TESIS DOCTORAL INTERNACIONAL

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VNIVERSITAT DE VALÈNCIA

Facultat de Farmàcia

REVALORIZACIÓN DE RESIDUOS DE LA INDUSTRIA ALIMENTARIA COMO AGENTES ANTIFÚNGICOS MEDIANTE LA FERMENTACIÓN CON BACTERIAS ÁCIDO LÁCTICAS

REVALORIZATION OF FOOD WASTE FROM INDUSTRY AS ANTIFUNGAL AGENTS THROUGH FERMENTATION WITH LACTIC ACID BACTERIA

TESIS DOCTORAL INTERNACIONAL

PROGRAMA DE DOCTORADO EN CIENCIAS DE LA ALIMENTACIÓN

Valencia, mayo 2024

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El Dr. Giuseppe Meca de Caro, Catedrático de Universidad de la Universitat de Valencia y el Dr. Carlos Luz Minguez, Profesor Ayudante Doctor de Universidad de la Universitat de Valencia, 20 de mayo de 2024

CERTIFICAN QUE:

La D./Da Victor D'Opazo Taberner ha realizado bajo su dirección la Tesis Doctoral que lleva por título REVALORIZACIÓN DE RESIDUOS DE LA INDUSTRIA ALIMENTARIA COMO AGENTES ANTIFÚNGICOS MEDIANTE LA FERMENTACIÓN CON BACTERIAS ÁCIDO LÁCTICAS, y autorizan su presentación para optar al título de Doctor por la Universitat de Valencia.

Y para que así conste, expiden y firman el siguiente certificado.

Burjassot, 20 de mayo de 2024

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Aquesta tesi doctoral ha donat lloc a 5 articles, publicats a la següents revistes:

1. Antifungal properties of whey fermented by lactic acid bacteria in films for the preservation of cheese slices. *International Journal of Dairy Technology*, *75*(3), 619-629 (2022). Impact factor: 4.374
2. Revalorization by lactic acid bacterial fermentation of goat whey from cheese industry as a potential antifungal agent. *Food Bioscience*, *53*, 102586 (2023). Impact factor: 4.240
3. Evaluation of shelf life and technological properties of bread elaborated with lactic acid bacteria fermented whey as a bio-preservation ingredient. LWT, 174, 114427 (2023). Impact factor: 4.952
4. Revalorization of beer brewing waste as an antifungal ingredient for bread biopreservation. *Food Bioscience,* 58, 103588, (2024). Impact factor: 4.374
5. Revalorization of rice bran as a potential ingredient for reducing fungal contamination in bread by lactic acid bacterial fermentation. *Food Bioscience,* 58, 103703, (2024). Impact factor: 4.374

El doctorand ha gaudit de la beca predoctoral “Formación de Personal investigador” (FPI) amb referencia PRE2020-093996, dins del proejcte PID2019-108070RB-I00, del *Ministerio de Ciencia, Innovación y Universidades* i la *Agencia Estatal de Investigación.* Així com l’ajuda de mobilitat (2023) per la menció internacional del títol de Doctor, la qual es va realitzar en la en el *Dipartimento di Scienze e Tecnologie Agro*-Alimentari, en la Alma *Mater Studiorum - Università di Bologna* baix la direcció del Dr. Antonio Prodi.

Aixi mateix, aquesta Tesi Doctoral Internacional s’engloba dins dels següents projectes:

- Reducción de aditivos en queso mediante soluciones de envasado activo – NaturCheese (AVI 2019-2020).
- Biopreservación de pan de molde con suero de leche fermentado frente a micotoxinas y hongos toxigénicos. Seguridad de uso en presencia de carotenoides (SAFEBIOBREAD) (PID2019-108070RB-I00).
- Enhancing Research and Innovation Capacity of Tubitak MAM Food Institute on Managment of Mycotoxigenic Fungi and Mycotoxins (MycoTWIN) (GA 952337).
- Smart and innovative packaging, postharvest rot management and shipping of organic citrus fruit (BiOrangePack) ((PRIMA)-H2020).
- ValOrización de Residuos Lácteos mediante el desarrollo de EnvASe bioactivo (Go Orleans) (Ministerio de Agricultura, Pesca y Alimentación).
- Obtención de aditivos funcionales y agro nutrientes a partir de residuos de la molienda de arroz (BALBRAN) (Proyectos de I+D+I para la realización de «Pruebas de Concepto». Ministerio de Ciencia e Innovación).
- Título del proyecto: Biotecnología microbiana para la valorización de residuos agroalimentarios: un paso adelante en el aprovechamiento del salvado de arroz (BioValRice) (Programa de I+D+I de agroalimentación (AGROALNEXT) de la Generalitat Valenciana).

The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' but 'That's funny...'

Isaac Asimov (1920 – 1992)

AGRADECIMIENTOS

Quisiera dedicar este espacio para expresar mi más profundo agradecimiento a todas las personas que han contribuido de manera significativa a la realización de esta tesis doctoral.

En primer lugar, deseo expresar mi gratitud hacia mis directores de tesis, el Dr. Giuseppe Meca y el Dr. Carlos Luz. Por su orientación, paciencia y apoyo a lo largo de este proceso. Su sabiduría y dedicación fueron fundamentales para el desarrollo y la finalización de este trabajo.

Agradezco también al departamento de por brindarme acceso a los recursos y facilidades necesarias para llevar a cabo esta investigación de manera efectiva. El ambiente académico y las oportunidades de aprendizaje que ofrece esta institución han sido fundamentales para mi desarrollo profesional y académico. Al personal del departamento, especialmente a Carmen Escriva y Amparo Gonzalez, por ayudarme con tantas dudas que he tenido. Además de los otros profesores que me han guiado y aconsejado a lo largo del camino como el Dr. Jordi Mañes o la Dra. Guillermina Font.

Grazie al Prof. Antonio Prodi del Dipartimento di Scienze e Tecnologie Agro-Alimentari per avermi accolto nel suo gruppo di ricerca e avermi fatto sentire uno di voi. L'esperienza con voi ha ampliato le mie conoscenze, che sono andate oltre alla sola esperienza scientifica. Ricorderò questo periodo con voi con un sorriso e con affetto.

Muchas gracias también a los estudiantes del grado, de máster, de doctorado y los erasmus que han pasado por este laboratorio y con los que he compartido esta experiencia. Ha sido un placer conoceros, poder enseñaros y aprender de vosotros también. Me gustaría agradecer a especialmente a los compañeros con los que he compartido más tiempo y rutina: Claudia, Mario, Ana, Jorge, Juanma, Carlos y otros muchos que me faltan por mencionar, muchas gracias.

Gracias a mis padres, Ana Taberner y Joaquín D'Opazo, ya que en cierta forma esta tesis ha sido gracias a ellos al haberme inculcado el interés y la curiosidad desde mi infancia. Por el apoyo y la educación que me habéis dado. Y al resto de mi familia que siempre ha estado ahí para ayudarme.

Finalmente, no me puedo olvidar de mis amigos, el grupo del Sanvi, los osos y todos los demás que he ido haciendo a lo largo de estos años. Me habéis ayudado y cuidado siempre cuando lo necesitaba. Y también sois con los que he compartido mis momentos de alegría. Muchas gracias por estar ahí.

Muchas gracias por todo.

Víctor

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ACN	*Acetonitrile*
AFB1	*Aflatoxin B1*
AFB2	*Aflatoxin B2*
AFG1	*Aflatoxin G1*
AFG2	*Aflatoxin G2*
BAL	Bacterias ácido lácticas
BB	Bagazo de cerveza/*Beer bagasse*
CECT	Colección Española de Cultivos Tipo
CFS	*Cell free supernatant*
CSIC	Consejo Superior de Investigaciones Científicas
DPPH	*1, 1-diphenyl-2-picrylhydrazyl*
EFSA	Autoridad Europea de Seguridad Alimentaria/ *The European Food Safety Authority*
FDA	*Food and Drug Administration*
GRAS	*Generally Recognized as Safe*
HPLC	*High-performance liquid chromatography*
IARC	Agencia Internacional de Investigación del Cáncer
LAB	*Lactic acid bacteria*
MeOH	*Methanol*
MFC	*Minimum fungicidal concentration*
MgSO4	*Magnesium sulphate*
MIC	*Minimum inhibitory concentration*
MRS-A	*Man Rogosa and Sharpe agar*
MRS-B	*Man Rogosa and Sharpe broth*

NaCl	*Sodium Chloride*
ONU	Organización de las Naciones Unidas
PDB	*Potato dextrose agar*
PDB	*Phosphate buffer saline*
PET	*Polyethylene terephthalate*
PVOH	*Polyvinyl alcohol*
QPS	*Qualified presumption of safety*
RB	Salvado de arroz/*Rice bran*
SLB	Sobrenadante libre de bacterias
VOC	Compuestos orgánicos volátiles
WHO	Organización Mundial de la Salud

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RESUMEN

El crecimiento de hongos contaminantes en materias primas, alimentos y piensos es una de las principales causas de pérdidas económicas del sector agroalimentario. La presencia de hongos puede provocar cambios en las características organolépticas del producto y reducir su valor nutricional. Además, algunas especies de hongos son capaces de producir una serie de toxinas, las micotoxinas, que tienen efectos perjudiciales para la salud de personas y animales. Todos esto desencadena en perdidas economías cuantiosas al tener que retirar el alimento y también en un malgasto de recursos al haber producido un alimento que no se ha podido utilizar. Existe una continua búsqueda de nuevos métodos que profilácticos contra la contaminación de hongos, ejemplo de ello es el uso de microrganismo para la conservación de alimentos, la biopreservación.

Otro gran problema al que se enfrenta la industria alimentaria es la elevada cantidad de residuos sin uso que se genera de forma regular en la producción de alimentos. La gran huella de carbono que se genera durante el tratamiento de estos residuos hace que continuamente se deba encontrar formas de darles un nuevo valor añadido.

En la presente Tesis Doctoral se ha buscado estudiar como mitigar ambos problemas mediante el desarrollo de estrategias que emplean residuos de la industria alimentaria para la protección de alimentos frente la contaminación de hongos y sus toxinas, dentro del enfoque de la economía circular. Los residuos con los que se trabajó fueron suero lácteo, bagazo de cerveza y salvado de arroz, que tras su fermentación con bacterias ácido lácticas se caracterizaron en diversas pruebas *in vitro*, se analizó y cuantificó la presencia de metabolitos producidos por las bacterias con capacidad antifúngica y se estudió su relación con test estadísticos.

En base a los resultados obtenidos se desarrollados diversas estrategias para la aplicación de las sustancias a base de residuos alimentarios fermentadas por las bacterias ácido lácticas. El primero de ellos fue la preparación de un separador de lonchas de queso que incorporaba una película con suero lácteo fermentado. Los resultados evidenciaban que el separador de queso logró alargar la vida útil del alimento ante la contaminación por *Penicillium commune, Penicillium verrucosum y Penicillium solitum*.

La segunda aplicación estudiada fue el uso de suero lácteo, bagazo de cerveza y salvado de arroz fermentado como ingrediente activo contra la contaminación de hongos en pan. El suero lácteo fermentado evidencio el mayor potencial al mostrar resultados mejores que los obtenidos por un pan con un conservante comercial en panes contaminados por *Aspergillus flavus y P. verrucosum*. Por su parte, bagazo de cerveza y salvado de arroz fermentado también evidenciaban un aumento en la vida útil de panes contaminados por *A. flavus* y *P. commune*, próximos a los obtenidos con el conservante comercial. Además, redujeron de forma significativa la aparición de aflatoxinas en estos panes.

En conclusión, los hallazgos evidenciaron que los tres ingredientes activos preparados mediante la fermentación con bacterias ácidos lácticas de subproductos de la industria (suero lácteo, bagazo de cerveza y salvado de arroz) pueden cumplir su propósito como agentes contra la contaminación de hongos en alimentos. Estas aplicaciones han demostrado eficacia como ingrediente en pan y como coadyuvante en separadores de queso.

ABSTRACT

The growth of fungi in raw materials, food and feed is one of the main loss causes in the food sector. The occurrence of this microorganism produces changes in the organoleptic qualities of the product, reduce the nutritional value and some species can produce toxic metabolites, called mycotoxins, which have harmful effects on human and animal health. This leads to large economic losses by having to remove the contaminated product from the food chain and a waste of resources by having produced food that could not be used. Therefore, there is continuous research for counter measures against fungal contamination. For example, the use of microorganism for food preservation, the biopreservation.

Another major problem faced by the food industry is the elevated occurrence of different byproducts generally obtained during the food production. The treatment of this industrial residues generates a large carbon food print that researchers are trying to reduce.

This Doctoral Thesis seeks to study how to mitigate both problems through the development of strategies that will use waste from the food industry to develop novel application for food biopreservation against food contaminant fungi, a perspective within the circular economy. The byproducts used in the studies were milk whey, beer bagasse and rice bran. The residues were fermented with lactic acid bacteria, then the antifungal potential was determined by *in vitro* test, the antifungal metabolite occurrence was analyzed and quantified and finally, the link between the researched factor was studied with statistical tests.

Based on the results obtained, various strategies were developed for the application of substances based on food waste fermented by lactic acid bacteria. The first of them was the preparation of a cheese slice film that incorporated a coating with fermented whey. The results showed that the cheese separator managed to extend the shelf life of the food in the event of contamination by *P. commune*, *P. verrucosum* and *P. solitum*.

The second application studied was the use of whey, beer bagasse and fermented rice bran as active ingredient against fungal contamination in bread. Fermented whey evidenced the greatest potential by showing better results than those obtained by bread with a commercial preservative in breads contaminated by *A. flavus* and *P. verrucosum*. The beer bagasse and fermented rice bran also showed an increase in the shelf life of bread contaminated by *A. flavus* and *P. commune*, close to those obtained with the commercial preservative. In addition, they significantly reduced the appearance of aflatoxins in bread.

In conclusion, the findings evidenced that the three active ingredients prepared through fermentation with lactic acid bacteria from byproducts of the industry (whey, beer bagasse, and rice bran) can fulfill their purpose as agents against fungal contamination in food. These applications have shown effectiveness as an ingredient in bread and as an adjunct in cheese separators.

1. INTRODUCTION

INTRODUCCIÓN

1. INTRODUCCIÓN

1.1. Contaminación por hongos toxigénicos de alimentos

Según la Organización Mundial de la Salud (WHO), anualmente se pierde un tercio de los alimentos producidos, lo que corresponde aproximadamente a 2.5 billones de toneladas (Quintieri et al., 2023). De entre todas las causas, la contaminación por hongos es una de las primeras razones, especialmente en productos hortícolas y cereales (Garnier et al., 2017). Los hongos son capaces de afectar al producto en cualquier etapa desde su recolección, pasando por el procesado, hasta su lugar de consumo. Esta capacidad se debe, primero, a su habilidad de propagarse mediante el aire con esporas que producen en grandes cantidades, y segundo, por su capacidad de crecer en ambientes hostiles, como: pH bajos y altos, bajas actividades de agua, presencia de conservantes y bajas temperaturas (Pitt & Hocking, 2022)

Diferentes tipos de alimentos se ven afectados por distintos hongos, ya que estos suelen estar bastante adaptados a la matriz que contaminan. En general las frutas como cítricos o manzanas suelen verse afectadas por *Penicillium* spp. Las uvas, bayas y frutas solanáceas (tomate y berenjena) son generalmente contaminadas por hongos del género *Botritis*, *Rhizopus* y *Alternaria*. Las diferentes verduras y hortalizas como los tubérculos suelen ser contaminadas por las especies pertenecientes al género *Fusarium*. Mientras que los conocidos como vegetales de hoja son afectados por *Botritis cinérea*, *Rhizopus stolonifer*, *Rhizoctonia solani* y *Alternaria* spp. Los productos lácteos se ven afectados por algunas especies del género *Penicillium*. Respecto a los cereales, frutos secos y semillas, suelen ser contaminados por hongos del género *Aspergillus*, además, cabe puntualizar que ciertos cereales, como el maíz y el trigo, también se ven especialmente afectados por la contaminación de *Fusarium* spp. Los alimentos derivados de los anteriores suelen verse afectados por los mismos contaminantes. También existen otras clases de alimentos, como la carne, susceptibles a la contaminación por hongos, pero solo en condiciones muy específicas, ya que tienen una mayor propensión a ser contaminados por otro tipo de microorganismos (Kabak et al., 2006; Pitt & Hocking, 2022). En la **Tabla 1** se puede apreciar con más detalle la tendencia de los géneros y especies hongos a contaminar diferentes grupos de alimentos.

Tabla 1. Hongos clasificados en función a los grupos de alimentos más susceptibles a su contaminación.

Grupo de Alimentos	Hongos Contaminantes	Referencias
Cereales y Granos	*Fusarium* spp. *Aspergillus* spp. *Penicillium* spp.	Kursa et al. (2022); Mousavi Khaneghah et al. (2018).
Frutos Secos	*Aspergillus* spp. *Penicillium* spp.	Arrus et al. (2005); Spadaro et al. (2020); Tolosa et al. (2013).
Frutas y Verduras	*Aspergillus* spp. *Penicillium* spp. *Botrytis* spp. *Alternaria* spp.	Bartholomew et al. (2021); Kurniawan et al. (2018); Sylla et al. (2015).
Lácteos	*Penicillium* spp. *Aspergillus* spp. *Mucor* spp.	Morin-Sardin et al. (2016); Ture et al., (2011).
Carnes	*Aspergillus* spp. *Penicillium* spp.	López-Díaz et al. (2001); Peromingo et al. (2016).

Los hongos contaminantes de los alimentos pueden afectar al alimento de diversas maneras no deseadas. Su crecimiento está relacionado con la producción de diferentes cambios organolépticos como mocos, pelusas, polvo, acidificando, decolorando, sabores extraños (Pitt & Hocking, 2022; Sauer, 1988). También pueden afectar a la calidad del producto reduciendo sus nutrientes, poseen una gran variedad de actividades enzimáticas (en función a la especie y cepa) que les permite ser capaces de aprovechar prácticamente todas las matrices alimentarias (Leyva Salas et al., 2017). Además, son capaces de producir diversos metabolitos que pueden dañar nuestra salud. Ejemplos de estos compuestos son los alcaloides tóxicos como la conocida dietilamida del ácido lisérgico producido por *Claviceps purpurea* (Kodisch et al., 2020) o el grupo de sustancias conocido como micotoxinas (Richard, 2007).

1.1.1. Micotoxinas

Actualmente las micotoxinas están consideradas como un problema creciente para las autoridades sanitarias y para la industria alimentaria (Mazumder & Sasmal, 2001). Ya a principios de los años 2000, un total de 100 países habían instaurado regulaciones sobre los límites de tolerancia de estos compuestos en los alimentos para consumo humano y animal (Van Egmond et al., 2007). En la **Tabla 2** se aprecia, de forma detallada, ejemplos de micotoxinas.

Tabla 2. Micotoxinas, principales especies fúngicas que las producen, efectos tóxicos y su clasificación por la IARC (datos obtenidos de Bennett & Klich, 2003; Mazumder & Sasmal, 2001; Richard, 2007).

