b}	34.9±1.9^{c}	2.87±1.13^{a}	2.8±0.8$^{a\#}$	8.9±1.6$^{b\#}$
Ethyl decanoate	1.0±0.4^{a}	9.8±1.7^{b}	14±3^{b}	1.5±0.9^{a}	1.4±0.4$^{a\#}$	16±2^{b}
2-Hydroxyethyl oleate	0±0^{a}	0±0^{a}	0.55±0.09^{b}	0±0^{a}	0±0^{a}	0.14±0.04$^{b\#}$
Ethyl dodecanoate	0±0^{a}	0.8±0.2^{a}	4.2±0.7^{b}	0±0^{a}	0±0$^{a\#}$	7.2±0.9$^{b\#}$
TOTAL ethyl esters	60±8^{a}	131±9^{b}	151±3^{c}	44±9^{a}	18±4$^{b\#}$	64±7$^{c\#}$
2-Methyl-1-propanol	4.8±0.2^{a}	3.2±0.5^{b}	5.4±0.8^{a}	7.0±0.8^{a}	5.7±0.9$^{a\#}$	7.2±0.3$^{a\#}$
3-Methyl-1-butanol	64.6±1.4^{a}	68.1±1.2^{a}	70±12^{a}	62±6^{a}	44.4±1.7$^{b\#}$	57±9^{b}
2-Methyl-1-butanol	21.4±0.8^{a}	26.7±0.4^{a}	35±4^{b}	27±3^{a}	23.6±0.9$^{a\#}$	32.3±0.3^{a}
1-Heptanol	1.3±0.2^{a}	0±0^{b}	1.8±0.4^{a}	0±0^{a}	0±0^{a}	0±0$^{b\#}$
1-Octanol	0±0^{a}	1.2±0.3^{b}	0.7±0.3^{b}	0±0^{a}	0±0$^{a\#}$	0±0$^{b\#}$
Phenylethyl alcohol	113.4±12.3^{a}	123±18^{ab}	164±26^{b}	93±15^{a}	76±14$^{a\#}$	102±7$^{a\#}$
2-Decen-1-ol	0±0^{a}	0.33±0.06^{b}	0±0^{a}	0±0^{a}	0±0$^{a\#}$	0±0^{a}
TOTAL alcohols	205±11^{a}	222±19^{ab}	277±41^{b}	189±19^{ab}	151±15$^{b\#}$	198±16$^{a\#}$

Continuation Table 7

	Temperature					
	20°C			37°C		
Compound (mg/L)	**BRY-97**	**SDy01**	**SDy02**	**VOSS**	**SDy01**	**SDy02**
Hexanoic acid	0.89 ± 0.12^{a}	1.03 ± 0.07^{a}	0.6 ± 0.2^{b}	0.5 ± 0.3^{a}	$0\pm0^{b\#}$	0 ± 0^{b}
Octanoic acid	1.5 ± 0.3^{a}	12.3 ± 0.5^{b}	12.5 ± 0.9^{b}	4.5 ± 1.6^{a}	$1.9\pm0.3^{b\#}$	$6.0\pm0.3^{a\#}$
9-Decenoic acid	0.148 ± 0.013^{a}	3.0 ± 0.4^{b}	5.6 ± 0.3^{c}	0.3 ± 0.2^{a}	$0.37\pm0.05^{a\#}$	$1.5\pm0.2^{b\#}$
n-Decanoic acid	0.23 ± 0.03^{a}	2.11 ± 0.16^{b}	5.1 ± 0.7^{c}	0.7 ± 0.3^{a}	$0.7\pm0.3^{a\#}$	4.5 ± 0.5^{b}
TOTAL carboxyls	2.8 ± 0.3^{a}	18.42 ± 1.10^{b}	23.7 ± 1.4^{c}	6 ± 2^{a}	$2.9\pm0.6^{a\#}$	$12.30\pm1.03^{b\#}$
Nonanal	0.39 ± 0.07^{a}	0.51 ± 0.13^{a}	0.43 ± 0.09^{a}	0.478 ± 0.002^{a}	0.51 ± 0.03^{a}	$0.81\pm0.12^{b\#}$
Isopentyl-2,4,4-trimethyl-2-cyclohexen-1-one	0.116 ± 0.006^{a}	0.20 ± 0.02^{b}	0.19 ± 0.03^{b}	0.46 ± 0.07^{a}	$0\pm0^{b\#}$	$0.7\pm0.2^{a\#}$
2,2,4-Trimethyl-1,3-pentanediol diisobutyrate	0.41 ± 0.05^{a}	0.287 ± 0.012^{b}	1.52 ± 0.05^{c}	0.7 ± 0.3^{a}	0.27 ± 0.15^{ab}	$0\pm0^{b\#}$
TOTAL carbonyls	0.92 ± 0.11^{a}	1.00 ± 0.11^{a}	2.25 ± 0.15^{b}	1.7 ± 0.4^{a}	0.78 ± 0.17^{b}	$1.5\pm0.3^{ab\#}$
Styrene	0.117 ± 0.002^{a}	0.40 ± 0.12^{b}	0.48 ± 0.10^{b}	1.151 ± 0.009^{a}	0.59 ± 0.08^{b}	$0.26\pm0.05^{c\#}$
p-Vinylguaiacol	0.153 ± 0.006^{a}	2.0 ± 0.6^{b}	2.3 ± 0.3^{b}	0.17 ± 0.05^{a}	$0.17\pm0.03^{a\#}$	$0.33\pm0.02^{b\#}$
Tetradecane	0 ± 0^{a}	0.6 ± 0.2^{b}	0.80 ± 0.11^{b}	0.9 ± 0.2^{a}	$0.84\pm0.06^{a\#}$	0.92 ± 0.12^{a}
(E)-β-Farnesene	0.143 ± 0.013^{a}	0.24 ± 0.06^{a}	0.19 ± 0.02^{a}	0.31 ± 0.08^{a}	0.25 ± 0.03^{a}	$0.37\pm0.03^{a\#}$
TOTAL others	0.41 ± 0.02^{a}	3.2 ± 0.7^{b}	3.8 ± 0.2^{b}	2.6 ± 0.2^{a}	$1.85\pm0.16^{b\#}$	$1.9\pm0.2^{b\#}$
TOTAL VOCs	298 ± 20^{a}	495 ± 31^{b}	551 ± 51^{b}	275 ± 34^{ab}	$211\pm18^{b\#}$	$320\pm31^{a\#}$

***** values are means ± sd of at least three independent replicates (n = 3). Values at a given temperature and within a row showing different superscript letters are significantly different ($p<0.05$). **#** indicates a significant difference between VOCs values at 20 or 37ºC for SDy01 or SDy02 ($p<0.05$).

37ºC. In line with this, fermentation at 37ºC also reduced the content of 4-vinylguaiacol to levels of the commercial VOSS strain, with values that were 9 and 23% of those observed at 20ºC for SDy01 and SDy02, respectively (**Table 7**). The result suggests that the regulation and/or activity of the phenylacrylic acid decarboxylase (Pad1) of sourdough yeast strains, which transform ferulic acid in 4-vinylguaiacol (Clausen *et al*., 1994) could be temperature-sensitive. Altogether, our results stress the ability of sourdough strains to provide aroma diversity in a wide range of temperatures.

Principal component analysis (PCA), a heat map and a correlation matrix were conducted to assess the differences in flavour production among strains and fermentation temperature. PCA results showed that the first two components accounted for 68.7% of the total variance (**Figure 18A**). PC1 (45.7%) reflected mainly the high content of acetate and ethyl esters detected in samples fermented with SD strains, while PC2 (23%) explained the temperature variation. As the heatmap shows (**Figure 18B**), the hierarchical clustering grouped the wort samples fermented with SD strains at 20°C (cluster 1) separated from the rest (cluster 2), mainly by their higher content in 14 compounds, (lower part of the heatmap, **Figure 18B**), that represented around 37.5% of the total content of VOCs, versus 12.5-26.4% in samples from cluster 2. On the other hand, BRY-97 samples were placed in the negative values of PC1 (**Figure 18A**), due to the production of ethyl butanoate as exclusive VOC produced by this yeast, as reflected clearly in the correlation matrix (**Figure S7**). Finally, SDy02 samples were mainly placed in the positive values of PC1 (**Figure 18A**) due to their higher amount of 2-methylbuthyl acetate, 2-phenylethyl acetate, octanoic acid and ethyl-9-decenoate (**Figure 18B** and **Figure S7**).