Micotoxina	Hongos productores	Efectos en la Salud
Aflatoxinas	*Aspergillus flavus* *Aspergillus parasiticus*	Carcinogénico, hepatotóxico, inmunosupresor, IARC 1.
Ochratoxina A	*Aspergillus ochraceus* *Penicillium verrucosum*	Nefrotóxico, carcinogénico, neurotóxico, IARC 2b.
Fumonisinas	*Fusarium verticillioides* *Fusarium proliferatum*	Hepatotóxico, carcinogénico, neurotóxico, IARC 2b.
Deoxinivalenol	*Fusarium graminearum* *Fusarium culmorum* *Fusarium cerealis*	Gastrointestinal, inmunosupresor, neurotóxico, IARC 3.
Zearalenona	*Fusarium graminearum* *Fusarium culmorum* *Fusarium equiseti* *Fusarium verticillioides* *Fusarium incarnatum* *Fusarium cerealis*	Reproductivo, hepatotóxico.
Patulina	*Penicillium expansum*, *Aspergillus clavatus*	Gastrointestinal, genotóxico, neurotóxico, IARC 3.
Citrinina	*Penicillium citrinum*, *Aspergillus terreus*	Nefrotóxico, hepatotóxico, carcinogénico.
T-2 Toxina	*Fusarium acuminatum* *Fusarium poae* *Fusarium sporotrichioides* *Fusarium langsethiae*	Dermatotóxico, inmunosupresor, gastrointestinales, IARC 3.
Enniatina	*Fusarium avenaceum* *Fusarium tricintum*	Neurotóxico, citotóxico, hepatotóxico.
Alternariol Alternariol Metil Éter	*Alternaria alternata* *Alternaria arborescens* *Alternaria brassicae* *Alternaria citri* *Alternaria solani*	Genotóxico, citotóxico, hepatotóxico.
Beauvericina	*Beauveria bassiana* *Fusarium sporotrichioides* *Fusarium poae* *Fusarium langsethiae* *Fusarium avenaceum*	Citotóxico, neurotóxico, inmunosupresor.

Las micotoxinas son metabolitos secundarios liberados por los hongos en condiciones de estrés. La exposición continuada a micotoxinas a través de alimentos contaminados puede producir toxicidad crónica tanto en humanos como en animales. También su a ingesta altas dosis de puede provocar toxicidad aguda (Stark, 2005). No fue hasta principios de la década de 1960 cuando se relacionó la presencia de micotoxinas en alimentos contaminados por hongos con daños severos en la salud con el caso, probablemente más famoso de contaminación por micotoxinas, la "enfermedad del pavo X", donde hasta un total de 100.000 pavos murieron debido a piensos contaminados por aflatoxinas (Pickova et al., 2021). Lo que conlleva grandes gastos para el sistema de salud pública y en el caso de los animales a las empresas que se ven afectadas (Phillips et al., 2008).

Dentro del conglomerado de moléculas que componen las micotoxinas, las más estudiadas por su alta tasa de aparición en alimentos y elevada toxicidad son las aflatoxinas y la ochratoxina A. Las aflatoxinas son el grupo de mayor riesgo debido a que su consumo prolongado está relacionado con efectos hepatotóxicos, cancerígenos e inmunodepresores. En la naturaleza se encuentran sobre todo en forma de aflatoxina B1 y aflatoxina B2. Siendo la primera la más tóxica, considerada cancerígeno del grupo 1 por la Agencia Internacional de Investigación del Cáncer (IARC). Algunos hongos del género *Aspergillus* son productores de estas toxinas (Ekwomadu et al., 2022). La ochratoxina A está relacionada con efectos nefrotóxicos, teratogénicos, genotoxicidad y cancerígenos (dentro del grupo 2B según la IARC). La producen ciertos hongos pertenecientes al género *Aspergillus* y *Penicillium* (Bui-Klimke & Wu, 2015).

Existen otros tipos de micotoxinas cuya aparición y virulencia es menor que las dos mencionadas anteriormente, aunque, por esto son menos importantes. Ejemplos de ellos son la citrina, producida por diversas especies pertenecientes a los géneros *Aspergillus*, *Penicillium*, y *Monascus*, que tienen efectos genotóxicos, teratogénicos, y nefrotóxicos (Zargar & Wani, 2023). Los alcaloides tóxicos producidos por el género *Claviceps* que tienen actividades alucinógenas, vómitos y gangrena (Schiff, 2006). Las fumonisinas, producidas por diversas especies pertenecientes al género *Fusarium* que al consumirse producen un efecto hepatotóxico y nefrotóxico (Kamle et al., 2019). Sobre la patulina, metabolito fúngico producido por *P. expansum*, *Aspergillus* spp. y *Byssochlamys fulva*, se han registrado efectos genotóxicos, nefrotóxicos, toxicidad e inhibición del sistema inmune y teratogénicos (Zhong et al., 2018). Las micotoxinas descritas en este párrafo están entre otras muchas, cuyos efectos son conocidos, en la actualidad y se conocen sus efectos y diversas legislaciones controlan ya su concentración en los alimentos.

Por último, están las conocidas como micotoxinas emergentes y las enmascaradas. Las micotoxinas emergentes son los metabolitos producidos por los hongos que, pese a tener una actividad dañina para la salud humana y animal, aún no se ha caracterizado toxicológicamente sus efectos. Ejemplos de este grupo son algunos precursores de las aflatoxinas o las beauvericinas (Scarpino et al., 2021). Por su parte, las micotoxinas enmascaradas son las que han sufrido alguna modificación por parte del procesad industrial, procesado culinario, o del metabolismo de algún ser vivo, principalmente plantas, pero la modificación puede ser de origen animal, fúngico, o bacteriano. Estas micotoxinas modificadas no son detectadas por los métodos tradicionales y no tienen la toxicidad de sus predecesores, no obstante, algunas de ellas todavía conservan parte de su toxicidad o al ser metabolizadas por los humanos vuelven a su forma original. La sulfato-zealenona o el glucosido-3-deoxidivanelon son alguna de las que se pude encontrar dentro de este grupo (Agriopoulou et al., 2020).

La Agencia Internacional de Investigación sobre el Cáncer (IARC), un organismo de la WHO, ha evaluado que la ingesta de ciertas micotoxinas puede aumentar el riesgo de desarrollar ciertos tipos de cáncer. En consecuencia, algunas de estas micotoxinas han sido clasificadas por la IARC en su lista, donde las aflatoxinas se sitúan en el Grupo 1 debido a las evidencias que sugieren su capacidad carcinogénica en los humanos. Por otro lado, Grupo 2a, catalogadas como probablemente carcinogénicas para los humanos, la fumonisina, fusarina y ochratoxina A se encuentran en el grupo 2b, posiblemente carcinógenos para humanos. El siguiente grupo es el 3 no clasificables como carcinógenos para humanos donde se encuentran el deoxinivanelon, la zearolona, la paturilna y la citrina. Y finalmente, dentro del Grupo 4, las consideradas probablemente no carcinógenos para humanos (Ekwomadu et al., 2022).

Una de las características más problemáticas de las micotoxinas es su alta resistencia a los tratamientos de esterilización utilizados de forma regular en humamos. Los tratamientos usados típicamente para la preservación de los alimentos no aseguran la eliminación de estos metabolitos secundarios de los hongos. Un ejemplo de ello es la alta resistencia de la ochratoxina A y la aflatoxina B1 que son capaces de resistir temperaturas de hasta 160 ºC (Kabak, 2009). O como el caso del deoxynivalenol, que en la bibliografía se reporta su alta resistencia a tratamientos con ácidos y álcalis, además de temperaturas superiores a los 100 ºC (Karlovsky et al., 2016). Otro factor muy importante que se ha de tener en cuenta con relación a la presencia de las micotoxinas es el hecho de que la ausencia de contaminación fúngica de un alimento no implica la ausencia de micotoxinas en el mismo. Estos metabolitos son resistentes a los procesos de desinfección. Esto quiere decir que si las materias primas han estado contaminadas por hongos productores de micotoxinas los tratamientos higiénico-sanitarios utilizados comúnmente por la industria no se servirían en la eliminación de estos metabolitos fúngicos (Oliveira et al., 2014).

En los alimentos preparados de segunda a quinta gama el análisis de estas toxinas fúngicas en las materias primas es un factor muy importante a tener en cuenta en su recepción. Se han reportado en diversas ocasiones la contaminación biológica de pan por debajo de los limites regulados, pero donde se detectaba la presencia de ochratoxina A, aflatoxinas, fumonisinas y zealenona, entre otras muchas, debido que se han usado harinas contaminadas para su producción (De Koe & Juodeikiene, 2012; Sacco et al., 2020). Es posible encontrarlos incluso en ciertas materias primas no contaminadas directamente por hongos. La leche es la principal materia prima donde, en ciertas ocasiones, existe la presencia de estas toxinas, pero también se pueden encontrar en la propia carne y otros productos derivados de la producción animal como el huevo, debido a la contaminación de los piensos utilizados en la alimentación del ganado y de las aves (Flores-Flores et al., 2015; Pleadin et al., 2021). Estos piensos contaminados pueden tener dosis de micotoxinas inocuas para el animal, pero la acumulación de estas toxinas en alimentos como leche o carne puede ser motivo de contaminación crónica para el consumidor final en la cadena trófica, es decir, el ser humano (Duarte et al., 2011). El caso más recurrente de este tipo se da en leches contaminadas con aflatoxina M1 y M2. La presencia de esta toxina se debe al metabolismo de los animales rumiantes productores de leche como la vaca, cabra u oveja. Cuando consumen piensos contaminados con aflatoxina B1 y aflatoxina B2, su organismo para eliminarla las metaboliza en el hígado a aflatoxina M1 y aflatoxina M2 y se excretan a través de la leche de estos animales. Estas micotoxinas, pese a tener un grado de toxicidad diez veces menor al de sus precursores, aún poseen una gran capacidad de ejercer efectos nocivos en seres vivos, y están clasificadas en el mismo nivel de carcinogenicidad por la IARC, es decir en el grupo 1 (Becker-Algeri et al., 2016).

1.1.2. Legislación hongos en alimentos

La regulación de los hongos y las micotoxinas se realiza siempre en base a hechos resultados de investigaciones científicas, como los diversos resultados obtenidos de estas, la capacidad de análisis de muestra y que cantidad a analizar para qué los resultados sean significativos. También se suelen tener en cuenta las consecuencias socioeconómicas resultantes de las distintas regulaciones impuestas (Udomkun et al., 2017).

Europa tiene uno de los sistemas alimentarios con mayores exigencias de seguridad a nivel mundial. Dentro de esta regulación, se incluye también la presencia de las micotoxinas y los hongos. Estos hongos están normalmente regulados para cada tipo de alimentos en función a la susceptibilidad de ser contaminados por los mismos (Kabak et al., 2006).

Respecto a las micotoxinas, en la actualidad existe una legislación severa. Ya a principio de los años 2000 en Europa había un total de 40 regulaciones de límites de micotoxina-alimento establecidos (Kabak et al., 2006). Con el paso de los años, la legislación vigente no ha dejado de crecer ante las nuevas evidencias científicas sobre la presencia de micotoxinas en alimentos y su efecto dañino. Ejemplo de ello es la reciente actualización por parte de la Agencia Europea de Seguridad Alimentaria (EFSA, *European Food Safety Authority*), agencia europea que regula los temas de seguridad alimentaria, que ha revisado recientemente los límites de ochratoxina A en alimentos consumidos habitualmente dentro de esta región (Schrenk et al., 2020). No solo eso, sabiendo que las micotoxinas pueden afectar a través de la alimentación animal, la unión europea legisla también los límites de micotoxinas en los piensos y alimentación animal (Schrenk et al., 2023). En la **Tabla 3** se pueden aprecian algunas de las 40 regulaciones determinadas por la Unión Europea.

Tabla 3. Las micotoxinas más comunes legisladas por la Unión Europea, junto con sus límites máximos permitidos en diferentes tipos de alimentos. Estos valores pueden variar según la legislación específica de cada país dentro de la Unión Europea. Datos obtenidos de Official Journal of the European Union (2023).

Micotoxina	Límite máximo permitido en alimentos según la legislación de la UE
Aflatoxinas B1	Cereales y productos derivados: 2-5 µg/kg Frutos secos, frutas secas y productos derivados: 2-12 µg/kg
Aflatoxinas totales	Cereales y productos derivados: 10 µg/kg Frutos secos, frutas secas y productos derivados: 4-15 µg/kg
Ochratoxina A	Cereales y productos derivados: 5 µg/kg Café: 3-5 µg/kg Vinos: 2 µg/kg
Deoxinivalenol	Otros cereales y productos derivados: 200-1750 µg/kg Alimentos para bebés: 20 µg/kg
Zearalenona	Cereales y productos derivados: 100-300 µg/kg Alimentos para bebés: 200 µg/kg
Fumonisinas B1 + B2	Maíz y productos derivados: 800-4000 µg/kg Otros cereales y productos derivados: 1400-2000 µg/kg

En otros países también existe una regulación, no tan rigurosa, de estos metabolitos. Por ejemplo, en Estados Unidos, su agencia reguladora de alimentos, la *Food and Drug Administration* (FDA) limita solo la presencia de aflatoxinas, fumonisinas, patulina y ochratoxina

A en alimentos (FDA, 2000, 2010). También está el ejemplo de China, donde la regulación también existe una similar a la estadounidense tanto para la alimentación humana como animal (Ma et al., 2018; Mazumder & Sasmal, 2001).

1.2. Control del crecimiento de hongos toxigénicos en alimentos

El control del crecimiento fúngico indeseado se realiza a lo largo de toda la cadena de producción de los alimentos. Las buenas prácticas de manipulación, lavado, tratamientos térmicos y envasado al vacío son ejemplos de métodos para prevenir o reducir el crecimiento de los hongos (Ribes et al., 2018).

Emplear únicamente las buenas prácticas de manipulación no es suficiente para prevenir la contaminación de hongos en nuestros alimentos. Se han de emplear de forma correcta otras técnicas para incrementar la seguridad frente a la contaminación. La más común es el empleo en todas las etapas de la producción de antifúngicos de origen sintético. Debido a su alta efectividad y la creciente demanda de alimentos a nivel mundial, su uso sigue aumentando continuamente, no obstante, esto conlleva una serie de problemas (Kathiravan et al., 2012). El primero es la aparición de resistencias a antifúngicos, es decir, la capacidad de algunos hongos de adquirir resistencia a moléculas con actividad contra su desarrollo. Ejemplos de ello son la aparición de alteraciones en la proteína diana del antifúngico o desarrollo de procesos de detoxificación de los compuestos antifúngicos. Esta capacidad ocurre por selección natural, por lo tanto, las resistencias son heredadas. En los años 60 comenzaron a aparecer estudios que demostraban que la capacidad de ciertas especies de hongos a crecer en presencia de los principales compuestos antifúngicos, como el ejemplo, las desarrolladas al metil benzimidazol carbamato en 30 hongos de distintas especies (Dalhoff, 2018; Fillinger et al., 2016). Otro de los inconvenientes de la utilización de compuestos antifúngicos sintéticos es su relación con la aparición de problemas de salud, ya que, esta puede verse afectada por una exposición continuada a este tipo de compuestos. Precisamente la exposición de trabajadores del sector agrícola demostró la relación de este tipo de pesticidas con problemas endocrinos, inmunológicos, neurológicos, teratogénicos y cancerígenos (Brauer et al., 2019; Sumalan et al., 2019).

Por último, se han relacionado también con efectos perjudiciales para el medio ambiente, son capaces de alterar el ecosistema de las zonas donde se aplican como tratamiento. Además, debido a que su estructura molecular les confiere una gran estabilidad, son muy dañinos ya que pueden permanecer activos en el medio ambiente durante largos periodos de tiempo (Brauer et al., 2019; Mukhopadhyay & Kumar, 2020; Sumalan et al., 2019). Estas razones, sumadas a un uso irresponsable de estos productos por cierta parte del sector alimentario, acarrean serios

problemas que se están tratando de solucionar mediante nuevas técnicas de procesado y cuidado de los alimentos (Bunker & Mathur, 2001).

Se ha documentado la reducción de micotoxinas mediante diversas técnicas como: la limpieza, molienda, fermentación, horneado, fritura, cocción, asado, extrusión, tratamiento con alcalinos y ácidos. No obstante, como se ha puntualizado en anteriores apartados la completa eliminación de estas toxinas en una ardua tarea para la industria alimentaria y estas técnicas no son totalmente eficaces para reducir la presencia de estos metabolitos hasta concentraciones que no presenten riesgo para su consumo (Karlovsky et al., 2016). En la **Tabla 4** se recogen los diversos métodos utilizados para evitar el crecimiento de hongo en alimentos.

Tabla 4. Principales métodos y técnicas conservación alimentos empleadas frente al crecimiento de hongos no deseados.

Método	Técnica utilizada	Referencias
Control de Humedad	Monitoreo y control de la humedad ambiental	Sterflinger (2010)
Higiene y limpieza	Limpieza regular de áreas de almacenamiento, equipos y utensilios	Liu et al. (2020)
Buena Práctica Agrícola	Rotación de cultivos, selección de semillas y material vegetal sano	Sarrocco & Vannacci (2018)
Tratamiento Postcosecha	Lavado, secado y tratamiento térmico de frutas y vegetales	Gonçalves et al. (2019)
Uso de Fungicidas	Mancozeb Azoxistrobina Tebuconazol Captan	Hrabětová et al. (2017)
Uso de conservantes alimentarios	Sorbato de Potasio Benzoato de Sodio Propionato de Calcio Natamicina Ácido sórbico	Lima et al. (2005)
Nuevas técnicas de conservación	Biopreservación Organismos genéticamente modificados Nanopartículas Aceites esenciales	Crowley et al. (2013); Fernandez Bidondo et al. (2019); Herron & Thorn (1998); Tongnuanchan & Benjakul (2014)

1.2.1. Nuevas técnicas de conservación

El desarrollo de nuevas metodologías está cobrando cada vez mayor fuerza a nivel de investigación para reducir el uso de fungicidas y la utilización de otro tipo de sustancias más sostenibles con el fin de evitar el crecimiento de contaminantes en los alimentos (Tao et al., 2018). En menor medida y todavía con pocas aplicaciones en el mercado se encuentra el uso de nuevos metabolitos de diferentes fuentes, como el uso de péptidos y proteínas derivados de la hidrolisis enzimática o con microorganismos ente otros (Martínez-Culebras et al., 2021; Mishra et al., 2021). También se ha estudiado la utilización de biopolímeros a base de polisacáridos, lípidos, proteínas y sus mezclas con ciertos conservantes sintéticos adheridos a ellos para que no migren al alimento (Jafarzadeh et al., 2023). Pero estas aplicaciones están pasando desapercibidas a nivel de interés de investigación y por parte de la industria en comparación a las siguientes.

La primera de estas nuevas opciones es el desarrollo de organismos genéticamente modificados. Los avances en la ingeniería genética han permitido la aparición de microorganismos capaces de sintetizar compuestos que de forma natural no producen. Su empleo es muy frecuente en la industria farmacéutica. No obstante, se pueden emplear en otros ámbitos como el de la seguridad alimentaria. Ejemplo de ello es el desarrollo de una cepa de patata (*Solanum tuberosum*) capaz de expresar genes que codifican metabolitos antifúngicos frente al crecimiento de *Rhizoctonia solani*, uno de los principales hongos que ocasionan daños a su cultivo (Fernandez Bidondo et al., 2019).