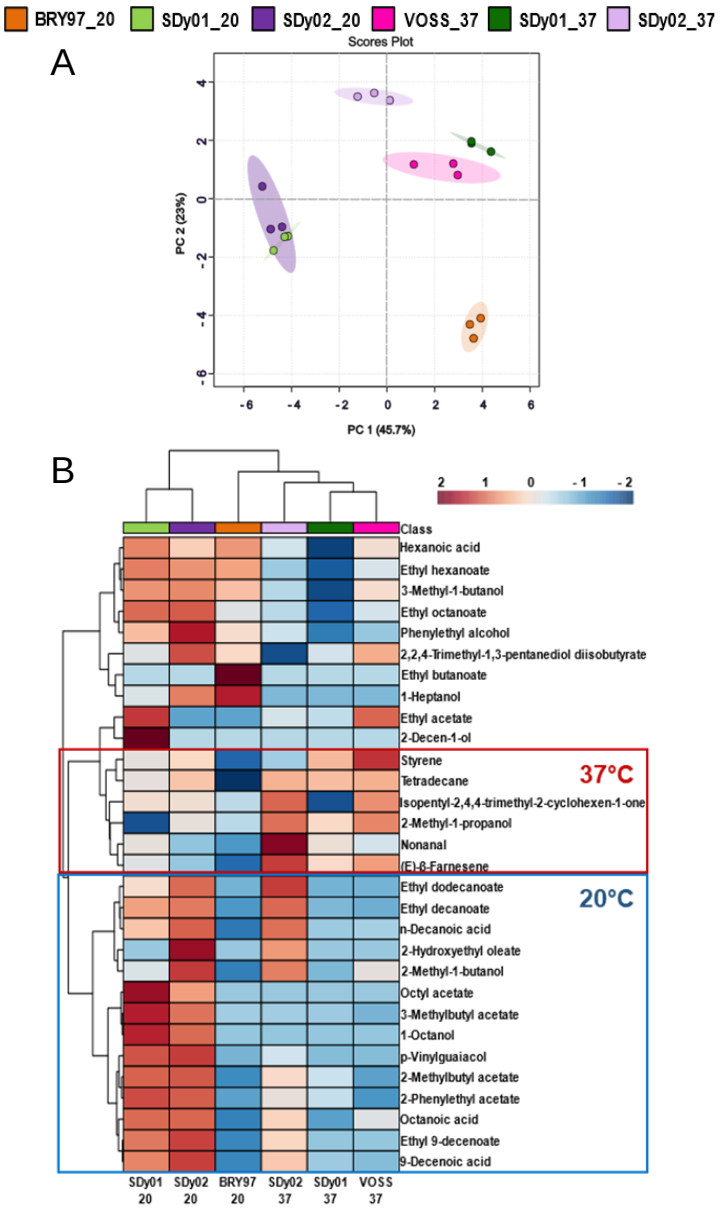


Figure 18. Principal component analysis (PCA) and heat map of VOCs from maturated wort. (A) PCA of volatile compounds in maturated wort fermented by sourdough strains SDy01 and SDy02, and commercial reference strains, BRY-97 and VOSS, at low (20°C; _20) and/or high (37°C; _37) temperature. **(B)** Heatmap of VOCs in maturated wort. Columns represent the different samples fermented at 20°C or 37°C, and the rows, the relative abundance and distribution of the 30 VOCs identified. Colors correspond with relative abundance values, where dark red indicates high levels, and dark blue indicates low levels. At least three biological replicates were analyzed.

4. Conclusions

The present work has provided evidence of the potential of *S. cerevisiae* yeast strains from sourdough environment to reduce brewing fermentation times at a wide range of temperatures with no apparent detriment to the main beer's biochemical features. The improved fermentation kinetics and increased thermal tolerance of sourdough strains provides microbiological stability to the process and brewer flexibility in allowing their use in typical ale-beer styles as well as in warmer-temperature brewing processes like Kvass and Finland's sahti, among others. Results also demonstrate the ability of sourdough strains to provide the highest overall level of aroma compounds at 20ºC, in particular of esters and acids, and a specific temperature-driven flavour profile. This is also a desirable characteristic as allows targeting beer fermentation for tailored aroma impact.

Our results have examined the potential for brewing of sourdough yeast isolates under lab-scale conditions. Considering the number of ingredients, wort preparation and fermentation conditions used in industrial and craft brewing, additional experiments are still needed to assess the practical applicability of these sourdough strains at a brewery scale. The sourdough strains analysed could lack some brewing-related features and would need to be improved before their introduction as starters at the industrial beer production scale. Previous work in our lab has demonstrated the ability of sourdough baker's yeast isolates to evolve by adaptive laboratory evolution approaches and acquire phenotypes like increased robustness and stress tolerance (Sánchez-Adriá *et al*., 2023b), which fits well with non-GMOs preferences in food biotechnology. Additionally, the remarkable and unique yeast strain diversity that sourdough offers can be further utilized to choose the best novel strains to meet the needs of the craft beer market and consumers seeking out new, distinctive flavours and experiences in beer.

Supplementary material

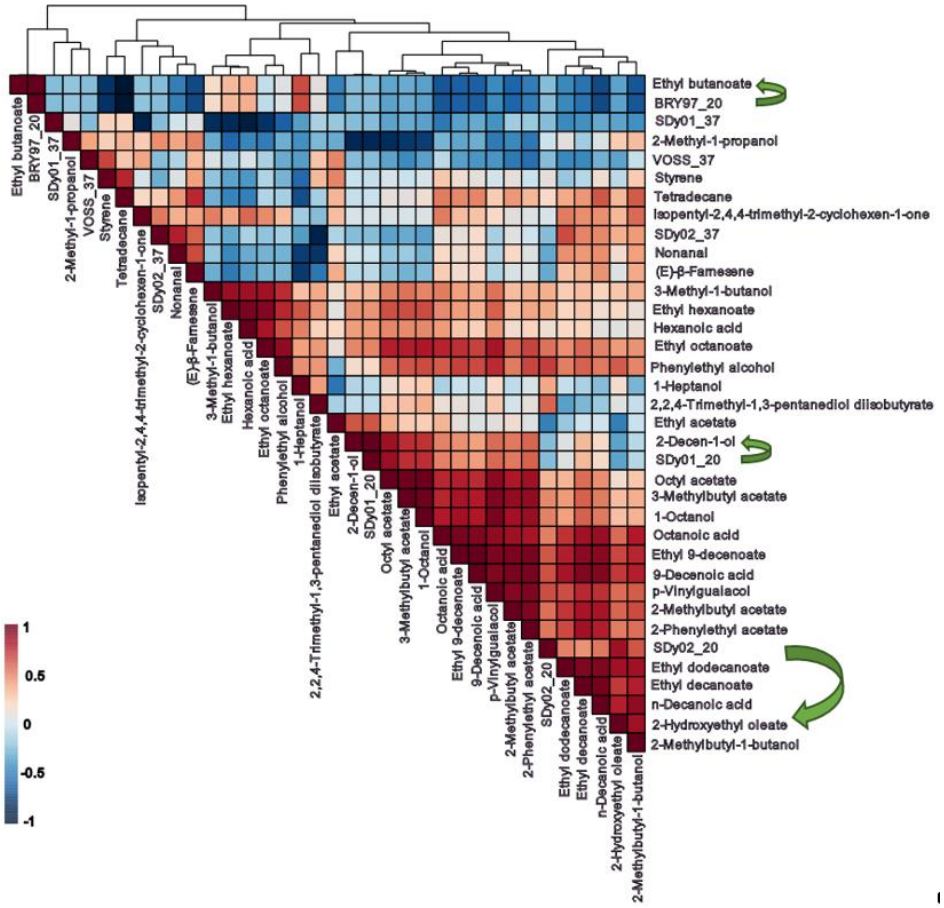
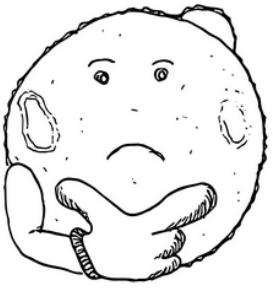


Figure S7. Correlation plot of VOCs from maturated wort. Pearson's correlation, between pairs of yeast strains and 30 identified volatile compounds in maturated wort samples fermented by sourdough strains SDy01 and SDy02, and commercial reference strains, BRY-97 and VOSS, at low (20°C; _20) and/or high (37°C; _37) temperature. The Pearson correlation index is indicated at the scale bar, denoting the nature of the correlation with 1 for perfect positive correlation (dark red) and -1 for perfect negative correlation (dark blue). Exclusive VOCs produced by a concrete strain are denoted by a green arrow.

Discusión general



"Sea el alimento tu medicina y la medicina tu alimento"

Hipócrates

La producción de panes con MM ha experimentado una tendencia al alza dentro del mercado de productos de panadería tras el confinamiento durante la pandemia. Encajan perfectamente con los conceptos de innovación y artesanía del sector panadero, así como con su política de sostenibilidad y eliminación de aditivos en sus formulaciones. Esto, unido a que los consumidores perciben el método de elaboración de pan con MM como natural y saludable, hace de estos productos una clara apuesta, tanto para las panaderías tradicionales, como para las industriales. En este escenario, destacar la demanda creciente de una gama más amplia de MMs, por ejemplo, sin gluten o integrales, entre otras, y de productos innovadores basados en el empleo de MM (Ramos *et al*., 2021). Una estrategia para cubrir dicha demanda es el aislamiento de levaduras endógenas de la MM y su caracterización en base a propiedades como elevada actividad fermentativa, resistencia a estrés o capacidad para potenciar el aroma y el sabor de estos productos. Las cepas que cumplen estos requisitos son claros candidatos a formar parte de cultivos iniciadores.

En el marco de la colaboración con la empresa Europastry, se han puesto a nuestra disposición, durante más de dos años, muestras de seis MM elaboradas en dos plantas industriales, Paterna (P) y Sant Joan Despí (SJ). Se trata de MM tipo 1, es decir, iniciadas por fermentación espontánea después de la hidratación de una variedad específica de harina y refrescadas diariamente hasta que alcanzan la madurez (6-7 refrescos a 28ºC cada 24h), tras lo que se escalan a nivel industrial. A partir de ese momento, se refrescan y mantienen siguiendo parámetros similares. Nuestros resultados mostraron que las seis MM presentaban valores de pH semejantes (±0,2 unidades), aunque dos de ellas (P1 y P3), destacaron por su mayor contenido en ácidos orgánicos, concretamente ácido láctico. Este resultado podría explicarse por el uso de harinas integrales en su elaboración, ya que poseen mayor capacidad tamponante que las harinas refinadas (Taccari *et al*., 2016) y, por tanto, acumulan más cantidad de ácidos para alcanzar valores similares de acidez. Este efecto favorece la imposición de

levaduras sensibles al pH en las primeras etapas de maduración, como *K. humilis* (Carbonetto *et al*., 2020). En consonancia con esto, nuestros resultados mostraron que *K. humilis* predomina en las muestras de MM de Paterna, mientras que *S. cerevisiae* es la levadura mayoritaria en las de Sant Joan Despí. Sin embargo, no podemos obviar la influencia que otros factores, como la interacción con *F. sanfranciscensis*, la LAB que predomina en las seis MM analizadas, o el entorno de la planta industrial donde se producen, pueden tener en la composición (distribución/ presencia) de levaduras de estas masas.