Otra opción que se ha tenido en alta consideración es el empleo de aceites esenciales. La investigación Lorán et al. (2022) prueba, con técnicas *in vitro*, la capacidad de inhibir el crecimiento *A. parasiticus* y de las aflatoxinas producidas por esta especie de hongos (aflatoxina B1, B2, G1 y G2) mediante el uso de aceites esenciales de romero (*Rosmarinus officinalis),* orégano (*Origanum virens*) y dos clases de lavanda (*Lavandins Grosso* y *Lavandins Abrial*). No obstante, existen una serie de problemas asociados a los aceites esenciales, el primero de ellos es la metodología de extracción de estos. Normalmente está relacionada con el uso de disolventes orgánicos que no se pueden emplear en la industria alimentaria, como el ciclohexano, diclorometano, o etil acetato (Sarikurkcu et al., 2009). Además, en la actualidad, se están comenzando a emplear nuevas técnicas como la extracción por supercríticos con CO_2 o agua, pero de nuevo aparece una complicación para la industria alimentaria, los equipos asociados a este tipo de extracción son demasiado caros para su empleo por la industria (Araus et al., 2009; Eikani et al., 2007). La aplicación de aceites esenciales conlleva un segundo problema, este tipo de agente antifúngico es un extracto concentrado y suele tener un sabor semejante al compuesto del que se ha extraído, es decir, va a influir en la calidad organoléptica del producto al que se añade ya que deben utilizarse a concentraciones muy altas para ejercer el efecto. Esta característica limita

mucho su uso ya que ciertos sabores y olores son solo deseados en asociación a cierto tipo de alimentos (Mariod, 2016). También se ha buscado su aplicación a través de compuestos orgánicos volátiles (COV) sin llegar a entrar en contacto con el alimento, lamentablemente, esta estrategia solo ha resultado útil en silos (Chaves et al., 2012).

El empleo de nanocompuestos o nanopartículas también se postula como técnica de conservación novedosa. Se considera nanocompuestos a las partículas con un tamaño inferior a 100 nm. Las partículas de este tamaño tienen la peculiaridad de tener una serie de características físico-químicas diferentes a las partículas macroscópicas y microscópicas, incluso cuando están compuestas por los mismos materiales (Herron & Thorn, 1998). En la bibliografía existen diversos artículos que exponen su uso como agente antifúngico, como en los trabajos de Agnihotri et al. (2014) y Panda et al. (2018) donde se demuestra que el uso de nanopartículas de grafeno o de plata evidenciaban actividad antimicrobiana contra un gran espectro de microorganismos, entre ellos hongos contaminantes de alimentos. La más destacada de estas son las producidas con ZnO, están registradas por Europa como *Generally Recognized as Safe* (GRAS), un término utilizado para indicar que su uso está aprobado en alimentos dentro de la Comunidad Europea (Sun et al., 2018). Pero al igual que el anterior método, su forma de aplicación es muy limitada. Estas partículas han de estar adheridos al envase o a alguna estructura en contacto con el alimento, como los separadores de lonchas de queso, para hacer su ejercer su actividad antifúngica de forma correcta (Rahman et al., 2018; Siegrist et al., 2007). Por esta razón, si se añadieran únicamente como ingredientes no se alcanzaría el objetivo deseado (Rogers, 2016).

Y la última de estas técnicas que ha cobrado interés es la bioconservación, la utilización de microorganismos para la preservación de alimentos.

1.2.1.1. Bioconservación

La bioconservación se define, de forma simple, como el empleo de microorganismos para la conservación de alimentos (Singh, 2018). Este método se reconoce en la bibliografía por varios términos: biopreservación, biocontrol o bioconservación (Leyva Salas et al., 2017). El concepto de bioconservación abarca diferentes vías, desde la fermentación directa del alimento, a la aplicación de metabolitos producidos por estos microorganismos (Pisoschi et al., 2018). Pese a ser una técnica que está cobrando importancia en la actualidad, se lleva realizando desde la antigüedad. Ejemplos de ello son alimentos tan conocidos como las leches fermentadas (yogur, kéfir, kumis, stragisto, entre otros). Hay evidencias de textos en Asia que datan del 6000 antes de Cristo donde se menciona el consumo de este tipo de alimentos. Ya en esa época era conocido que, mediante la fermentación bajo ciertas condiciones, algunos alimentos podían incrementar su vida útil, pese al parcial desconocimiento de los procesos que ocurrían detrás de la conservación

(Fisberg & Machado, 2015). También la fermentación alcohólica es otro ejemplo de la transformación de alimentos para la producción de alimentos pocos perecederos. Hay evidencias de residuos de fermentados alcohólicos en vasijas que datan del 9000 antes de Cristo (Liu et al., 2019).

A lo largo del mundo y diferentes culturas se pueden observar diversos alimentos con este mismo principio, como la salsa de soja en Japón, el pan se masa madre en Europa y parte de Asia, el ogi (un pure de cereales fermentados) en el este de África, o el atole agrio (una bebida de maíz fermentado) en Centro América (Cuamatzin-garcía et al., 2022). Todos estos productos son en la actualidad comidas o bebidas consideradas como "típicas", no obstante, comparten algo en su origen: todas ellas fueron métodos del ser humano para alargar la vida útil de alimentos perecederos gracias a la fermentación con microorganismos (Sharma et al., 2020).

El principio por el que este método de conservación funciona es el hecho de que existe una competición continua que ocurre entre los diferentes microrganismos de forma natural con el objetivo de poder ser el organismo que pueda aprovechar mejor el sustrato donde crece (Bollenbach, 2015). Esta competición y continua adaptación puede ser aprovechada por el ser humano para inhibir o entorpecer el crecimiento de microrganismos no deseados en el alimento (Heydari & Pessarakli, 2010).

La bioconservación no solo contempla el uso de microorganismos para fermentar el alimento, también implica el uso de agentes derivados o purificados obtenidos durante la fermentación. Para el desarrollo de esta técnica no es necesario fermentar el alimento en sí, sino que se pueden fermentar otras matrices aptas para el crecimiento del microorganismo y aplicarlas como agente antimicrobiano de formas diversas (Topisirovic et al., 2006). Se han estudiado diversas estrategias para la aplicación en alimentos de estos agentes antifúngicos con diferentes microorganismos bioprotectores, se puede apreciar ejemplos en la **Tabla 5**.

Tabla 5. Ejemplos de microrganismos utilizados para la biopreservación de alimentos contra hongos contaminantes.

Microorganismo Protector	Mecanismo de acción	Hongo alterante	Referencias
Bacillus subtilis	Antagonismo competitivo	*Botrytis cinerea*	Sylla et al. (2015)
Pseudomonas fluorescens	Producción de antibióticos	*Fusarium oxysporum*	Ramamoorthy et al. (2002)
Trichoderma spp.	Competencia y producción de enzimas	*Rhizoctonia solani*	Anees et al. (2010)
Lactiplantibacillus plantarum	Producción de ácido láctico	*Penicillium expansum*	Chen et al. (2021)
Streptomyces spp.	Producción de metabolitos activos	*Aspergillus flavus*	Sari et al. (2021)
Lactobacillus acidophilus	Producción de bacteriocinas	*Fusarium graminearum*	Franco et al. (2011)
Bacillus amyloliquefaciens	Producción de péptidos antimicrobianos	*Aspergillus niger*	Kadaikunnan et al. (2015)
Streptomyces halstedii	Producción de antibióticos	*Fusarium oxysporium*	Joo (2005)

Entre los muchos ejemplos que encontramos se puede apreciar la mejora de métodos más antiguos. Como en la investigación de Illueca et al. (2023) donde se evidenció que con un cultivo iniciador de masa madre controlado añadido a producto panario pudo alargar la vida útil ante la contaminación de hongos y micotoxinas. Todo esto en comparación a otros tipos de masa madre comerciales y también a una masa madre hecha de la microbiota autóctona de la harina.

Otro ejemplo de uso es la aplicación del microorganismo directa al alimento, pero sin necesidad de transformarlo en un derivado mediante una fermentación previa. En el estudio Pantelides et al. (2015) analizó la actividad de diferentes cepas de levaduras aisladas de uvas para la elaboración de vino (*Vitis vinifera*) frente al crecimiento de hongos contaminantes de este alimento. Tras una selección *in vitro* de las cepas de levadura con mayor actividad antagonista se evidenció que un recubrimiento con una cepa de la levadura *Aureobasidium pullulans* lograba reducir la contaminación de *Aspergillus tubingensis* hasta un 94%. Este incremento en la preservación se conseguía sin alcanzar alteraciones notables en el alimento. Otro ejemplo es el uso de microorganismos contaminantes que son una mejor alternativa a la contaminación natural, es decir, cepas de microorganismos contaminantes que compiten por el sustrato, pero no crean ningún tipo de metabolito dañino para el ser humano. En estos casos la aplicación es especialmente útil en países en vías de desarrollo. En algunos lugares de África se emplean cepas no toxigénicas de *Aspergillus* para evitar el crecimiento de cepas toxigénicas en los cultivos de trigo. Estas cepas no toxigénicas se han seleccionado especialmente por su alta competición por el sustrato y la capacidad de no hibridar con las cepas toxigénicas autóctonas. Gracias a ello se consiguen cultivos sin presencia de toxinas y a un coste muy bajo (Kagot et al., 2019).

Finalmente, la última categoría clasificada como métodos de bioconservación es la aplicación no del microorganismo, sino, de los metabolitos o productos metabolizados. Este tipo de bioconservación es la más recurrente en la bibliografía en comparación a las anteriores. Mediante esta técnica se ha logrado biopreservar diferentes matrices como frutas, panes y otros alimentos. Izzo et al. (2020) evidenció que el empleo de un sobrenadante libre de bacterias (SLB) proveniente de un suero de leche fermentado por bacterias ácido lácticas (BAL) presentaba actividad contra hongos contaminantes. Este SLB cuando era aplicado en pruebas *in vitro* lograba detener el crecimiento de hongos contaminantes de alimentos, pertenecientes a los géneros *Aspergillus*, *Penicillium*, and *Fusarium*. También se han desarrollado técnicas donde interviene la purificación de un metabolito para su empleo. Como el uso de un ciclo-péptido, "cyclo-(l-leucyl-trans-4-hydroxy-l-prolyl-d-leucyl-trans-4-hydroxy-l-proline", producido del cocultivo de una cepa de *Alternaria* spp*. y Phomopsis* spp. Con el que se pudo de inhibir el crecimiento de *Gaeumannomyces graminis, Rhizoctonia cerealis, Helminthosporium sativum* y *Fusarium graminearum* (Li et al., 2014).

Otra herramienta importante en la que bioconservación que también puede ayudar es la detoxificación de los alimentos reduciendo la presencia de las micotoxinas. Como se ha comentado en anteriores puntos la ausencia de crecimiento fúngico no implica la ausencia de micotoxinas. En ciertos alimentos susceptibles a este tipo de contaminación por toxinas ya se ha logrado encontrar microorganismos capaces de eliminar la presencia de estos metabolitos. Como las cepas de *Lactiplantibacillus plantarum* aisladas y estudiadas por El oirdi et al. (2023), donde se demostró su capacidad para degradar patulina presente en zumo de manzana. O el estudio Hua et al. (2014) donde se determinó la capacidad de un COV producido por una cepa de la levadura, *Pichia anomala,* de inhibir la producción y expresión de aflatoxina por *A. flavus*. No obstante, cabe mencionar que en la Unión Europea tan solo hay dos patente aprobadas para este uso y todas se limitan a alimentación animal (Official Journal of the European Union, 2009).

Entre todos los tipos de microorganismos estudiados con este objetivo, en la bibliografía destaca uno por encima del resto, las bacterias ácido lácticas. Diversos estudios recogen que estos microrganismos son posiblemente los más eficaces para aplicaciones de bioconservación. Es más, también poseen una serie de características que las convierten en agentes de interés para su aplicación de forma directa e indirecta en alimentos de consumo humano (Barcenilla et al., 2022; Ibrahim et al., 2021; Leyva Salas et al., 2017).

1.3. Bacterias ácido lácticas (BAL)

Las bacterias ácido lácticas se comprenden como grupo heterogéneo de bacterias que comparten una serie de características: forma de coco o bacilo, Gram positivas, catalasa negativo, inmóviles, no esporuladas, anaeróbicas facultativas y productoras de ácido láctico, metabolito que le da el nombre a este grupo de microorganismos (Munoz et al., 2011).

Todos los géneros que comprenden este grupo están clasificados taxonómicamente dentro del filo Firmicutes, clase *Bacilli*, orden *Lactobacillales* y familia *Lactobacillaceae*. Antiguamente solo se reconocían 4 géneros distintos dentro de este grupo de bacterias, *Lactobacillus*, *Pediococcus*, *Leuconostoc*, y *Streptococcus*. Esta clasificación fue realizada en función a su capacidad de fermentar distintos azucares y a su temperatura optima de crecimiento (Ayivi et al., 2020). Gracias a los avances en la genética y la taxonomía en la actualidad se reconocen un total de 23 géneros destinitos, muchos de ellos han surgido de estudios más exhaustivos que han logrado diferenciar genéticamente los antiguos 4 géneros en más: *Amylolactobacillus*, *Acetilactobacillus*, *Agrilactobacillus*, *Apilactobacillus*, *Bombilactobacillus*, *Companilactobacillus*, *Dellaglioa*, *Fructilactobacillus*, *Furfurilactobacillus*, *Holzapfelia*, *Lacticaseibacillus*, *Lactiplantibacillus*, *Lapidilactobacillus*, *Latilactobacillus*, *Lentilactobacillus*, *Levilactobacillus*, *Ligilactobacillus*, *Limosilactobacillus*, *Liquorilactobacillus*, *Loigolactobacilus*, *Paucilactobacillus*, *Schleiferilactobacillus*, y *Secundilactobacillus* (Zheng et al., 2020).

Las BAL son bacterias ubicuas, presentes de forma habitual en prácticamente toda la naturaleza. En el suelo, agua, en los alimentos de origen vegetal y de origen animal además conviven en el tracto intestinal y urogenital de humanos y otros animales (Liu et al., 2014). Su presencia en todos estos nichos biológicos se debe a su gran adaptación a todos ellos, su rapidez a la hora de colonizar estas matrices y finalmente que producen una serie de metabolitos y alteraciones en el medio que impiden o dificultan la colonización por otros microorganismos (Stellato et al., 2015).

1.3.1. Empleo de las bacterias acido lácticas en la conservación frente a hongos

Las BAL han sido empleadas en los alimentos desde la antigüedad. Su crecimiento en ellos los modifica de tal forma que adquieren nuevas cualidades organolépticas mediante distintos procesos bioquímicos como la liberación de lipasas o de enzimas proteolíticas. El resultado son productos con unos sabores y texturas muy característicos como el yogur, tofu o la salsa de soja (Tangyu et al., 2023). Pero como se ha mencionado anteriormente, esta modificación del alimento por parte del microorganismo no se limita al cambio en organolepsia de este, sino que alarga la

vida útil frente a contaminantes no deseados, es decir, las BAL son también capaces de ser utilizadas como agentes de bioconservación (Hernández-Figueroa et al., 2024).

Su alta presencia en diversos alimentos con un alto historial de consumición sin efectos adversos le confiere una gran ventaja respecto a otro tipo de agentes de biopreservación. Estos microorganismos son considerados QPS (*qualified presumption of safety*) en la Unión Europea por la Autoridad Europea de Seguridad Alimentaria (EFSA) y GRAS en Estados Unidos por la FDA (Cai et al., 2019). Por lo tanto, su empleo y adición en alimentos está considerado como seguro dentro de estas regiones, lo que facilita a la industria el escalado de los resultados experimentales a la aprobación y venta de productos innovadores dentro del mercado. Además, la etiqueta de QPS y GRAS no se limita a la aplicación propia de la bacteria, sino que también se puede extrapolar al empleo de matrices fermentadas por las BAL que su uso este aprobado en el mercado (Ryan et al., 2015).

Se considera que las BAL son capaces de evitar, reducir o retrasar, dependiendo del caso, la contaminación fúngica de los alimentos mediante varias vías que se pueden apreciar esquematizadas en **Figura 1** (Carr et al., 2002). La primera de ellas es la competición por la matriz. Su gran adaptación a los diversos sustratos en los que se encuentran les ha permitido desarrollar una serie de herramientas para evitar la colonización de la matriz por otros microorganismos. Además, tienen una tasa de crecimiento rápida en comparación con los hongos, lo cual les confiere una ventaja al colonizar las matrices (Canon et al., 2020). Dentro de esta primera vía de conservación empleada por las BAL se encuentra el aprovechamiento de los nutrientes del alimento, ya que consumen las fuentes de carbono y de nitrógeno de metabolización rápida como los monosacáridos y ciertas proteínas o péptidos. La ausencia de estos nutrientes impide o retrasa la contaminación por otros microorganismos (Lorn et al., 2021). También son capaces la colonizar sin alterar el sustrato. El ejemplo más conocido sería su presencia en el sistema intestinal de muchas especies de animales. Estos microorganismos no atacan el intestino del animal, sino que forman una sinergia con este mismo donde ellos colonizan el tracto y que aporta beneficios para ambas partes (Luz et al., 2021). Pero esta colonización sin alteración también se da en otras matrices, como los alimentos actuando como agente de conservación de frente a otros microorganismos (Rodrigues et al., 2021).

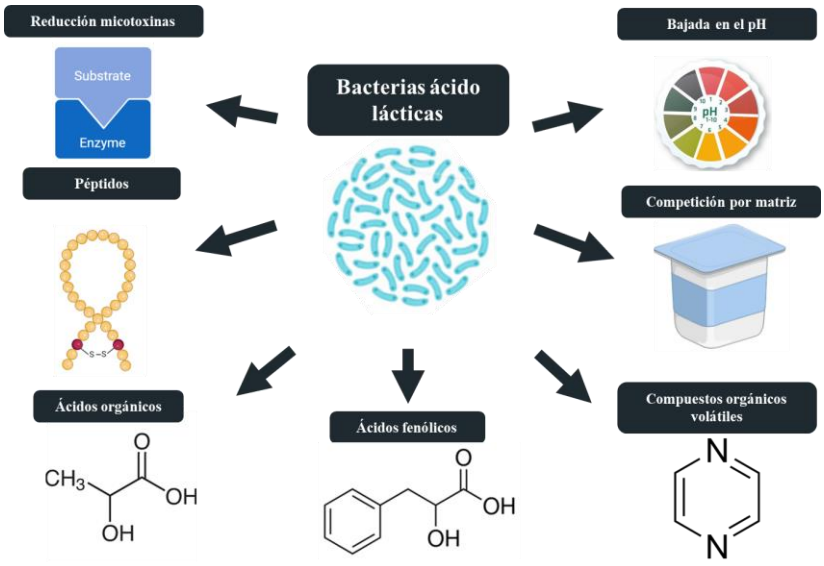

Figura 1. Esquema de las vías por las que las bacterias ácido lácticas ejercen su efecto de biopreservación contra hongos contaminantes de alimentos.