La utilización de métodos de cultivo complementó la información obtenida mediante secuenciación masiva en la identificación de la microbiota de las MM analizadas y permitió aislar microorganismos poco abundantes en estas comunidades. Estos microorganismos satélite han suscitado recientemente el interés de la comunidad científica, ya que se consideran responsables de parte de los atributos sensoriales que la MM confiere a los productos de panadería y que los hacen únicos (Gänzel *et al*., 2023). En este sentido, la valiosa colección de 200 aislados (entre levaduras y bacterias), que hemos identificado y conservado, servirá para ampliar nuestro conocimiento acerca de los mecanismos que rigen las interacciones microbianas y de las que depende el establecimiento de una microbiota estable que contribuye al sabor del pan. De hecho, otro aspecto diferenciador de las seis MM analizadas es su perfil de compuestos volátiles. Así, las MM de Paterna destacan por la presencia mayoritaria de alcoholes (con notas herbáceas) en su perfil aromático, mientras que en las de Sant Joan cobran importancia los ésteres (aromas afrutados), lo que concuerda con la correlación establecida con anterioridad entre *K. humilis* y *S. cerevisiae* y la acumulación de iso-alcoholes o ésteres, respectivamente (Xu *et al*., 2019). Por último, el análisis de 45 aislados cultivados en medio de melaza permitió elaborar un pequeño catálogo de levaduras con información relativa a sus características fenotípicas y tecnológicas, lo que nos ayudó a identificar las cepas de *S. cerevisiae, K. humilis* y *T. delbrueckii* con mejor comportamiento y, por tanto, más adecuadas

para ser transferidas a la industria como cultivos iniciadores o utilizadas como parentales para la obtención de derivados con mayor tolerancia a estrés.

La aplicación de diferentes estrategias de adapatación evolutiva dirigidas a mejorar la tolerancia a ácido acético de dos aislados de masa madre (S06 y S13), rindió poblaciones heterogéneas que mostraron un alto poder fermentativo, tanto en condiciones control, como de estrés, de acuerdo con los resultados descritos anteriormente en cepas de laboratorio (González-Ramos *et al*., 2016). Como cabía esperar, la exposición a miriocina como motor evolutivo en alternancia con concentraciones crecientes de acético, aceleró la adquisición de tolerancia a este ácido. El resultado es consistente con trabajos anteriores que muestran que la exposición continua a este fármaco, un inhibidor de la síntesis de esfingolípidos (Wadsworth *et al*., 2013), induce la aparición de clones con mayor contenido en esfingolípidos (Randez-Gil *et al*., 2020). Los lípidos son componentes esenciales de las membranas y los principales determinantes de su funcionalidad como barreras celulares. Concretamente, los esfingolípidos complejos promueven, junto con los esteroles, la formación de una membrana plasmática gruesa y compacta (Lester *et al*., 2013), menos permeable a los ácidos orgánicos (Lindberg *et al*., 2013; Lindahl *et al*., 2016). Sin embargo, la adquisición de este fenotipo redujo, en mayor o menor medida, la tolerancia intrínseca a ácido láctico de las cepas parentales, en las poblaciones evolucionadas. Tradicionalmente, la inhibición microbiana por ácidos débiles se ha relacionado con su habilidad, común en todos ellos, para acidificar y reducir el pH intracelular (Palma *et al*., 2018; Peetermans *et al*., 2021). No obstante, existen evidencias de que la exposición a distintos ácidos genera en *S. cerevisiae* respuestas específicas, tanto a nivel fisiológico, como transcripcional (Narendranath *et al*., 2001a; Thomas *et al*., 2002; Lee *et al*., 2017; Fletcher *et al*., 2017); si bien, por lo que nosotros sabemos, esta es la primera vez que se describe un efecto negativo sobre la tolerancia a ácido láctico en células de levadura adaptadas a ácido acético.

El aislamiento y la caracterización fermentativa de clones individuales nos llevó a seleccionar un total de cuatro, dos de cada ancestro, que mostraron diferencias importantes en cuanto a su crecimiento en presencia de láctico y acético. Esto no nos resultó sorprendente, ya que estudios previos habían hecho hincapié en la importante heterogeneidad observada en la respuesta de cultivos genéticamente homogéneos de *S. cerevisiae*, expuestos a concentraciones elevadas de ácido acético (Swinnen *et al*., 2014; Franco-Duarte *et al*., 2015; Palma *et al*., 2018). En consecuencia, se llevó a cabo la secuenciación masiva y el análisis del nivel de ploidía de las cepas evolucionadas y parentales, con la idea de profundizar en los mecanismos de adaptación al estrés ácido. Cabe destacar que las cepas parentales de este trabajo son diploides, como la mayoría de las cepas de *S. cerevisiae* que han sido domesticadas en entornos generados por el hombre (Liti *et al*., 2009; Wang *et al*., 2012; Gallone *et al*., 2018). A diferencia de los aislados silvestres, que acumulan SNPs, las cepas domesticadas se caracterizan por alteraciones en el contenido de su genoma, tales como una alta ploidía, aneuploidía y variaciones en el número de copias de genes (Yue *et al*., 2017; Peter *et al*., 2018; De Chiara *et al*., 2022). En efecto, la secuenciación masiva reveló variaciones en el número de copias (CNVs) similares a las descritas para cepas comerciales de panadería y otros aislados de masa madre. En concreto, el aumento del número de copias de genes implicados en el metabolismo de la maltosa explica su excelente capacidad fermentativa en alimentos y bebidas a base de cereales (Bigey *et al*., 2021). Además, también se observaron cambios en el nivel de aneuploidía, lo que sugiere su implicación en los mecanismos de adaptación impuestos por la evolución experimental. A modo de ejemplo, los clones S06A/M_{13}, S13A_{14} y S13A/M_{14} perdieron una copia del cromosoma I y los dos primeros (que son más sensibles a ácido láctico) mostraron una copia adicional de los cromosomas XV o XII, respectivamente. Cambios en el número de copias de genes localizados en estos cromosomas, como *ISW2* (Chr. XV), implicado en la remodelación de la cromatina y la

regulación de la expresión génica (Donovan *et al*., 2021), o *ACE2* (Chr. XII), un activador transcripcional implicado en la tolerancia al ácido láctico y ácidos dicarboxílicos (Ratcliff *et al*., 2015; Fletcher *et al*., 2017), podrían explicar la variación fenotípica de estos clones en medios conteniendo ácido láctico.

Por otro lado, cabe destacar la identificación de SNPs exclusivos en las cepas evolucionadas. Algunos de ellos se localizan en genes previamente relacionados con la tolerancia a ácido acético, lo que da validez a las estrategias evolutivas ensayadas en este trabajo y subraya la similitud en la respuesta adaptativa entre cepas de *S. cerevisiae* de laboratorio y domesticadas. Entre estos genes, cabe destacar *IRA2,* que presenta, en el clon $S06A_{15}$, un codón de parada en la posición 772, en coincidencia con el encontrado por Stojiljkovic *et al*. (2020) en un derivado haploide tolerante a acético de una cepa aislada de mosto fermentado. Esta forma truncada de Ira2 podría reducir la actividad de *RAS* y, con ella, la de *PKA*, favoreciendo la respuesta general a estrés en estas levaduras (Estruch, 2000). También observamos que el clon $S13A/M_{14}$ contiene una mutación (N880S) en el gen *ASG1*, que codifica para un factor transcripcional que coordina la gluconeogénesis y el metabolismo lipídico. Las células que carecen de Asg1 muestran una mayor acumulación de triglicéridos (Jansuriyakul *et al*., 2016), lo que cabe esperar que altere la fluidez y la permeabilidad de la membrana. Además, en ambos clones, se encontró una inserción de un solo nucleótido en *GIS4,* que codifca para una proteína truncada en la posición 364, un cambio también observado por González-Ramos *et al*. (2016) en derivados tolerantes a acético de una cepa de laboratorio (CEN.PK113-7D). Gis4 es una proteína de membrana implicada en la homeostasis iónica a través de la proteína quinasa Snf1 (Ye *et al*., 2006), que regula la expresión de *ENA1*, la principal bomba de Na^+ en *S. cerevisiae,* y su deleción proporciona tolerancia a ácido acético, como fue demostrado por Sousa *et al*. (2013), y confirmado en nuestro estudio utilizando un mutante nulo de la colección Euroscarft. Por último, todas las cepas secuenciadas mostraron un único SNP en ambas copias de *CRZ1*, el

factor de transcripción que responde al estrés iónico vía calcineurina (Stathopoulos y Cyert, 1997), e implicado en la inducción de *ENA1* (Mendizabal *et al*., 2001). En nuestras manos, un mutante *crz1* mostró sensibilidad tanto a ácido acético como a láctico, un fenotipo compartido por *kex1*; mientras que otros mutantes nulos en genes como *SPT20*, *CTK1* o *ARG82*, en los que se encontraron SNPs en alguna o todas las cepas analizadas, solo mostraron sensibilidad a láctico. Investigaciones adicionales son necesarias para dilucidar el efecto concreto de estos SNPs sobre la funcionalidad de estos genes y su implicación en los fenotipos descritos en este estudio.