La segunda de las vías de bioconservación contra otros microrganismos es la bajada del pH. Las BAL tienen un crecimiento optimo a pH entre 6.0 y 7.0, algunas especies como Oenococcus *oeni* son capaces de crecer en pH entre 4.2 a 4.8, o ciertas cepas de *L. plantarum* entre valores de pH de 4.5 a 5.5; incluso ya en ejemplos más específicos se ha observado el crecimiento de algunas cepas de *Lactobacillus sakei*, *Lactobacillus brevis* y *Lactiplantibacillus plantarum* en valores por debajo de 3.0 (Muñoz et al., 2011). No obstante, tienen en común la capacidad de sobrevivir largos periodos de tiempo en pH por debajo de 4, pese a que su crecimiento se haya detenido (Śliżewska & Chlebicz-Wójcik, 2020). Durante su crecimiento las bacterias ácido lácticas producen una gran cantidad de metabolitos que bajan el pH del sustrato en el que habitan. Esta bajada del pH es un método de defensa contra la contaminación de otros microrganismos a este sustrato ya que inhibe el crecimiento de algunos de ellos y, en ciertos casos, hasta es capaz de tener actividad biocida (Schillinger et al., 2006). Respecto a la contaminación por los hongos, que es el tema que atañe a esta tesis, esta bajada de pH les afecta igual que al resto de microorganismos. Es cierto que algunas especies como *Aspergillus flavus* o *Penicillium italicum* presentan una alta capacidad de adaptación a pH bajos, no obstante, su germinación y crecimiento se ve retardada de forma significativa (Pitt & Hocking, 2022).

Finalmente, la última vía de preservación es mediante la producción de diversos metabolitos con capacidad antifúngica, que se va a explicar de forma más detalla en el siguiente punto.

1.3.2. Metabolitos con propiedades antifúngicas

La bibliografía recoge una gran cantidad de investigaciones donde se ha relacionado metabolitos producidos por las BAL con la actividad inhibitoria o antifúngica contra hongos contaminantes (Ibrahim et al., 2021). Para algunos de ellos, los mecanismos de acción se han elucidado y entendido de forma detallada, como es el caso del ácido láctico, pero para otros todavía se desconoce el método de acción exacto (Dalié et al., 2010). Cabe mencionar, que ninguno de estos metabolitos en las cantidades que son producidos por las BAL, ni ninguna de las tres vías de inhibición descritas tiene de forma individual la capacidad de tener la actividad antifúngica necesaria para preservar alimentos. Esta actividad contra los hongos surge de la sinergia entre las tres vías de acción (Muhialdin et al., 2020). En la **Tabla 6** se aprecian ejemplos de estos compuestos. A lo largo de los siguientes subapartados se va a comentar de forma más detallada los diferentes grupos de metabolitos antifúngicos que se muestran más en la bibliografía.

Tabla 6. Ejemplos de metabolitos registrados en la bibliografía producidos por las BAL y con actividad antifúngica o fungistática.

Compuesto Antifúngico	Bacteria Ácido Láctica Productora	Actividad Antifúngica	Referencias
Ácido láctico	Orden *Lactobacillales*	Inhibe el crecimiento y la germinación de esporas de hongos	Schillinger et al. (2006)
Ácido feniláctico	*Lactobacillus* spp. *Lactiplantibacillus plantarum*	Acción bactericida y fungicida contra hongos patógenos	Yang et al. (2019)
Bacteriocinas	Orden *Lactobacillales*	Acción bactericida y fungicida contra hongos patógenos	Siiskovic et al. (2010)
Nisina	*Lactococcus lactis*	Inhibe el crecimiento de bacterias Gram-positivas y hongos	Sung et al. (2023)
Ácido acético	*Lactobacillus* spp.	Actúa como conservante natural, inhibiendo el crecimiento de hongos	Anand (2011)
Acetato de Etilo	*Lactobacillus* spp. *Leuconostoc* spp.	Inhibe el crecimiento de hongos y levaduras	Abdel-Nasser et al., 2023)
Reuterina	*Lactobacillus reuteri*	Inhibe el crecimiento de hongos y levaduras	Pilote-Fortin et al. (2021)
Ácido propiónico	*Lactobacillus* spp.	Actúa como conservante natural, inhibiendo el crecimiento de hongos	Dagnas et al. (2015)
Pirazinas	*Lactobacillus* spp.	Actúa como conservante natural volátil, inhibiendo el crecimiento de hongos	Kratky et al. (2012)
Ácido benzoico	*Lactobacillus* spp.	Actúa como conservante natural, inhibiendo el crecimiento de hongos	Liu et al. (2018)

1.3.2.1. Ácidos orgánicos

Los ácidos orgánicos son los que mayor actividad se ha reportado en la bibliografía y de alguno de ellos se ha logrado entender completamente su mecanismo de acción en la actividad antimicrobiana. Los que son producidos en cantidades más abundantes por las BAL (el ácido láctico y en segundo lugar el ácido acético) tienen un método de acción similar (Oude et al., 2001).

Estos ácidos en su forma no disociada son hidrofóbicos y tienden a difundirse a través de la membrana plasmática, una vez dentro del citoplasma se disocian y bajan el pH intracelular. Esto les permite actuar de dos formas. Primero, la bajada del pH dificulta las diversas reacciones intracelulares del organismo. En segundo lugar, afecta causando un desequilibrio en el potencial electroquímico de la membrana, lo que aumenta la permeabilidad a otros compuestos y dificulta el buen funcionamiento de la célula. El mal funcionamiento de las reacciones intracelulares, sumado a la perdida de la correcta actividad de la membrana plasmática, puede inducir a la muerte celular (Dalié et al., 2010). Cabe mencionar que no es la única vía de acción de los ácidos orgánicos producidos por las BAL, un ejemplo es el ácido sórbico que inhibe la bomba de protones con actividad ATPasa de la membrana celular de ciertos microorganismos (Stratford et al., 2013).

Estudios como Guimarães et al. (2018) evidencian la capacidad de los ácidos láctico, acético e indolacético producidos por una cepa de *Lentilactobacillus buchneri* de presentar actividad antifúngica a contra *P. nordicum*. Los autores llegaron a la conclusión a la conclusión de que el ácido acético era el más eficaz de ellos. También se ha reportado esta capacidad antifúngica de otros ácidos orgánicos producidos por las BAL como el ácido propiónico. Artículos como Dagnas et al. (2015) muestran que también es capaz de inhibir el crecimiento de *Aspergillus* spp. y de *Penicillium* spp. y hasta tener actividades antifúngicas contra estos hongos.

1.3.3.2. Compuestos fenólicos

La actividad fungistática de los compuestos fenólicos es otra de las más estudiadas en la literatura dentro del campo de la bioconservación. La gran mayoría de ellos podrían estar considerados dentro del grupo de los ácidos orgánicos al presentar uno o más grupos “C(=O) OH” en su estructura molecular, como por ejemplo el ácido DL-3-fenilactico, el ácido benzoico (Badawi et al., 2015). Y ya en menor medida existen algunos que no presentan este grupo químico, como la quercetina (Aghababaei & Hadidi, 2023). La característica estructural común de estos compuestos es la presencia de un anillo benzoico, un grupo carboxílico y uno o más grupos hidroxilo o metoxilo en su estructura (Badawi et al., 2015).

En cuanto a este tipo de compuestos, todavía se desconoce su mecanismo de acción, no obstante, una mayor producción de algunos de estos compuestos por parte de las BAL (ácido DL-3-fenilactico, ácido benzoico y ácido 3-4-dihidroxihidrocinámico entre otros pocos) está intrínsecamente relacionada con una mayor actividad antifúngica. La bibliografía recoge diversos ejemplos de ello. Al que mayor actividad se le atribuye es al ácido DL-3-fenilactico (Mun et al., 2019). Se ha reportado actividades inhibitorias del crecimiento de hongos asociadas a este metabolito en concentraciones de 39–84 mmol/kg contra *Aspergillus* spp. y *Penicillium* spp. en

pan (Debonne et al., 2020). Actividades similares han sido evidenciadas de otros ácidos fenólicos tales como el benzoico que inhibe el crecimiento de *Fusarium* spp. en maíz. O pruebas *in vitro* que demuestran las bajas concentraciones necesarias del ácido 3-hidroxidecanoico y otros para alcanzar a tener actividad antifúngica contra hongos del género *Aspergillus*, *Pichia* y *Penicillium* (Broberg et al., 2007; Peyer et al., 2016).

1.3.3.3. Otros compuestos con carácter antifúngico

Además de los ya mencionados anteriormente, las BAL producen otros muchos compuestos con actividad contra los hongos. A ser catalasa negativos, las BAL deben expulsar continuamente todo el peróxido de hidrogeno producido al medio, lo cual, también interviene en su mecanismo de defensa (Schnürer & Magnusson, 2005). Algunas de ellas son capaces de producir etanol, diacetilo, acetoína y acetaldehído para fortalecer su actividad de inhibición contra otros posibles competidores microbianos como hongos y bacterias (Adeniyi et al., 2015). La reuterina es otro ejemplo de compuesto antifúngico con una gran actividad producido solo por algunas cepas de la especie *Lactobacillus reuteri*, este metabolito es reconocido por tener una gran actividad contra la proliferación de diferentes hongos. La reuterina, en alimentos como el yogur ha demostrado tener actividades antifúngicas en concentraciones 6.9 mM y se ha utilizado como agente antifúngico en el campo de forma eficaz (M. C. Sun et al., 2022; Vimont et al., 2019).

Por último, se han relacionado otros grupos de moléculas producidos por las BAL con actividad antifúngica, pero todavía se desconoce bastante sus mecanismos de acción y formas de aplicar o aprovechar estos metabolitos. Hay ejemplos en la bibliografía de ácidos grasos de cadena larga producidos por las BAL con actividad contra el crecimiento de hongos, como los ácidos 3-(R)-hidroxidecanoico, 3-hidroxi-5-cis-dodecenoico, 3-(R)-hidroxidodecanoico y ácido 3-(R)-hidroxitetradecanoico (Siiskovic et al., 2010). También se ha reportado el efecto contra el crecimiento de hongos de ciertos COV, como las pirazinas (Kratky et al., 2012). Los péptidos cíclicos son otro grupo de compuestos producidos por las BAL que han demostrado ser buenos agentes antifúngicos. Son un método de defensa similar a las bacteriocinas, solo que el objetivo de su acción no son bacterias sino los hongos (Bojarska et al., 2021). Diversos autores han sido capaces de relacionar la producción de estos péptidos en distintos medios de cultivo con actividades antifúngicas contra *Fusarium*, *Botritis*, *Alternaria*, *Penicillium*, *Aspergillus* y *Fusarium* (Crowley et al., 2013; Ström et al., 2002). Todavía queda por descubrir el complejo sistema por el cual, las BAL evitan, reducen o retrasar la contaminación fúngica y, por ende, se ha de investigar más profundamente. No obstante, queda evidenciado que esta faceta de las BAL es real y que pueden ser utilizadas en favor de la seguridad alimentaria como agentes contra la contaminación de hongos.

1.3.4. Reducción de micotoxinas mediante la acción de las BAL

Otro de los factores que hace interesante el estudio de las BAL en la lucha contra los hongos contaminantes de alimentos es su capacidad para reducir las concentraciones de micotoxinas en las matrices alimentarias. Se ha reportado un gran número de cepas que presentan esta actividad mediante varios mecanismos. El primero de ellos es la inhibición de la biosíntesis de las micotoxinas. Más allá de simplemente reducir el crecimiento del hongo y, a consecuencia, la aparición de micotoxinas, las BAL producen también una serie de metabolitos que inhiben la producción de las toxinas por parte de los hongos. Este fenómeno se ha constatado desde los años 90 y de forma consistente se ha podido replicar en investigaciones, ejemplo de ello es como se ha relacionado, mediante técnicas de expresión genética, la presencia de ácidos orgánicos en medios fermentados por BAL con la reducción en la síntesis de micotoxinas (Hassan & Bullerman, 1995; Moon et al., 2018).

La vía enzimática es otro de los mecanismos mediante los que se ha conseguido reducir el contenido en micotoxinas en ensayos *in vitro* y en ciertas matrices alimentarias. Se observan ejemplos de la reducción de aflatoxinas, zealenona y ochratoxina A entre otras. En algunos de ellos, se ha logrado determinar la actividad enzimática tras el efecto en la reducción de la micotoxina, como es el caso de la zealenona donde se ha reportado que ciertas cepas de BAL con alta actividades hidrolíticas sobre el grupo éster son capaces de eliminar este metabolito fúngico en ensayos *in vitro* (Chen et al., 2018). En otros ensayos no se ha logrado descubrir el mecanismo detrás de la degradación de las micotoxinas, no obstante, sigue siendo un resultado positivo y a tener en cuenta. Algunos estudios han evidenciado la capacidad de reducir la presencia de aflatoxinas hasta un 45% y 77% (Oluwafemi et al., 2010; Verheecke et al., 2016).

El último de los mecanismos es la adhesión de estos metabolitos a su pared celular de las BAL. Es una buena técnica de detoxificación, ya que una vez unidos a la pared mediante la eliminación de las bacterias con ciertos tratamientos, se ha logrado reducir drásticamente la biodisponibilidad de las micotoxinas en los alimentos en varios estudios (Dalié et al., 2010; Oliveira et al., 2014). Este mecanismo de eliminación se ha demostrado eficaz contra una gran variedad de micotoxinas como: aflatoxinas, ochratoxina A, tricotecenos y fumonisinas, entre otras (Bueno et al., 2007; El-Nezami et al., 2002; Niderkorn et al., 2006; Piotrowska & Zakowska, 2005).

1.4. Problemática de los residuos de la industria alimentaria

La industria alimentaria de forma intrínseca, durante la producción de los alimentos que suministra para su uso, genera una serie de residuos que no son de fácil eliminación a los niveles de producción que se alcanzan en la actualidad (Sattari et al., 2016). Muchos de estos residuos tienen grandes cantidades de nitrógeno, fósforo y potasio además de otros componentes con un gran impacto medioambiental. Sí no son tratados de forma correcta, suponen un gran daño para la naturaleza (Metson et al., 2021). Una de las formas más típicas de calcular el impacto de estos residuos es medir la huella de carbono. Las medidas de tratamiento de residuos que normalmente se emplean en la actualidad producen elevadas emisiones de CO_2 (Lal, 2020). Estos métodos de eliminación de residuos necesitan una descontaminación apropiada para cada tipo de producto (Gaspar & Braga, 2023). Existe una gran cantidad de residuos industriales que con el tratamiento correcto pueden llegar a tener un valor adicional. En los siguientes subapartados se verán ejemplos de esto.

1.4.1. El suero de leche

El suero de leche es el principal residuo generado por la industria láctea. Es un desecho formado durante la producción del queso. Es producido en la etapa del desuerado del cuajo, donde se separa la masa que va a ser el futuro queso del resto de componentes de la leche. El suero lácteo es el efluente producido de esta separación (Prazeres et al., 2012). De media, por cada 1 L de leche empleada en la producción de queso se producen un total de 0.9 L de suero lácteo (Luján-Facundo et al., 2017). El queso es uno de los alimentos más consumidos a lo largo del mundo, se estima que la industria genera unos 11 millones de toneladas anuales de este producto. Existen alternativas a su depurado, no obstante, prácticamente la mitad de estas toneladas ha de tratarse como aguas residuales, dejando una gran huella de CO_2 (Verma & Subudhi, 2021a).

El suero lácteo suele clasificarse en dos tipos: suero dulce, cuando su pH oscila entre 6.2 y 6.4, normalmente está formado en procesos donde para la elaboración del cuajo se han empelado solo enzimas. El segundo de los tipos es el suero acido, con un pH entre 4.6 y 5.0, en estos casos además de las enzimas, se emplean bacterias ácido lácticas en la producción del cuajo, las cuales bajan este pH (Lavelli & Beccalli, 2022). Suele tener una composición nutricional que varía entre un 93 a 94% de agua, 4.4 a 5.4% azucares (principalmente lactosa), un contenido entre 0.6 a 1% de proteínas y finalmente un contenido en minerales entre 0.5 a 0.8% (este último varía mucho dependiendo del tipo de queso que se busque obtener (Jelen, 2002). Este alto contenido en nutrientes y micronutrientes de interés es lo que convierte a este residuo en un gran problema si se vierte de forma no controlada en la naturaleza, ya que altera los nutrientes presentes en la zona

de vertidos y, por lo tanto, el ecosistema. No obstante, esta misma cualidad es la que puede permitir su aprovechamiento en nuevos usos de interés (Casallas-Ojeda et al., 2021).

1.4.2. Bagazo de cerveza

Se conoce bagazo como el conjunto de residuos formados del triturado, macerado o prensado de frutos, semillas, tallos. Por ende, este residuo de obtiene de muchos procesos diferentes como la elaboración de azúcar, vino y diferentes tipos de bebidas (Farinella et al., 2007; Parameswaran, 2009). La cerveza es uno de estos alimentos que durante su producción se genera gran cantidad de bagazo. Dentro de la industria cervecera ese bagazo se compone principalmente de los restos generados tras el prensado de la malta macerada (Martínez et al., 2012). Las toneladas generadas de este residuo fluctúan mucho dependiendo de la metodología empleada por la industria para el filtrado, variando en cantidades de humedad de entre 65 a 80%. Se calcula que de cada hectolitro producido de cerveza se generan una media de 20 kg de este residuo (Castro-Criado et al., 2023). Esto supone un gran problema ya que el reaprovechamiento del bagazo para su uso en campo o alimentación animal sería posible a pequeña escala, no obstante, la cerveza es la tercera bebida más consumida en el mundo, solo después del agua y el té, y encabeza la lista entre las bebidas alcohólica más consumidas mundialmente (Habschied et al., 2020).

La composición del bagazo de cerveza es altamente irregular debido a las diferentes técnicas y materias primas empleadas en su composición. Pese a que la malta es su principal materia prima existen también variaciones que emplean en mayor o menor cantidad otros ingredientes como el arroz, trigo y lúpulo. Estos detalles son importantes ya que cambian la composición del bagazo y, por lo tanto, los tratamientos a los que se le puede someter (Baiano, 2021). Sin embargo, generalmente se considera que la composición de macronutrientes del bagazo seco tiene de media un 20% de proteínas, un 70% de fibra (principalmente compuesto por lignocelulosa), un 13% de lípidos y un 3 % de cenizas. Respecto a otros compuestos de interés que se pueden encontrar existen diferentes vitaminas, amino ácidos esenciales y una gran cantidad de compuestos fenólicos útiles por su gran capacidad antioxidante (Mussatto, 2009). Por lo tanto, el bagazo de cerveza es también una matriz de gran valor para su reaprovechamiento, incluso dentro de la propia industria alimentaria.

1.4.3. Salvado de arroz

El arroz es un alimento básico para aproximadamente la mitad de la población mundial. Es producido en grandes cantidades en Oceanía, América, Europa, África y Asia. Se estima que debido al continuo incremento de la población mundial su producción y uso va a crecer (Prasad et al., 2017). La producción de este alimento genera una serie de subproductos no deseados en el tipo grano de arroz consumido generalmente. Tras la separación de la espiga, el grano de arroz continúa cubierto por una capa formada por diferentes componentes de la semilla: el pericarpio, la aleurona, la testa, junto con el germen y una pequeña porción de endospermo. Esta última capa es el salvado de arroz que de forma usual se elimina debido a que, por la oxidación lipídica, adquiere un tono marrón no deseado en el arroz. Cuando no es retirada se obtiene el conocido como arroz integral (Sharif et al., 2014). Debido a la alta preferencia del consumidor por el arroz blanco frente al integral, en la producción de este alimento se estima que anualmente se generan unos 30 millones de toneladas de este residuo (Sohail et al., 2017).