Es de gran interés también, la identificación de cambios en la secuencia de genes que no habían sido relacionados previamente con el estrés ácido. El hallazgo de que las dos copias de *PSP2*, que codifica una proteína que regula la autofagia (Yin *et al*., 2019), contienen el mismo SNP en las seis cepas estudiadas es destacable y podría explicar, junto con otros cambios genómicos, la alta tolerancia intrínseca de las cepas de masa madre analizadas. Además, *MSN5*, una exportina implicada en diversas rutas de señalización (Alepuz *et al*., 1999), aparece mutada en la cepa $S06A_{15}$. En consonancia con esto, descubrimos que la deleción de este gen confiere tolerancia a ácido acético en la cepa BY4741. La pérdida de protección frente a ácido láctico que acompaña a este fenotipo en el mutante *msn5* es curiosa y sugiere de nuevo que ambos ácidos desencadenan una respuesta distinta en la levadura. Además, en un trabajo reciente, Mormino *et al*. (2022) mostró que la regulación a la baja de *MSN5* mediante la tecnología CRISPRi, aumenta la sensibilidad a ácido acético. Es necesario aclarar esta discrepancia y estudiar más a fondo el papel dual de Msn5 en la respuesta a ácido acético y láctico.

En relación con el tercer capítulo de este trabajo de doctorado, hemos examinado algunas características relevantes en cervecería de dos cepas de *S. cerevisiae* aisladas de MM, tras compararlas con cepas cerveceras comerciales. Hemos centrado nuestra atención en parámetros tecnológicos como la velocidad

de fermentación y la temperatura, ya que han recibido poca o ninguna atención en trabajos anteriores, pero también se han analizado las principales características de calidad del mosto fermentado, incluido el perfil bioquímico y volátil. Las cepas de masa madre SDy01 y SDy02, mostraron mayor tasa de crecimiento específico y menor tiempo de latencia que las cepas de cerveza de referencia, BRY-97 y VOSS, a 20 y 37ºC, respectivamente, lo que confirma resultados anteriores de Marongiu *et al*. (2015) para algunas levaduras de panadería en fermentaciones llevadas a cabo en mosto macerado con lúpulo a 20ºC. Los resultados son relevantes, ya que pequeños retrasos en el crecimiento tras la inoculación o una tasa de crecimiento reducida pueden dar lugar a contaminación microbiológica y comprometer la calidad del producto final. Además, nuestro trabajo pone de manifiesto la amplia tolerancia térmica de las cepas de masa madre. La fermentación a temperaturas altas acorta el proceso de elaboración y ahorra costes de refrigeración.

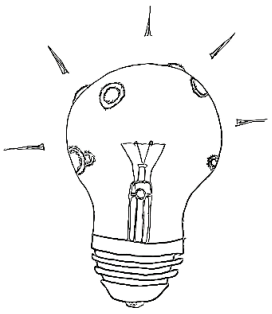
Como era de esperar, las cepas SDy01 y SDy02 lograron agotar el contenido inicial de maltosa del mosto en un tiempo más corto que las cepas comerciales, independientemente de la temperatura de fermentación. Sin embargo, mostraron una escasa habilidad para utilizar maltotriosa, el segundo azúcar más abundante en el mosto de cerveza. El fenotipo maltotriosa-positivo está muy extendido entre las levaduras cerveceras (Magalhães *et al*., 2016; Cubillos *et al*., 2019), pero en las levaduras panaderas varía en función de la cepa (Catallo *et al*., 2020). Por ejemplo, Galli *et al*. (2023) han descrito, recientemente, el uso de una cepa de *S. cerevisiae* totalmente competente en maltotriosa, aislada de una MM italiana, para la producción de una cerveza afrutada con frambuesa. Por contra, Pietrafesa *et al*. (2023) comentan la incapacidad para matabolizar maltoriosa de una cepa de MM utilizada en la elaboración de cerveza artesanal tipo "Italian Grape Ale". La fermentación ineficiente de maltotriosa reduce el rendimiento de etanol, un parámetro económico importante, en particular para producir cervezas de tipo lager (Zheng

et al., 1994). Sin embargo, la selección de cepas de levadura maltotriosa-negativa ha ganado interés como rasgo deseado para la producción de cervezas no sólo de bajo contenido alcohólico (Simões *et al*., 2023), sino también por un dulzor residual, mayor cuerpo y sensación en boca (Zhao *et al*., 2015).

A pesar de la incompetencia de las cepas de masa madre SDy01 y SDy02 para la utilización de maltotriosa, el rendimiento en etanol, tras la maduración a 4ºC del mosto, fue sólo un 15% inferior en las fermentaciones realizadas a 20ºC y similar a 37ºC que el obtenido empleando las cepas de referencia BRY-97 y VOSS, respectivamente. Más importantes fueron las diferencias en la producción de glicerol y ácido acético, claramente superiores en el mosto fermentado con las levaduras de masa madre. Las variaciones en estos metabolitos, junto con el perfil de compuestos volátiles, son determinantes en las características organolépticas de la cerveza (Vrzal *et al*., 2023). Cabe destacar que las cepas SDy01 y SDy02, aumentaron la cantidad total de volátiles en el mosto fermentado, en comparación con la cepa de referencia BRY-97 a 20ºC, especialmente de ésteres y ácidos. Las diferencias fueron mayores para las especies moleculares más abundantes de ésteres de acetato y ésteres de ácidos grasos de cadena media, que aportan notas de sabor a frutas y rosas. En comparación, el aumento de la temperatura de fermentación, dio lugar a una drástica reducción del rendimiento en volátiles por parte de las levaduras de MM, que mostraron un perfil de volátiles similar al de VOSS, la cepa de referencia a 37ºC, con alcoholes como principal grupo químico. Es bien conocido que la relación entre alcoholes superiores y ésteres influye en el equilibrio aromático de las cervezas (Pires *et al*., 2014), así como que numerosas condiciones del proceso (oxígeno disuelto, geometría del fermentador, agitación, etc.) afectan a esta relación, para la que algunos autores consideran óptimos valores de 2,5 a 3,0 (Šmogrovičová and Dömény, 1999). En nuestras manos, sólo las fermentaciones realizadas con BRY-97 a 20ºC y SDy01 a 37°C presentan una relación similar a ésta, mientras que el resto de las muestras tienen valores en el

rango de 0,9 a 1,8. Por tanto, es difícil en este momento aventurar cómo esta inusual relación influirá en el sabor y el aroma de nuestras cervezas experimentales. Además, la presencia de diferentes ésteres tiene efectos sinérgicos que afectan al sabor por debajo de sus umbrales individuales, lo que hace necesario efectuar un análisis sensorial. Por último, destacar que, incluso con el mismo mosto, se pueden producir cervezas con un perfil de aroma distintivo utilizando la combinación adecuada de temperatura y cepa de masa madre.

Conclusiones generales



“Un alma no es más que la última burbuja de
una larga fermentación en el mundo”
George Santayana

1) Las masas madre analizadas se caracterizaron por el predominio de *K. humilis* o *S. cerevisiae* entre las levaduras y de *F. sanfranciscensis* como principal bacteria láctica.

2) La especie de levadura predominante y el tipo de harina utilizados fueron factores determinantes en el perfil de compuestos volátiles de las masas madre. Se detectó una mayor abundancia relativa de alcoholes en las muestras de Paterna, mientras que, en las de Sant Joan Despí, son los ésteres los compuestos mayoritarios.

3) En general, los aislados de levaduras de masa madre muestran una elevada capacidad fermentativa, pero difieren mucho en su tolerancia a condiciones de estrés relevantes para su utilización biotecnológica, lo que pone de manifiesto su diversidad genética.

4) Las cepas de *K. humilis* muestran un gran potencial para su uso como cultivos iniciadores. Exhiben mayor capacidad leudante que las cepas de *S. cerevisiae*, un perfil aromático característico y elevada tolerancia a las condiciones de estrés habituales en masas madre.

5) Se han obtenido clones tolerantes a ácido acético a partir de dos aislados de masa madre de *S. cerevisiae*, mediante adaptación evolutiva en presencia de concentraciones crecientes de ácido acético, o en combinación con miriocina, una alternativa que aceleró la generación de poblaciones evolucionadas.

6) La tolerancia a acético, en algunos clones evolucionados, se ve acompañada por una mayor sensibilidad a ácido láctico, lo que sugiere que las levaduras desarrollan una respuesta diferente a ambos ácidos.

7) La secuenciación del genoma y el análisis del nivel de ploidía, tanto de las cepas parentales, como de los clones evolucionados, reveló la existencia de aneuploidías, variaciones en el número de copias (CNVs) y polimorfismos de un único nucleótido (SNPs) que podrían explicar su heterogeneidad fenotípica.

8) La tolerancia intrínseca a ácido acético de los aislados de masa madre puede explicarse por CNVs de genes, principalmente infrarrepresentados, con funciones en respiración y equilibrio iónico-rédox; ambos procesos relacionados anteriormente con la protección frente a estrés ácido.

9) La identificación de SNPs únicos en los clones evolucionados confirma la implicación, tanto de determinantes ya conocidos (Arg82, Kex1, Ctk1, Spt20, Ira2, Asg1 y Gis4), como de otros nuevos (Msn5 y Psp2), en la tolerancia a ácido acético.

10) La fermentación de mosto de malta por aislados de masa madre de *S. cerevisiae* muestra la versatilidad de estas cepas para su utilización a diferentes temperaturas, permitiendo además reducir el tiempo de fermentación, en comparación con las cepas cerveceras comerciales.

11) Las cepas de masa madre generan más compuestos volátiles que las cepas comerciales, en particular ésteres y ácidos, y muestran un perfil aromático específico dependiente de la temperatura de fermentación.

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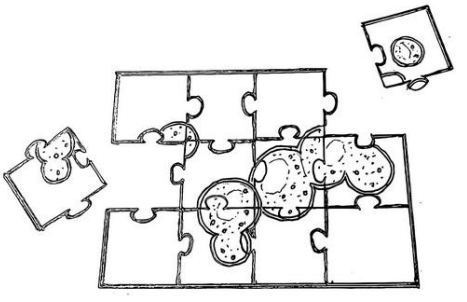

“La mente es igual que un paracaídas. Sólo funciona si se abre”

Albert Einstein

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