Parte del salvado se destina a alimentación animal, pero, de nuevo se presenta el mismo problema que con los anteriores residuos mencionados, su producción a nivel mundial es tan elevada que la generación del residuo supera con creces su eliminación mediante este método (Sohail et al., 2017). El salvado de arroz es una matriz rica en nutrientes. Su composición, que varía según el tipo de arroz, suele oscilar entre un 12 a 22% de grasas, 11 a 17% de proteínas, 6 a 14% de fibra, 10 a 15% de agua y de 8 a 17% cenizas. Al formar parte de la estructura de una semilla, también, es rica en diferentes micronutrientes y compuestos activos que pueden ser de interés para su explotación como vitaminas, niacinas y minerales tales como hierro y magnesio (Sharif et al., 2014). Por lo tanto, es importante el estudio de nuevas vías de utilización.

1.4.4. Aprovechamiento de los residuos de la industria alimentaria

Entre los objetivos propuestos por la Organización de las Naciones Unidas (ONU) a cumplir mundialmente de forma previa al 2030, el conocido como horizonte 2030, está la búsqueda de nuevos procesos que faciliten esta descontaminación y/o reducción de los residuos producidos por la industria, entre ellos la industria alimentaria (United Nations, 2015). Las soluciones propuestas para la reducción de residuos son la implementación de nuevos métodos más efectivos que ayuden a reducir las pérdidas intrínsecas a la producción en todos los puntos de la cadena. Es decir, mejor la protección del producto a nivel de campo, optimizar desde las técnicas de recolección, pasando por diversas etapas, hasta el punto final en super mercado y perfeccionar el envasado y almacenamiento de producto (Bhaskar et al., 2016). No obstante, por muchos adelantos que se realicen en el primer punto, va a haber una considerable generación de residuos industriales. A modo de ejemplo, en la producción del vino se genera gran cantidad de

residuos como el hollejo, residuo de piel, huesos, pulpas y tallos de la uva. Todos estos residuos no son fruto de unas malas prácticas en la producción, sino residuos que se generan inevitablemente por el procesado de este alimento (Farinella et al., 2007).

Muchos de estos residuos intrínsecos a la producción de alimentos tienen diferentes usos para su reincorporación dentro de la industria alimentaria u otros fines de provecho. Si los residuos alimentarios todavía contienen algún valor nutricional, como es el caso de los tres ejemplos descritos anteriormente, se procura que su valorización dentro de la economía circular se centre en la producción de piensos para animales (tanto ganadería como piscifactoría) o para la producción de abono (Cheng et al., 2021; Elferink et al., 2008). Otra opción es su utilización para la producción de compuestos de interés para la industria, ejemplo de esto es el uso de residuos con un alto grado de carbohidratos para la producción de etanol (Panahi et al., 2022). Finalmente, la última de las alternativas que más se emplea es su utilización como biocombustibles. Existen industrias que incluso han incorporado a su línea de producción el quemado de sus residuos para la generación de energía que utiliza la propia empresa. Sin embargo, esta alternativa incrementa la huella de CO_2 y, por lo tanto, su utilización no está especialmente recomendada.

Las producciones a escala no industrial como en el caso de las queserías o cervecerías artesanales pueden ser capaces de gestionar estos residuos mediante estas vías. No obstante, cuando se llega a las escalas de producción alimentaria para satisfacer las demandas de producción del mercado, la producción de residuos que se alcanza se incrementa tanto que siempre existirá un excedente que se ha de descontaminar mediante procesos menos dañinos para el medio ambiente (Ogino et al., 2007).

En este punto es donde entra en juego la investigación para poder ofrecer nuevas opciones a cómo emplear estos residuos de tal forma que entren dentro de un modelo de economía circular. Se han abierto muchos frentes de investigación para el uso de estos residuos. Un grupo de investigadores del Consejo Superior de Investigaciones Científicas (CSIC) ideó la forma de aprovechar el bagazo de cerveza para la producción de ladrillos utilizados en la construcción (Martínez et al., 2012). O en la revisión bibliográfica Gaspar & Braga (2023) se recogen muchos ejemplos de plásticos comestibles que han sido desarrollados a partir de residuos de la industria alimentaria. Dentro de estos posibles usos, los microrganismos fermentativos también pueden jugar un papel interesante, que no solo se limita a la producción de biocombustibles o etanol. Se han llegado a identificar cepas microbianas con capacidad de descontaminación de residuos y la producción de metabolitos de interés para la industria (Ma et al., 2021; Si et al., 2023).

1.4.5. Aprovechamiento de residuos para biocontrol contra hogos

Una de las vías de investigación dentro del aprovechamiento de residuos que más despiertan el interés en la actualidad es combatir la contaminación de los alimentos. Mediante el desarrollo de estas técnicas se puede mitigar muchos de los problemas asociados a la producción alimentaria. Se reduce la cantidad de residuos generados y se crean alternativas a la utilización de los conservantes de origen químico que el consumidor rechaza. Además, al estar en muchas ocasiones empleando alimentos en su reincorporación como ingredientes con capacidad antifúngica es más posible que se apruebe de forma legal y que no exista toxicidad asociada a su consumo (Leyva Salas et al., 2017; Si et al., 2023).

En la literatura se muestran diversos ejemplos que prueban la alta viabilidad de estos usos. En Yaseen (2020) describen el empleo de un fermentado con *Bosea thiooxidans* de diversos residuos de la agricultura que es capaz de generar compuestos de interés para el control de hongos a nivel de campo. A excepción de algunos ejemplos como este, lo más común es que esta vía de investigación utilice microrganismo dentro de las listas GRAS o QPS, como *Rhizopus oryzae*, *Saccharomyces cerevisiae* o las BAL (Afroz et al., 2021; Denardi-Souza et al., 2022; EFSA, 2024; FDA, 2024).

1.4.5.1. Empleo de bacterias ácido lácticas

Como ya se ha explicado anteriormente en anteriores apartados, las BAL son un excelente productor de metabolitos antifúngicos. Muchos artículos se centran en la producción de esos metabolitos en medios de cultivo desarrollados para una alta producción de estos o tan solo optimizar el crecimiento de la bacteria (Chen et al., 2021; Scano et al., 2021). Pero estos microorganismos pueden usar muchos sustratos diferentes para la metabolización de estos metabolitos, normalmente, la única condición para su crecimiento es un medio donde haya accesibilidad a monómeros o dímeros de hidratos de carbono fermentables y una alta cantidad de agua (Holzapfel & Wood, 2014).

Los residuos provenientes de la industria láctica cumplen estos requisitos y ya se pueden apreciar ejemplos en la literatura de su uso como agente antifúngico en ensayos, principalmente *in vitro* y algunos sobre alimentos donde se ha demostrado su efectividad contra el crecimiento de hongos contaminantes (Chen et al., 2021; Izzo et al., 2020). En los casos en los que no existen hidratos de carbono simples, con un ligero enriquecimiento del medio de cultivo, se puede lograr el crecimiento y posterior metabolización del residuo. Como en el estudio Ghosh et al. (2021) donde tras el desarrollo de un medio de cultivo de residuos de patata, se obtuvo un biopolímero con cualidades antifúngicas. También se ha conseguido dar uso a residuos de la industria del café

con un método similar (Alvarado-Ambriz et al., 2020). Con todos estos antecedentes, el desarrollo de nuevos agentes de bioconservación, mediante la fermentación por BAL de residuos de la alimentación, y las formas de aplicar estos agentes en los alimentos, se convierte en un objeto de estudio de interés con grandes perspectivas de unos buenos resultados.

2. OBJECTIVES

OBJETIVOS

2. OBJETIVOS

El **objetivo general** de esta tesis doctoral ha sido el uso de distintos residuos de la industria alimentaria (suero lácteo, bagazo de cerveza y salvado de arroz) para el desarrollo de agentes bioconservación contra hongos contaminantes de alimentos mediante la fermentación con bacterias ácido lácticas.

Para ello, se han planteado los siguientes **objetivos específicos**:

1. Evaluar en ensayos *in vitro* la actividad antifúngica de los diferentes residuos fermentados por las bacterias ácido lácticas contra distintos hongos contaminantes de alimentos pertenecientes a los géneros *Alternaria*, *Aspergillus*, *Botrytis*, *Fusarium* y *Penicillium*.
2. Caracterizar y cuantificar los principales metabolitos interviniendo en la actividad antifúngica presentada por el suero lácteo, bagazo de cerveza y salvado de arroz fermentados por las bacterias ácido lácticas para alcanzar una mayor comprensión del mecanismo de acción detrás.
3. Estudiar el empleo de el suero lácteo fermentado por bacterias ácido lácticas como agente de bioconservación mediante su adición un recubrimiento en separadores en lonchas de quesos contaminados por *P. commune, P. verrucosum y P. solitum*.
4. Estudiar la incorporación de suero lácteo, bagazo de cerveza y salvado de arroz fermentado por bacterias ácido lácticas como ingrediente en pan con el objetivo de alargar su vida útil frente a la contaminación de hongos pertenecientes a las especies *A. flavus*, *P. commune* y *P. verrucosum.*
5. Caracterizar la capacidad de los ingredientes a base de bagazo de cerveza y salvado de arroz fermentado por bacterias ácido lácticas de reducir la aparición de aflatoxina B1, B2, G1 y G2 en los panes contaminados por *A. flavus*.

2. OBJECTIVES

The general objective of this doctoral thesis has been the use of different waste from the food industry (whey, beer bagasse and rice bran) for the development of biopreservation agents against food contaminating fungi through fermentation with lactic acid bacteria.

To seek this objective, the following specific objectives have been set:

1. To evaluate by *in vitro* tests the antifungal activity of the different residues fermented by lactic acid bacteria against different food contaminating fungi belonging to the genera *Alternaria*, *Aspergillus*, *Botrytis*, *Fusarium* and *Penicillium*.

2. Characterize and quantify the main metabolites involved in the antifungal activity presented by whey, beer bagasse and rice bran fermented by lactic acid bacteria to achieve a greater understanding of the mechanism of action.

3. Study the use of whey fermented by lactic acid bacteria as a biopreservation agent by adding a coating to films in slices of cheese contaminated by *P. commune*, *P. verrucosum* and *P. solitum*.

4. Study the incorporation of whey, beer bagasse and rice bran fermented by lactic acid bacteria as an ingredient in bread with the aim of extending the shelf life against contamination by fungi belonging to the species *A. flavus*, *P. commune* and *P. verrucosum*.

5. Characterize the ability of ingredients based on beer bagasse and rice bran fermented by lactic acid bacteria to reduce the occurrence of aflatoxin B1, B2, G1 and G2 in breads contaminated by *A. flavus*.

3. RESULTS

RESULTADOS

International Journal of Dairy Technology, 75(3), 619-629 (2022)

3.1. Antifungal properties of whey fermented by lactic acid bacteria in films for the preservation of cheese slices

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1. INTRODUCTION

Currently, contamination by toxigenic fungi is one of the main interests for the food industry. Spoilage by these microorganisms not only deteriorates the food, but also releases a series of secondary metabolites, called mycotoxins, that are abundant in raw foods such as cereals and nuts (Fazekas, Tar and Zomborszky-Kovács, 2002). Within the group of toxigenic fungi, those belonging to the genus *Penicillium* are one of the main producers these metabolites. Mycotoxins have been related to a wide spectrum of pathophysiology, neurotoxic and nephrotoxic effects, lesions in the circulatory system, intestinal disorders, nephrotoxic, neurological, and genotoxic effects (Marin et al., 2013). Therefore, the preservation of food against these fungi is a major concern for the industry,

The use of synthetic antifungal compounds is the most common response from the industry to avoid fungal contamination. However, use of these compounds has been related to pathologies. Additionally, there is an increase of the consumer doubts towards this type of food preservatives and a rising demand for "healthier" foods (Hanafy et al., 2021). Therefore, new and safer methods are being studied to avoid fungal contamination of food.

The use of lactic acid bacteria (LAB) in the preservation against toxigenic fungi contamination is postulated as a great alternative to these synthetic origin compounds. Furthermore, the addition of LAB to food is allowed by the European Union, since they are classified as "Qualified Presumption of Safety" (QPS) by this entity (Leuschner et al., 2010). There are several examples in the bibliography of their application as preservatives against the toxigenic fungi and their toxins (Li et al., 2012; Mcnair et al., 2018; Ngolong Ngea et al., 2021). Such as the application from a *Lactobacillus plantarum* strain to bio-preserve grapes from *Aspergillus flavus* contamination (Lappa et al., 2018). Or the screening from several LAB for the preservation of bread against *Penicillium* genera contamination (Valerio et al., 2009).

Whey is one of the main wastes of the dairy industry. Processing of this leftover is a concerning problem for this industry. Whey is mainly processed as residual water or used as fertilizer and livestock feed (Verma and Subudhi, 2021). According to the objectives established by the World Health Organization (WHO) in the goals for 2030 for sustainable development, new methods must be found for the reuse of industrial waste, the circular economy (United Nations, 2015). Moreover, the use of whey fermented LAB as a bio-preservative against fungi is postulated as a correct recycle of this residue within the established objectives by the WHO. Additionally, the ideal method of preserving food is considered to be "easy to use" and economically viable (Leroy and De Vuyst, 2004). The combination of the use of whey (a waste from the food industry) as a culture medium for lactic acid bacteria is an option that falls within the considered ideal method to control fungal growth in food.

The objective of this study was to develop an active film with LAB fermented whey for a novel application as a bio-preservative of cheese. To achieve this aim a) the quantitative and qualitative antifungal activity of LAB fermented whey was tested, b) the different antifungal compounds present in the fermented culture were quantified; c) the antioxidative activities and total polyphenolic compound in the fermented culture mediums were studied; d) the antifungal potential of films with LAB fermented whey for the preservation of cheese slices.

2. MATERIALS AND METHODS

2.1. Chemicals

Media cultures used for the assays were Man Rogosa and Sharpe broth (MRS-B), Man Rogosa and Sharpe agar (MRS-A), potato dextrose broth (PDB) and potato dextrose agar (PDA) all purchased to Thermo Fisher Scientific Inc (Oxoid Limited, Hampshire, United Kingdom). The double deionised water was obtained from water purification system (Millipore Corp., Massachusetts, United States). Whey was whey provided by the ALCLIPOR company, S.A.L. (Benassal, Spain).

Acetic acid glacial (99%), Gallic acid (99%), and methanol (99.9%) (MeOH) were from VWR Chemicals (Radnor, United States). Lactic acid (99%), 2,2-Diphenyl-1-picrylhydrazyl and Folin Ciocalteu phenol reagent were purchased to Sigma-Aldrich (Missouri, United States). $Na2CO_3$ was obtained Millipore Corporation (Massachusetts, United States).

2.2. Microorganism

Bacteria used in this study were isolated and identified by the Colección Española de Cultivos Tipo (CECT) in Valencia, Spain, during previous investigations (Luz et al. 2020). Those microorganisms were: *Lactobacillus plantarum* 5L1 (5L1), *Lactobacillus plantarum* 5H1 (5H1), *Lactobacillus plantarum* HA1 (HA1), *Lactobacillus plantarum* HA2 (HA2), *Lactobacillus plantarum* F16 (F16), *Lactobacillus plantarum* F17 (F17) and *Lactobacillus plantarum* BN17 (BN17),

Fungi used were *Penicillium camemberti* CECT 2267, *Penicillium expansum* CECT 2278, *Penicillium roqueforti* CECT 2905, *Penicillium digitatum* CECT 2954, *Penicillium brevicopactum* CECT 2316, *Penicillium commune* CECT 20767, *Penicillium solitum* CECT 20818 from the CECT collection (Valencia, Spain), and *Penicillium verrucosum* VTT 01847, from the VTT Culture Collection (Espoo, Finland).

2.3. Preparation and fermentation of the whey

To obtain the cell-free supernatant first, a pre-inoculum of the bacteria was incubated in MRS-B for 24 h at 37 °C. After the incubation period, the pasteurised whey (70 °C for 20 min) was inoculated with a 5% (v/v) of the pre-inoculum and incubated for 72 h at 37 °C. Afterwards, the medium was centrifuged at 1500 g, 4 °C for 15 min. The cell-free supernatant was frozen at −20 °C and lyophilised (FreeZone 2.5 L, Labconco, MI, USA) for further use. The pH of the media was measured to ensure that the bacteria fermented the whey.

2.4. Antifungal activity assays by the qualitative method

The antifungal activity was evaluated as described in Dopazo et al. (2021) with a few modifications. Initially, the fermented whey was frozen, lyophilised and suspended to a 400 g/L concentration with sterile distilled water. Then, PDA plates were sown with fungal spores using sterile swabs, and wells were punched in the agar with sterile 1000-µL pipette tips. A 100 µL of the fermented whey was added to the wells, and plates were incubated for 48 h at 25 °C. After the incubation, the inhibition halos around each well were measured and marked as '−' when no inhibition was observed, '+' when inhibition was from 1 to 4 mm, '++'when inhibition was from 4 to 10 mm and '+++' when inhibition was greater than 10 mm. Whey at same concentrations was used as control.

2.5. Antifungal activity assays by the quantitative method

The quantitative antifungal activity of the fermented whey was measured using the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) methods as described in de Melo Nazareth et al. (2019) with some modifications. In 96-well plates, 100 µL of a fungal suspension of 10^5 spores/mL in PDB was mixed at 1:1 (v/v) ratio with different concentrations of the fermented whey (in final concentrations from 0.1 to 200 g/L). Simultaneously, a negative control (200 µL of sterile PDB) and a positive control (100 µL of sterile PDB mixed with 100 µL of the suspension of 10^5 spores/mL) were prepared. The plates were incubated for 72 h at 25 °C. Then, the MIC was identified as the minimal concentration in which there was a reduction in the fungal growth compared with the positive control. Four replicates were performed of each test.

To study the MFC, 10 µL of the MIC and higher concentrations were plated in PDA plates. The plates were incubated for 72 h at 25 °C. The MFC was determined as the minimal concentration in which an absence of fungal growth was observed.

2.6. Identification and quantification of organic acids in the fermented whey

For the determination of the main organic acids produced by the bacteria (acetic acid and lactic acid), the method described in Xu et al. (2021) was used with a few modifications. Samples of non-lyophilised fermented whey were diluted 1/20 with deionised water and filtered through a 0.22-µm pore size cellulose filter membrane before analysis. The high-performance liquid chromatography (HPLC) was equipped with a Jasco PU-4180 pump (Maryland, USA), a Jasco MD-4015 diode array detector (Maryland, USA), a Rezex ROA-Organic Acid (150 × 7.8 mm) reverse phase column (Phenomenex Inc., California, USA) and a 20-µL sample injection loop. The mobile phase was an isocratic solution of water and sulphuric acid 0.005 M, flowing at 0.8 mL/min for 10 min and a temperature of 40 °C. The detector was set at a wavelength of 214 nm for the quantification. All trials were performed per triplicate trials were conducted of each sample. Calibration curves were performed using lactic acid and acetic acid diluted in whey, at concentrations from 0 to 1000 mg/L. Results were expressed in g/L. Software used for the data acquisition and analyses was ChromNAV 2.0 HPLC (Jasco, Maryland, USA).

2.7. "In vitro" study of antioxidative activities and total phenolic compound present in the fermented whey

To study the antioxidant activity, 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay described by Khan et al. (2018) was used. Samples of non-lyophilised fermented whey were centrifuged at 12 000 g for 5 min, then filtered through 0.22-µm pore size cellulose filter membrane. The filtered samples were mixed with 1 mL of a 0.04 mM solution of DPPH in methanol and then vortexed for 1 min and incubated for 1 h. Finally, the absorbance of the samples was acquired at 517 nm in a LAMBDA 365 UV/Vis spectrophotometer (PerkinElmer Inc., MA, USA). Results were expressed as a percentage of the antioxidant activity. The estimation of the antioxidant activity 'P' was calculated with the equation 'P (%) = ((C–W)/C) × 100', in which C is the absorbance of the control, and W is the absorbance of the samples. The software used for the data acquisition and analyses was PerkinElmer UV WinLab (MA, USA).

The total phenolic compounds present in the fermented whey were analysed with the Folin–Ciocalteu method (Omedi et al. 2019). Samples were filtered following the steps described before. Then, 780 µL water was mixed with 130 µL of the fermented whey and 130 µL of Folin–Ciocalteu reagent and vortexed for 1 min. Afterwards, 130 µL of $NaCO_3$ solution at 20% (m/m) was added to the mixture and vortexed for 1 min, and tubes were incubated for 1 h in the absence of light. The absorbance was measured at 750 nm. Gallic acid at concentrations from 8.5 to 140 mg/L was used to calibrate the method. Results were expressed as equivalent mg of gallic acid/L.

2.8. Preparation of films carrying LAB-fermented whey

The fermented whey films were formed using the following steps. First, a polyvinyl alcohol (PVOH) water-soluble synthetic polymer was diluted at 15% (w/v) and mixed at continuous stirring conditions for 24 h, at a starting temperature of 90 °C, which decreased to 25 °C. After reaching 25 °C, the lyophilised fermented whey was added at a concentration of 20% (w/w) and kept at stirring conditions for 24 h. Then, the pH of the varnish was adjusted to 3.5 using lactic acid. The varnish was printed in a polyethylene terephthalate (PET) plate with a K control coater instrument (RK PrintCoat Instruments, Litlington, UK) with two different thickness: 12 µm (V1) and 24 µm (V2). For the control films the PVOH preparation pH was adjusted to 3.5 and printed following the same steps as the samples. Film are shown in **Figure 1**.

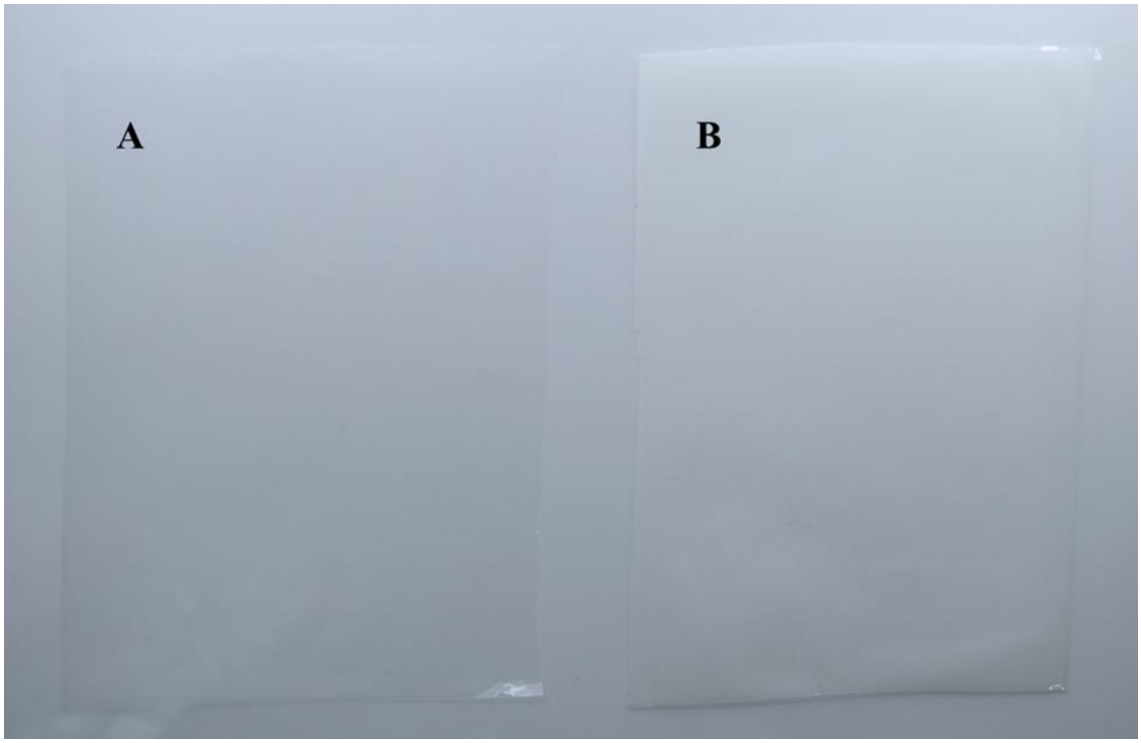

Figure 1. Film varnished with a coating thickness of 12 µm (a) and 24 µm (b). Coatings were prepared with 20% BN17-fermented whey (w/w) and 15% PVOH resin (w/w).

2.9. Evaluation of the antifungal activity of the active films carrying LAB-fermented whey

The antifungal activity of the antifungal separator was validated in solid medium following the steps exposed in Quiles et al. (2019) with some modifications. To carry out the test, the films were cut into squares of 1 cm^2 and placed in direct contact with plates of potato dextrose agar culture medium previously contaminated with 100 µL of a fungal suspension (10^5 spores/mL). After the inoculum had dried, the films were placed over the inoculum and the plates were incubated at 25 °C for 72 h. Fungal growth was observed each day.

2.10. Cheesemaking process

Cheese was manufactured under aseptic conditions in a Telstar MH 100 laminar flow cabinet (Terrassa, Spain). First, calcium chloride and citric acid were added at a concentration of 0.02% (v/v) to refrigerated milk (4 °C) and mixed until it reached a pH of 5.6. Then, the milk was heated to 30 °C and 0.1 mL of chymosin was added per liter of milk. The milk was left under stirring conditions until clotting occurred. After the coagulation, the curd was cut to increment the moisture loss and left to rest for 5 min. Finally, the shaping was performed in a circular mold and stored at 8 °C for 24 h for the maturation stage.

2.11. Evaluation of active films for the control of fungal decay of cheese slices

For the study of the antifungal activity of the films, the next steps were followed. First, the cheese was sliced under sterile conditions in a Telstar MH 100 laminar flow cabinet (Terrassa, Spain). Then, 10 µL of a conidial suspension (105 spores/mL) was inoculated in 9 spots over each cheese slice, after the spots dried films were placed over the slices. Afterwards, the cheese slices were sealed inside trays of ethylene vinyl alcohol and incubated at room temperature for 23 days. Each day, the number of spots with fungal colonies was observed. Results were expressed as '+' when more than a 50% of the spots presented fungal colonies and as '−' when less than a 50% of the spots presented fungal colonies. A total of 3 replicates were performed of each treatment.

2.12. Statistical analysis

The significant differences between the groups in the data have been analysed statistically using Tukey's multiple comparison test.

The software package used for the statistical analysis was InfoStat 2019 (Universidad Nacional de Córdoba, Córdoba, Argentina). The differences have been considered statistically significant at P values < 0.05.

3. RESULTS AND DISCUSSION

3.1. pH measurement

The pH values of the whey after 72 h at 37 °C fermentation are reported in Table 1. As expected, pH values of the LAB-fermented whey were significantly lower than the noninoculated control. The pH of the control sample was 6.19, while pH in the LAB-fermented samples reached a pH from 3.18 to 4.21. Many of the metabolites, such as lactic acid, acetic acid and phenyllactic acid, produced by this ferment reduce pH from the culture media and are reported to have antimicrobial activities (Dagnas *et al*. 2015; Le Lay *et al*. 2016; Luz *et al*. 2020).

Table 1. Measurement of the pH of the pasteurised whey fermented by LAB (72 h at 37 °C).

Samples	pH
Control whey	6.19 ± 0.01^{a}
L. plantarum BN17	4.21 ± 0.01^{b}
L. plantarum 5L1	3.60 ± 0.01^{bc}
L. plantarum 5H1	3.57 ± 0.01^{bc}
L. plantarum HA1	3.57 ± 0.01^{bc}
L. plantarum HA2	3.18 ± 0.01^{c}
L. plantarum F16	3.42 ± 0.01^{bc}
L. plantarum F17	3.68 ± 0.02^{bc}

3.2. Antifungal activity assays

The antifungal activity of the fermented whey was determined with a qualitative method, the agar diffusion test (**Table 2**). As it can be seen, the whey fermented by all 7 bacteria evidenced inhibition against a wide pool of *Penicillium* species. The whey fermented by the *L. plantarum* BN17 strain manifested the greatest antifungal potential by showing inhibition halos against 6 of the tested fungi. The greatest inhibition halos were also reported by the whey fermented by the *L. plantarum* BN17 reaching a diameter from 4 to 10 mm against *P. commune* and *P. verrucosum* species. The species *P. commune* and *P. verrucosum* were the most sensitive fungi against this treatment. On the contrary, *P. solitum* presented a high resistance to this application of the fermented whey as biocontrol agent, not being inhibited by any of the treatments. An example of the plates is shown in **Figure 2**.

Table 2. Inhibitory activity towards *Penicillium* genera of the whey fermented by different LAB (*L. plantarum* 5L1, *L. plantarum* 5H1, *L. plantarum* HA1, *L. plantarum* HA2, *L. plantarum* F16, *L. plantarum* F17 and *L. plantarum* BN17) determined by agar well diffusion assay. Inhibition halos were measured and marked as: '−' when no inhibition was observed, '+' when inhibition was from 1 to 4 mm and '++' when inhibition was from 4 to 10 mm.

Fungi	Samples							
	Whey	5L1	5H1	HA1	HA2	F16	F17	BN17
Penicillium camemberti	-	+	+	+	+	+	+	+
Penicillium expansum	-	+	+	+	+	+	+	+
Penicillium roqueforti	-	+	-	+	+	+	+	+
Penicillium digitatum	-	-	-	-	+	-	-	-
Penicillium brevicopactum	-	+	+	+	+	+	+	+
Penicillium commune	-	+	+	+	+	++	+	++
Penicillium solitum	-	-	-	-	-	-	-	-
Penicillium verrucosum	-	+	+	+	+	+	+	++

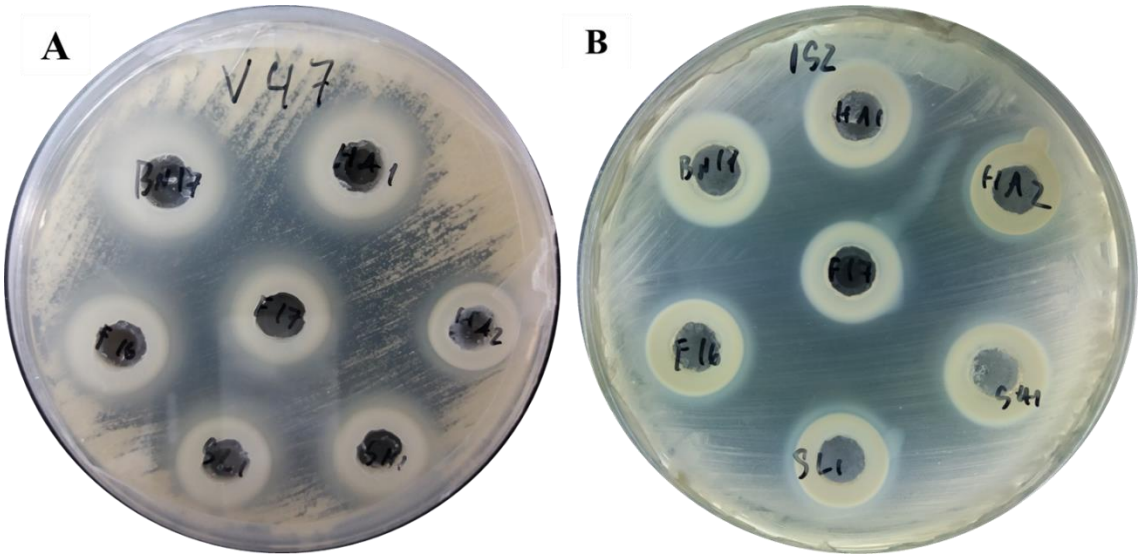

Figure 2. Agar well diffusion assay of the LAB-fermented whey against *Penicillium verrucosum* (a) and *Penicillium commune* (b) after incubation (48 h at 25 °C).

Table 3 shows the results of the MIC-MFC assay. The MIC values reported inhibition concentrations from 3.1 to 50 g/L and MFC values from 25 to >200 g/L. As in the results evidenced by agar diffusion test, *P. verrucosum* presented a high sensitivity against this the LAB fermented whey. Overall, the BN17 strain produced the most active fermented whey against *P. commune*, *P. solitum* and *P. verrucosum* reaching MIC from 3.1 to 25 g/L and MFC from 25 to 100 g/L. This 3 *Penicillium* spp. are typical fungal cheese contaminants (Pitt and Hocking 2009).

Table 3. Minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs) of the whey fermented by different LAB (*L. plantarum* 5L1, *L. plantarum* 5H1, *L. plantarum* HA1, *L. plantarum* HA2, *L. plantarum* F16, *L. plantarum* F17 and *L. plantarum* BN17) against several *Penicillium* spp.

Fungi	Sample													
	5L1		5H1		HA1		HA2		F16		F17		BN17	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
P. camemberti	6.3	200	3.1	100	3.1	100	25	100	3.1	200	12.5	>200	6.3	>200
P. expansum	12.5	200	6.3	25	12.5	200	12.5	25	6.3	>200	12.5	>200	6.3	>200
P. roqueforti	6.3	8	3.1	25	6.3	12.5	12.5	25	12.5	25	25	25	6.3	25
P. digitatum	3.1	50	6.3	50	12.5	50	12.5	50	25	100	12.5	100	12.5	>200
P. brevicopactum	6.3	50	6.3	25	3.1	25	12.5	25	25	25	25	25	6.3	12.5
P. commune	6.3	100	6.3	100	12.5	50	12.5	50	6.3	50	6.3	200	12.5	50
P. solitum	25	200	50	200	50	200	50	200	25	100	25	200	25	100
P. verrucosum	3.1	12.5	6.3	50	3.1	25	3.1	12.5	6.3	25	12.5	25	3.1	25

A total of 4 replicates were performed of each sample. MIC was determined as the concentration of fermented whey in which an absence of fungal growth was observed after the incubation (72 h 25 °C). MFC was considered as the concentration of fermented whey that presented an antifungal activity after 72 h at 25 °C incubation. Results are given in g/L.

The lactic acid bacteria have exhibited a great antifungal potential against different *Penicillium* species (Bianchini and Bullerman 2009; Lai et al. 2022). Overall, results obtained by other fermented mediums show similar or lower inhibitions against *Penicillium* genera (Ramos-Pereira et al. 2021). Uses of cell-free supernatants fermented by LAB, such as the MRS-B fermented by LAB in Magnusson and Schnürer (2001), evidence lower inhibition halos. In Yépez et al. (2017), a MRS-fermented cell-free supernatant by LAB isolated from vegetables evidenced halos around 5 mm. Other treatments, such as the purification of 2,4-di-tert-butylphenol, a volatile organic component with antifungal activity, produced by LAB showed inhibition halos of 15 mm against *Penicillium* spp. (Varsha et al. 2014). There are several reports of MRS-fermented cell-free supernatants reaching MFC from 12.5 to 100.0 g/L against *Penicillium* (de Melo Nazareth et al. 2019; Luz et al. 2020). Some purified natural compounds presented antifungal activities

against *Penicillium* genera such as the use of reuterin and natamycin that evidenced MFC 0.28 and 1.30 g/L, respectively (Pilote-Fortin et al. 2021).

3.3. Identification and quantification of organic acids and in vitro study of antioxidative activities and total phenolic compound present in the fermented whey

Organic acids were quantified in the whey fermented by the LAB. As it can be seen in **Table 4**, the concentration of lactic acid significantly increased from 4.01 to 6.98 g/L in all the fermented whey compared with the nonfermented control (0.27 g/L). Sample BN17 produced the highest concentration of this acid. Acetic acid was not detected in any of the samples.

Table 4. Detection and quantification of potential antifungal organic acids in the fermented whey by the different *Lactiplantibacillus plantarum* strains (*L. plantarum* 5L1, *L. plantarum* 5H1, *L. plantarum* HA1, *L. plantarum* HA2, *L. plantarum* F16, *L. plantarum* F17 and *L. plantarum* BN17).

Sample	Lactic acid	Acetic acid
Control	0.27 ± 0.15^{e}	<LOD
5L1	4.01 ± 0.22^{d}	<LOD
5H1	6.58 ± 0.02^{a}	<LOD
HA1	4.14 ± 0.01^{d}	<LOD
HA2	5.14 ± 0.09^{bc}	<LOD
F16	4.80 ± 0.05^{c}	<LOD
F17	5.95 ± 0.34^{b}	<LOD
BN17	6.98 ± 0.58^{a}	<LOD

Data under the limit of detection were marked as '<LOD'. Values are written as means ± standard errors (n = 3). Statistically significant differences for each sample are indicated with different letters ($P < 0.05$). Results are given in g/L.

The study of the total polyphenol content and antioxidant activity was performed on the whey fermented by *L. plantarum* BN17. As shown in **Table 5**, the LAB fermentation significantly increased the antioxidant activity and total phenolic content present in the media. The LAB-fermented medium incremented the DPPH free radical scavenging activity by a 22% compared with the nonfermented whey. BN17-fermented medium reached an 84% of antioxidant activity. The number of phenolic compounds in the fermented whey was 15.48 mg of gallic acid equivalents/L, a significantly greater amount than in the nonfermented control (3.31 mg/L).

Table 5. Results of the antioxidant activity obtained by the DPPH free radical scavenging assay and the total phenolic content studied by the Folin–Ciocalteu method of the *L. plantarum* BN17-fermented whey. Results of the DPPH free radical scavenging assay were expressed as the % of reduction of the DPPH+ and the results of the Folin–Ciocalteu method were expressed as mg equivalents of gallic acid per mL of sample.

Sample	% Reduction of DPPH radical	Total phenolic content (GA mg/L)
Whey	62.34 ± 3.45^{a}	3.31 ± 0.90^{a}
BN17	84.48 ± 0.80^{b}	15.48 ± 2.71^{b}

Statistically significant differences for each sample are indicated with different letters ($P < 0.05$).

Bibliography recognizes lactic acid as one of the main sources of the antifungal activity produced by the LAB. Magnusson et al. (2003) suggest that this activity is due to the synergistic activity of lactic acid with other LAB-produced acids. Other studies correlate higher concentrations of lactic acid with an improvement in the antifungal potential from different LAB strains (Schnürer and Magnusson 2005; Sadiq et al. 2019). The mechanism of action is well known that the lactic acid permeabilizes the membrane of different microorganisms; as a result of this permeabilization, other antifungal compounds can enter the cell (Alakomi et al. 2000). Moreover, in higher concentrations, the presence of lactic acid is related to a bacteriostasis and even leads to death of the microorganisms (Dalié et al. 2010).

Lactic acid bacteria produce a wide spectrum of different antioxidant compounds during their fermentation process (Qi et al. 2021). The production of these metabolites is the natural response of the LAB against the oxidative stress (Feng and Wang 2020). This activity is given by various molecules such as lactate oxidase, catalases, superoxide dismutase, exopolysaccharides, ferulic acid and carotenoids (Jeong et al. 2021). The study of the DPPH radical-scavenging activity performed on MRS broth by a different *L. plantarum* strain evidenced relative antioxidant activities of 76% (Won et al. 2021). Other authors report that the direct antioxidant activity of LAB ranged values from 20 to 30% (Jeong et al. (2021). Several studies also report the capacity of the LAB to increase the total phenolic compound concentration, such as Mantzourani et al. (2019), where the fermentation of a pomegranate juice with a *L. plantarum* evidenced higher phenolic concentrations than the nonfermented juice, or in Ferronatto et al. (2021), where also a LAB fermentation increased the presence of phenolic compounds in an ingredient made of rice. Moreover, the presence of these phenolic compounds selectively inhibits the growth of pathogenic microorganism (Pacheco-Ordaz et al. 2018).

3.4. Evaluation of the antifungal activity of the active films carrying LAB-fermented whey

Results of the antifungal evaluation of the films are shown in **Table 6**. An *in vitro* test showed values that correspond to the antifungal activity tests of agar diffusion and MIC-MFC. The studied film managed to delay the growth of *P. verrucosum* one day compared with the control film and 2 days compared to the control with no film. The antifungal effect of plates after 2 days of incubation is shown in **Figure 3**.

Table 6. Antifungal activity of the *Lactiplantibacillus plantarum* BN17-fermented whey films against *Penicillium* spp. Film control V1 had a PVOH coating acidified to a 3.5 pH. Film V1 has a PVOH + fermented whey coating. Both coatings were printed on the films with a 12 µm thickness.

Fungi	Samples		
	No film control	Film control V1	Film V1
P. commune	**1**	**2**	**2**
P. solitum	**1**	**2**	**2**
P. verrucosum	**1**	**2**	**3**

Films of 1 cm^2 were placed over a spore solution on the surface of the PDA plates. Results were expressed as number of days without visual fungal germination.

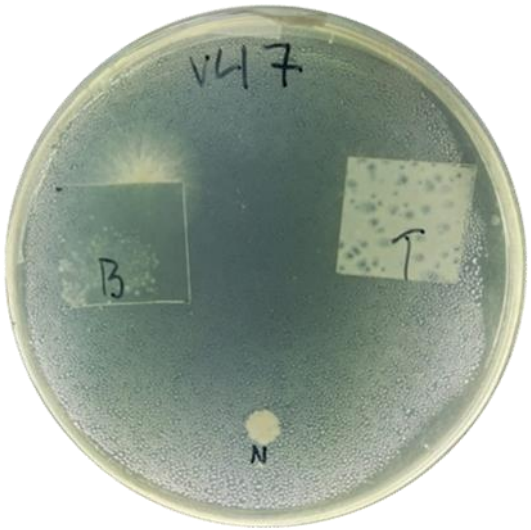

Figure 3. Antifungal effect of film V1 after 2 days of incubation at 25 °C against *Penicillium verrucosum*.

There are other examples of the use of other products as biopreservatives incorporated through film and tested by *in vitro* assays. Natamycin films have been proved to inhibit *Rhodotorula mucilaginosa*, or carvacrol, an essential oil from oregano and other plants, films evidenced an inhibition in the growth of some *Penicillium* and *Rhizopus* species (Klinmalai et al. 2021; Naderi Bab Anari et al. 2022).

3.5. Evaluation of active films for the control of fungal decay of cheese slices

The results of the evaluation of the potential of the films to control fungal contamination in cheese slices are shown in Table 7. The most effective film against all three *Penicillium* spp. was the V2 film (film coated with a 24 μm thickness). All films evidenced an average reduction of 11 days of the fungal growth, compared to the control without film. The film V2 managed to extend the shelf life of the cheese slices of 15 days in samples contaminated with *P. commune*, of 13 days in samples contaminated with *P. verrucosum* and of 14 days in samples contaminated with *P. solitum*, compared to the control slices without film. Moreover, film V2 exhibited an increase in the shelf life of 4 days in the samples contaminated with *P. commune*, 5 days in the samples contaminated with *P. verrucosum* and 10 days in the slices contaminated with *P. solitum*, compared with the control film V2. Overall, the film V2 delayed fungal growth for an average of 20 days against all 3 *Penicillium* species studied, especially against *P. commune* inhibiting the fungal occurrence of 23 days **Table 7**. **Figure 4** shows cheese slices inoculated with *P. commune* after 10 and 20 days.

Table 7. Effectiveness of the antifungal fermented whey films in cheese slices against (a) *Penicillium commune*, (b) *Penicillium solitum* and (c) *Penicillium verrucosum* for 23-day storage. Films V1 and V2 had a 12 and 24 μm coating thickness, respectively.

a)					
Days	**No film control**	**Film control V1**	**Film V1**	**Film control V2**	**Film V2**
1	-	-	-	-	-
2	-	-	-	-	-
3	-	-	-	-	-
4	-	-	-	-	-
5	-	-	-	-	-
6	-	-	-	-	-
7	-	-	-	-	-
8	+	-	-	-	-
9	+	-	-	-	-
10	+	-	-	-	-
11	+	-	-	-	-
12	+	-	-	-	-
13	+	-	-	-	-
14	+	-	-	-	-
15	+	-	-	-	-
16	+	-	-	-	-
17	+	-	-	-	-
18	+	-	-	-	-
19	+	-	-	-	-
20	+	+	-	+	-
21	+	+	+	+	-
22	+	+	+	+	-
23	+	+	+	+	+

b)					
Days	**No film control**	**Film control V1**	**Film V1**	**Film control V2**	**Film V2**
1	-	-	-	-	-
2	-	-	-	-	-
3	-	-	-	-	-
4	-	-	-	-	-
5	-	-	-	-	-
6	-	-	-	-	-
7	+	-	-	-	-
8	+	-	-	-	-
9	+	-	-	-	-
10	+	-	-	-	-
11	+	-	-	-	-
12	+	-	-	-	-
13	+	-	-	-	-
14	+	+	-	-	-
15	+	+	-	+	-
16	+	+	-	+	-
17	+	+	-	+	-
18	+	+	+	+	-
19	+	+	+	+	-
20	+	+	+	+	+
21	+	+	+	+	+
22	+	+	+	+	+
23	+	+	+	+	+

c)					
Days	**No film control**	**Film control V1**	**Film V1**	**Film control V2**	**Film V2**
1	-	-	-	-	-
2	-	-	-	-	-
3	-	-	-	-	-
4	+	-	-	-	-
5	+	-	-	-	-
6	+	-	-	-	-
7	+	+	+	+	-
8	+	+	+	+	-
9	+	+	+	+	-
10	+	+	+	+	-
11	+	+	+	+	-
12	+	+	+	+	-
13	+	+	+	+	-
14	+	+	+	+	-
15	+	+	+	+	-
16	+	+	+	+	-
17	+	+	+	+	+
18	+	+	+	+	+
19	+	+	+	+	+
20	+	+	+	+	+
21	+	+	+	+	+
22	+	+	+	+	+
23	+	+	+	+	+

Results were expressed as '+' when more than a 50% of the spots presented fungal colonies and as '−' when less than a 50% of the spots presented fungal colonies. Each experiment was replicated 3 times. A total of 9 spots of a fungal solution were inoculated over each replicate.

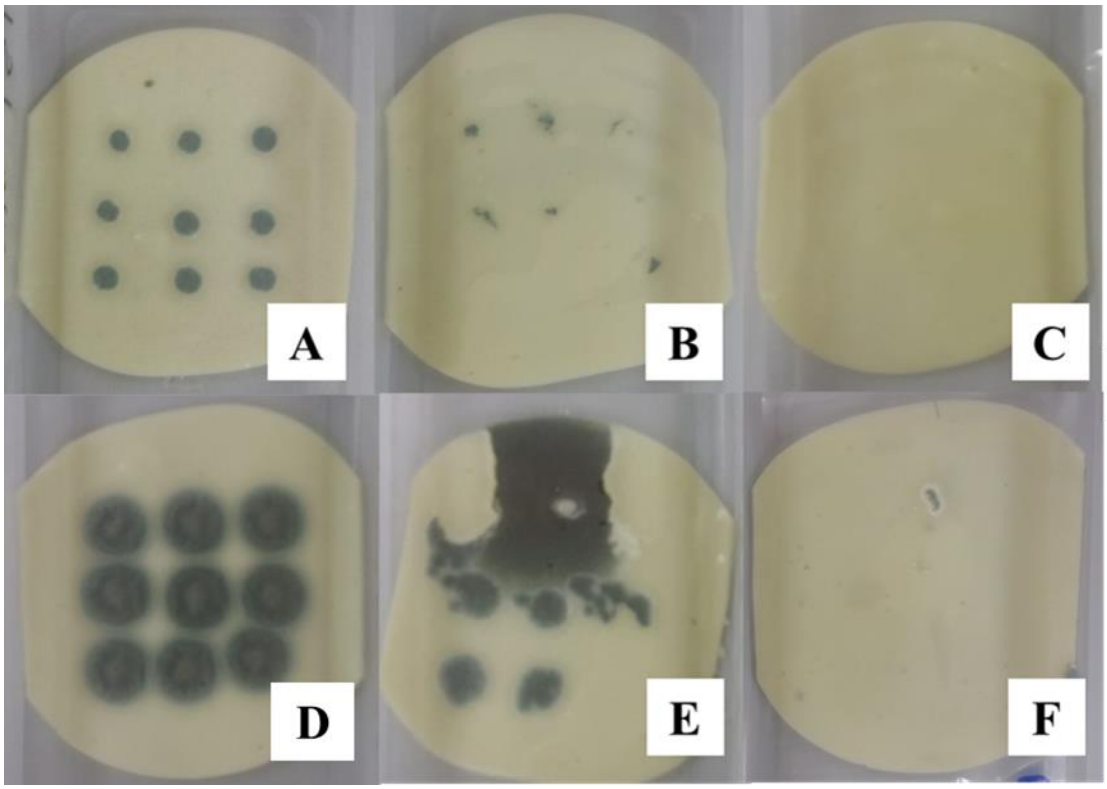

Figure 4. Cheese slices inoculated with *Penicillium solitum* after an incubation of 10 (a, b, c) and 15 (d, e, f) days covered with the whey-fermented films. (a) and (d) are nonfilm controls; (b) and (e) are control film V2; and finally, (c) and (f) are film V2.

Two factors are key in the shelf life prolongation of the sliced cheese produced by all films, even the control films, compared to the non-film control. First, PET plastic used for the film production has a low oxygen permeability. All *Penicillium* species are aerobic; therefore, the reduction in this gas contributes to a decrease in the spore germination (Pitt and Hocking 2009). Moreover, the low pH contributes to the reduction in the fungal germination (Pitt and Hocking 2009). This work evidence that the LAB-fermented whey may increase the shelf life of these foods compared with a regular film. This is the first report of a bioactive film with antifungal properties against *Penicillium* genera in cheese. In Lund et al. (1995), the identification of fungi from cheese samples from different countries such as Italy, Switzerland, Denmark, France, Greece and the United Kingdom evidenced that 38% of the isolates were *P. commune*. Also, Ramos-Pereira et al. (2021) concluded that *Penicillium* spp. was the main contaminant of cheese in Spain. Reports of other antifungal films and coatings incorporating other natural compounds can be found in the literature. Among them, natamycin is the most studied antifungal agent added to films; studies such as Ture et al. (2011) and Fajardo et al. (2010) did not evidence any inhibition of *Penicillium* spp. with natamycin and reported a delay in the fungal sporulation of 14 days, respectively. Many other compounds report lower or similar antifungal activities applied to films

or packaging such as chitosan (Ortiz de Elguea-Culebras et al. 2019), chestnut extract (Kõrge et al. 2020) or cinnamaldehyde (Balaguer et al. 2013). Nevertheless, the extraction of these bioactive molecules includes several steps for the compound purification, which is far more complicated in comparison with the preparation of the fermented whey that only needs to be centrifuged and dried.

4. CONCLUSIONS

Based on the results, LAB-fermented whey when incorporated into packaging film can prevent fungal contamination and may have the potential to reduce the use of synthetic antifungal compounds in foods. *In vitro* assays evidenced the antifungal activity of the fermented whey by the strain against different *Penicillium* species. Moreover, the *L. plantarum* BN17 fermentation incremented the antifungal metabolite occurrence in the whey and the antioxidant activity in comparison to the control. Finally, the use of *L. plantarum* BN17-fermented whey films managed to delay the fungal germination in contaminated cheese slices for 20 days against three different fungal contaminants. The use of LAB-fermented whey for food preservation is a promising way to solve two industrial problems: finding new approaches for recycling one of the major wastes of the dairy industry; and finding an easy-to-use and cheap method for food preservation.

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3.2. Revalorization by lactic acid bacterial fermentation of goat whey from cheese industry as a potential antifungal agent

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1. INTRODUCTION

Contamination by toxigenic fungi is one of the main concerns for the food industry. These fungi not only deteriorate the food, also releases a series of toxic secondary metabolites, called mycotoxins, that are abundant in raw foods such as cereals and nuts (Romero-Sánchez et al., 2022). Within the group of toxigenic fungi, those belonging to the genus *Aspergillus* and *Penicillium* are the main producers of the most common mycotoxins in foods, aflatoxins (AF), especially aflatoxin B1 (AFB1) and aflatoxin B2 (AFB2), and ochratoxin A (OTA). The consumption of these mycotoxins has been related to a wide spectrum of toxigenic effects. Carcinogenesis, genotoxicity and problems in the reproductive and immune system by the contamination of AF. In the case of OTA these effects are neurotoxic, teratogenic and nephrotoxic effects among many others. (Marin et al., 2013).

The use of antifungals of synthetic origin is the common industry response to avoid fungal contamination (Císarová et al., 2020). However, use of these compounds has been related to pathologies. New methods are being studied to avoid fungal contamination of food. Additionally, all this leads to an increase in consumer doubts towards this type of food preservative and an increase in the demand for "healthier" foods (Hanafy et al., 2021). Innovative techniques like fermentation or addition of lactic acid bacteria (LAB) for the preservation against toxigenic fungi contamination and the reduction of the toxins is postulated as a great alternative to these synthetic origin compounds (Freire et al., 2020; Murugesan et al., 2015; Zheng et al., 2020). Furthermore, the addition of LAB to food is allowed by the European Union, since they are classified as "Qualified Presumption of Safety" (QPS) by this entity (Leuschner et al., 2010). There are several examples in the bibliography of their application as preservatives against the toxigenic fungi and their toxins, especially from the *Lactiplantibacillus plantarum* (*L. plantarum*) species (Li et al., 2012; Mcnair et al., 2018; Ngolong et al., 2021). Such as the application from a *Lactiplantibacillus plantarum* strain to bio-preserve grapes from *Aspergillus flavus* contamination (Lappa et al., 2018).

Whey is one of the main wastes of the dairy industry. The processing of this waste is one of the principal problems for these factories. These by-products are mainly processed as residual water or used as fertilizer and livestock feed (Verma & Subudhi, 2021). According to the objectives established by the World Health Organization (WHO) in the goals for 2030 for sustainable development, new methods must be found for the reuse of industrial waste, the circular economy (United Nations, 2015). Therefore, the use of whey fermented LAB as a bio-preservative against fungi is an interesting revalorization of this residue within the established objectives by the WHO. Additionally, the ideal method of preserving food is considered to be "easy to use" and economically viable (Leroy & De Vuyst, 2004). The use of a waste from the

industry to develop an easy to produce and to use ingredient, in addition to cheap ingredient that can be used to reduce fungal contamination and synthetic preservatives is an innovative idea for the food industry.

The objective of this study was to develop an ingredient made of LAB fermented whey for further application in food. To achieve this aim a) to identify the different antifungal compounds present in the fermented culture; b) to determine the quantitative and qualitative antifungal activity of different fermented whey-made bacterial culture mediums against MRS medium c) and to determine how the quantified antifungal compounds influenced in the MFC from the different fermented samples with multivariate dataset analysis.

2. MATERIALS AND METHODS

2.1. Chemicals and microorganism

Media cultures used for the assays were MRS broth (MRS-B), MRS agar (MRS-A), potato dextrose broth (PDB) and potato dextrose agar (PDA) all purchased to Liofilchem (Teramo, Italy). The double deionised water was obtained from water purification system (Millipore Corp., Massachusetts, United States). Whey was whey provided by the ALCLIPOR company, S.A.L. (Benassal, Spain).

Acetic acid glacial (99%), acetonitrile (99.9%), ethyl acetate (99.9%), formic acid (99%), (AcOH) and methanol (99.9%) (MeOH) were from VWR Chemicals (Radnor, United States). Ammonium formate, C18, magnesium sulphate (MgSO4) and sodium chloride (NaCl) were acquired from Sigma–Aldrich (Missouri, United States). Glucose, tryptone, sodium acetate, yeast extract, dipotassium phosphate and ammonium ferric acetic and lactic acid were provided from Sigma–Aldrich (Missouri, United States). Ferulic acid was purchased from MP Biomedicals (California, United States). Phenyllactic acid was obtained from BaChem (Weil am Rhein, Germany). All analytes had a purity of 95%.

Bacteria used in this study were isolated and identified in previous investigations by the Colección Española de Cultivos Tipo (CECT) in Valencia, Spain (Luz et al., 2020). Those microorganisms were: *L. plantarum* SC2, *L. plantarum* SC3, *L. plantarum* SC6, *L. plantarum* SC7, *L. plantarum* SC9, *L. plantarum* SC13 and *L. plantarum* SC20. The identification of the strains was performed from an active culture of the strain as described by Arahal et al. (2008) where an amplification of a specific section of the DNA by PCR of the isolate is performed, then analysed in EzTaxon and BLAST platforms and the identification is performed by the level of similarity to the closest already isolated strain in the data bases.

Fungi used were *Penicillium expansum* CECT 2278, *Penicillium digitatum* CECT 2954, *Penicillium commune* CECT 20767 from the CECT collection, *Penicillium verrucosum* VTT 01847, from the VTT Culture Collection (Espoo, Finland), and *Aspergillus flavus* ISPA 8111, from *Istituto di Scienze delle Produzioni Alimentari* (Bari, Italy).

2.2. Preparation and fermentation of the cell free supernatant (CFS)

The antifungal and metabolite production of three different mediums was studied and compared to fermented MRS-B. First tested culture media was whey (W1). Second media (W2) was whey complemented with 1% (w/V) glucose, 1% (w/V) tryptone, 0.5% (w/V) sodium acetate, 0.4% (w/V) yeast extract, 0.2 dipotassium phosphate, 0.2% (w/V) ammonium ferric citrate, 0.02% (w/V) magnesium sulphate and 0.005% (w/V) manganese sulphate. Finally, the third culture media (W3) was composed by whey diluted in distilled water 1/5 (v/V) complemented with the same nutrients as W2. After the preparation, the mediums were pasteurized for 30 min at 70 °C. The MRS-B was prepared as described by the producer.

To the production of the cell free supernatant first a pre-inoculum of the bacteria was incubated in MRS-B for 24 h at 37 °C. After the incubation period all four tested media were inoculated with a 5% (v/V) of the pre-inoculum and incubated for 72 h at 37 °C. Afterwards, the medium was centrifuged at 3000×g, 4 °C for 15 min in a 5810 R de Eppendorf centrifuge (Madrid, España). The cell free supernatant was frozen at −20 °C for further uses.

2.3. Identification and quantification of organic acids, phenolic compounds in the CFS

For the determination of the main organic acids produced by the bacteria, acetic acid and lactic acid, samples were diluted in double deionized water from an equipment Milli-Q (Millipore Corp., Massachusetts, United States) and filtered through a 0.22 μm pore size. Afterwards, the identifications and quantification were performed as described in Dopazo et al. (2023). All samples were prepared and injected per triplicate.

The analysis of the phenolic compounds was performed as described in after a QuEChERS extraction, as described in Brosnan et al. (2014) from 10 mL of the liquid samples. Then the dried extract were suspended un 1 mL of acetonitrile/water 1:9 (v/V) and analysed as described in (Dopazo et al., 2022). A total of 3 replicas of each sample were purified and injected.

2.4. Antifungal activity assays by qualitative method

The antifungal activity was evaluated following the next steps. Initially, the CFS was dried by lyophilization with a FreeZone 2.5 L equipmentmen (Labconco, Missouri, USA) and then suspended with distilled water to reach a final concentration 5 times higher than the original CFS. In PDA plates were sow with fungal spores using sterile swabs, and several wells were punched in the agar with sterile 1000 µL pipette tips, the wells had a dimension of 4 mm of heigh and a diameter on 9 mm. Then 100 µL from the concentrated CFS were added in the wells and plates were incubated for 48 h at 25 °C. After the incubation, the inhibition halos around the end of each well were measured and marked as: "-" when no inhibition was observed, "+" for inhibitions from 1 to 2 mm, "++" from 2 to 5 mm "+++" from 5 to 10 mm and "++++" for inhibitions superior 1 cm. Each culture medium at same concentrations was used as control (Dopazo et al., 2021).

2.5. Antifungal activity assays by quantitative method

The quantitative antifungal activity from the CFS was measured using the Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) method as described in de Melo Nazareth et al. (2019) adapted to this study. In 96-well plates 100 µL a fungal suspension of 105 spores/mL in PDB was mixed at 1:1 (v/V) ratio with different concentrations of de CFS, from 200 to 0.1 g/L. Simultaneously, A negative control (200 µL of sterile PDB) and a positive control (100 µL of sterile PDB mixed with 100 µL of the 105 spores/mL suspension), were prepared. Plates were incubated for 72 h at 25 °C. Then, the MIC was identified as the minimal concentration in which there was a reduction of the fungal grow compared to the positive control. Four replicas were performed of each test.

To the study of the MFC 10 µL of the MIC concentration and higher concentrations were plated in PDA plates, incubated for 72 h at 25 °C and the observed. The MFC was determined as the smallest concentration in which an absence of fungal grow was observed.

2.6. Statistical and data analysis

To evaluate significant differences among each sample a multiple comparation analysis was performed with the software InfoStat 2019 (Universidad Nacional de Córdoba, Córdoba, Argentina). A one-way analysis of variance (ANOVA) test followed by Tukey's HSD post-test was used to sport the differences between means ($p < 0.05$).

The principal component analysis (PCA) and the biplot was performed using the software MetaboAnalyst 5.0 (Wishart Research Group, Edmonton, Canada). This two statistical methods were adopted for a further understanding of the link between the detected antifungal compounds and the studied fungicidal activity from the CFS (Wang et al., 2023). The correlation analysis was performed with software MetaboAnalyst 5.0 (Wishart Research Group, Edmonton, Canada), it was conducted with a Pearson r test which compared the correlation of the obtained results in the experiments against an expected result from a calculated pattern. Only correlation patterns bellow - 0.8 were interpreted as strong negative correlations.

3. RESULTS AND DISCUSSION

3.1. Identification and quantification of organic acids, phenolic compounds in the CFS

The results obtained from the analysis of the main antifungal organic acids produced by the LAB can be seen in the **Table 1**. The concentration of lactic acid was significantly greater in the MRS-B medium in comparation to the whey mediums. Reaching concentrations from 15 to 18 g/L. The concentration of this acid among the different whey mediums was significantly lower. After the fermented MRS-B medium the culture medium W2 evidenced the second greatest concentration of lactic acid, followed by the culture medium W1. The culture medium W3 evidenced the lowest concentration of this acid. It can be also observed that the control from the whey-based medium already presents a concentration of this acid. Since the moment the whey is extracted from the milk transported and frozen for future fermentation the started cultures and autochthonous bacteria ferment the whey (Cosentino et al., 2018). The bacterial strain *L. plantarum* SC7 and *L. plantarum* SC13 evidenced the highest lactic acid production among all the LAB strains. The presence of acetic acid was below the limits of detection of the equipment for all the samples. The production of lactic acid is highly related to the sugar metabolization; therefore, it is an expected conclusion that among the whey based culture mediums the ones with more sugar had rising quantities of this metabolite (Carr et al., 2002). Also, the absence of lactose (as a result of the dilution of the whey) decreased the lactic acid production. These bacteria isolates were obtained from goat milk, usually LAB are adapted to the matrix they inhabit and their metabolism there is more synergized with the use of lactose as a main sugar.

Table 1. Results of the quantification from the analysis of lactic acid present in the fermented mediums analysed with a HPLC-DAD equipment. Data was displayed as mean ± SD of lactic acid g/L.

Sample	Lactic acid (g/L)			
	MRS	**W1**	**W2**	**W3**
Control	nd	2.27 ± 0.15 ^{dA}	2.31 ± 0.20 ^{fA}	0.67 ± 0.13 ^{dB}
SC2	16.39 ± 0.33 ^{bA}	5.51 ± 0.32 ^{abcC}	7.91 ± 0.23 ^{eB}	4.01 ± 0.04 ^{abD}
SC3	15.83 ± 0.43 ^{bcA}	5.43 ± 0.25 ^{abcC}	10.95 ± 0.34 ^{abB}	3.95 ± 0.29 ^{bD}
SC6	16.23 ± 0.34 ^{bA}	6.52 ± 0.68 ^{aC}	9.98 ± 0.58 ^{bcB}	3.98 ± 0.11 ^{bD}
SC7	18.02 ± 0.10 ^{aA}	6.08 ± 0.32 ^{abC}	11.70 ± 0.12 ^{aB}	4.58 ± 0.34 ^{abD}
SC9	15.38 ± 0.20 ^{cdA}	5.10 ± 0.01 ^{bcC}	8.40 ± 0.11 ^{deB}	3.14 ± 0.00 ^{cD}
SC13	17.43 ± 0.15 ^{aA}	6.54 ± 0.19 ^{aC}	8.58 ± 0.90 ^{cdeB}	4.14 ± 0.43 ^{abD}
SC20	15.03 ± 0.13 ^{dA}	4.89 ± 0.25 ^{cC}	9.80 ± 0.15 ^{bcdB}	4.80 ± 0.07 ^{aC}

Statistically significant differences among each culture medium fermented by the different bacteria and control were indicated with different lower-case letters (columns), differences between different culture medium fermented by the same bacteria and control were indicated with different capital letters (rows) ($p < 0.05$). Quantification below the level of detection were marked as nd. Culture mediums studied were MRS-B, W1 (whey), W2 (whey complemented with nutrients and salts) and W3 (diluted whey complemented with nutrients and salts). The bacteria used were *L. plantarum* SC2 (SC2), *L. plantarum* SC3 (SC3), *L. plantarum* SC6 (SC6), *L. plantar*um SC7 (SC7), *L. plantarum* SC9 (SC9), *L. plantarum* SC13 (SC13) and *L. plantarum* SC20 (SC20).

The lactic acid is the main metabolite produced by all bacteria considered as LAB. Usually in the bibliography the production of this compound is related to antimicrobial activities. This relation has been described by Awah et al. (2018) where after the screening of different isolates the one with a higher production of lactic acid was proved to be effective in the biopreservation of fruits, or in El oirdi et al. (2021) were following a similar screening the biopreservation against fungal contaminants of different foods was achieved.

The results regarding the presence or absence of various phenolic compounds in the fermented CFS are presented in **Fig. 1**. The most abundant ones are quantified in **Table 2**. **Fig. 1** shows that only 3-4-dihydroxyhydrocinnamic, benzoic acid, and DL-3-phenyllactic acid were observed in great relative abundance. *L. plantarum* SC13 exhibited an absence of these compounds in comparation to the other bacteria. The highest abundance of phenolic compounds was observed in the MRS-B and W2 culture media. By fermenting MRS-B or the W2 culture media 4 LAB produced 3-4-dihydroxyhydrocinnamic, benzoic acid, and DL-3-phenyllactic acid in detectable quantities. After the fermentation of the W1 and W3 mediums the relative abundance of phenolic compounds was low. The strain *L. plantarum* SC6 demonstrated the highest production of antifungal phenolic metabolites overall. All controls evidenced low abundances of these compounds.

a) MRS

Compounds	Control	SC2	SC3	SC6	SC7	SC9	SC13	SC20
1-2-Dihydroxybenzene								
3-(4-hydroxy-3-methoxyphenyl) propionic								
3-4-Dihydroxyhydrocinnamic								
Benzoic acid								
Caffeic acid								
Chlorogenic acid								
DL-3-Phenyllactic acid								
Ellagic acid								
Ferulic acid								
Gallic acid								
Hydroxycinnamic acid								
P-Coumaric acid								
Protocatechuic								
Quercetin								
Salicylic acid								
Sinapic acid								
Syringic acid								
Vanillic acid								
Vanillin								

b) W1

Compounds	Control	SC2	SC3	SC6	SC7	SC9	SC13	SC20
1-2-dihydroxybenzene								
3-(4-hydroxy-3-methoxyphenyl) propionic								
3-4-dihydroxyhydrocinnamic								
Benzoic acid								
Caffeic acid								
Chlorogenic acid								
DL-3-phenyllactic acid								
Ellagic acid								
Ferulic acid								
Gallic acid								
Hydroxycinnamic acid								
P-coumaric acid								
Protocatechuic								
Quercetin								
Salicylic acid								
Sinapic acid								
Syringic acid								
Vanillic acid								
Vanillin								

c) W2

Compounds	Control	SC2	SC3	SC6	SC7	SC9	SC13	SC20
1-2-dihydroxybenzene								
3-(4-hydroxy-3-methoxyphenyl) propionic								
3-4-Dihydroxyhydrocinnamic								
Benzoic acid								
Caffeic acid								
Chlorogenic acid								
DL-3-phenyllactic acid								
Ellagic acid								
Ferulic acid								
Gallic acid								
Hydroxycinnamic acid								
P-coumaric acid								
Protocatechuic								
Quercetin								
Salicylic acid								
Sinapic acid								
Syringic acid								
Vanillic acid								
Vanillin								

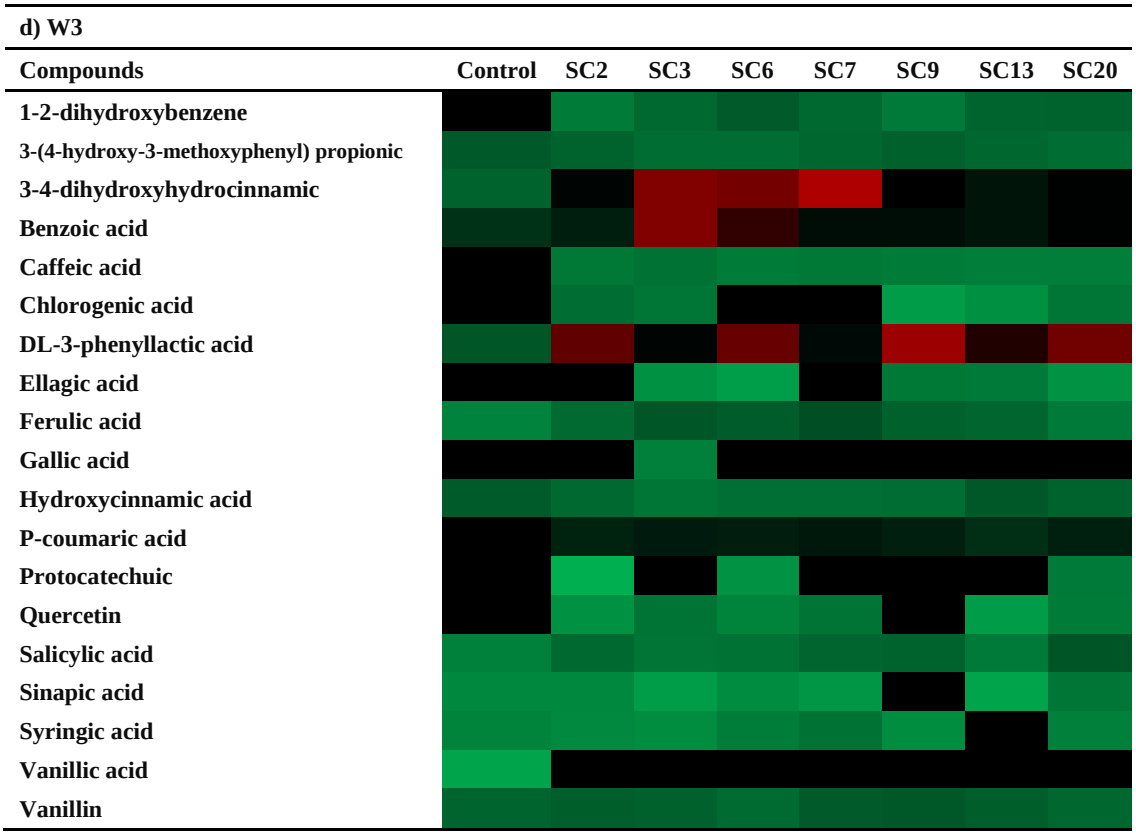

Fig. 1. Results of the abundancy of antifungal the main LAB antifungal metabolites present in the fermented culture mediums. The CFS studied were a) MRS-B, b) W1 (whey), c) W2 (whey complemented with nutrients and salts) and d) W3 (diluted whey complemented with nutrients and salts). Results were expressed as more abundancy (red) to less abundancy (green). All samples were studied by triplicate. The bacteria used were *L. plantarum* SC2 (SC2), *L. plantarum* SC3 (SC3), *L. plantarum* SC6 (SC6), *L. plantarum* SC7 (SC7), *L. plantarum* SC9 (SC9), *L. plantarum* SC13 (SC13) and *L. plantarum* SC20 (SC20).

Table 2. Results of the quantification most abundant antifungal phenolic compounds produced by LAB present in the fermented mediums analysed with a LC-MS/MS equipment. Data was displayed as mean ± SD of lactic acid mg/L.

a) DL-3-phenyllactic acid (mg/L)				
Sample	**MRS**	**W1**	**W2**	**W3**
Control	nd	nd	nd	nd
SC2	25.75 ± 0.37 ^{bA}	8.61 ± 0.33 ^{bcD}	21.25 ± 0.78 ^{bcB}	15.43 ± 1.05 ^{abC}
SC3	30.00 ± 1.42 ^{aA}	8.36 ± 0.37 ^{cB}	29.35 ± 2.19 ^{aA}	8.41 ± 1.76 ^{cB}
SC6	30.64 ± 1.69 ^{cdA}	8.29 ± 0.65 ^{cC}	27.94 ± 1.43 ^{aA}	15.91 ± 1.56 ^{abB}
SC7	19.86 ± 0.66 ^{cdA}	10.46 ± 0.38 ^{bB}	23.80 ± 1.56 ^{abA}	6.33 ± 1.75 ^{cB}
SC9	23.14 ± 2.20 ^{bcA}	6.78 ± 0.32 ^{cB}	15.85 ± 1.24 ^{cdA}	20.10 ± 0.45 ^{aB}
SC13	4.97 ± 0.81 ^{eB}	2.70 ± 0.29 ^{dB}	11.95 ± 2.85 ^{dB}	11.78 ± 2.56 ^{bcA}
SC20	16.54 ± 0.55 ^{dAB}	14.78 ± 1.17 ^{aB}	20.88 ± 1.25 ^{bcA}	16.64 ± 2.07 ^{abAB}

b) 3-4-dihydroxyhydrocinnamic (mg/L)				
Sample	**MRS**	**W1**	**W2**	**W3**
Control	nd	nd	nd	nd
SC2	13.69 ± 0.53 ^{bA}	2.24 ± 0.03 ^{cD}	11.88 ± 0.72 ^{bB}	8.01 ± 0.06 ^{cC}
SC3	18.02 ± 0.61 ^{aA}	2.20 ± 0.28 ^{cB}	17.45 ± 0.37 ^{aA}	17.81 ± 0.52 ^{bA}
SC6	19.19 ± 0.91 ^{aA}	1.84 ± 0.26 ^{cdB}	16.74 ± 3.45 ^{aA}	16.93 ± 0.84 ^{bA}
SC7	9.19 ± 0.53 ^{cB}	3.73 ± 0.17 ^{bC}	20.43 ± 2.54 ^{aA}	21.76 ± 0.66 ^{aA}
SC9	13.55 ± 1.59 ^{bA}	0.90 ± 0.11 ^{eC}	10.80 ± 1.12 ^{bB}	10.27 ± 0.29 ^{cB}
SC13	1.46 ± 0.24 ^{eB}	1.37 ± 0.04 ^{deB}	3.26 ± 0.45 ^{cA}	3.50 ± 0.28 ^{dA}
SC20	6.28 ± 0.58 ^{dB}	4.81 ± 0.20 ^{aB}	11.10 ± 0.36 ^{bA}	9.77 ± 1.44 ^{cA}

c) Benzoic acid (mg/L)				
Sample	**MRS**	**W1**	**W2**	**W3**
Control	nd	nd	nd	nd
SC2	10.37 ± 1.64 ^{abA}	2.37 ± 0.04 ^{cdB}	12.02 ± 0.72 ^{bcA}	2.08 ± 0.90 ^{deB}
SC3	13.67 ± 2.86 ^{aB}	2.29 ± 0.28 ^{bcC}	17.65 ± 0.36 ^{aA}	17.83 ± 0.57 ^{aA}
SC6	14.23 ± 3.23 ^{aA}	2.00 ± 0.22 ^{bcB}	16.92 ± 1.46 ^{aA}	12.61 ± 2.34 ^{bA}
SC7	6.71 ± 1.41 ^{bB}	2.93 ± 1.90 ^{abB}	15.90 ± 1.70 ^{abA}	5.62 ± 1.00 ^{cB}
SC9	13.41 ± 1.54 ^{aA}	0.78 ± 0.47 ^{bcC}	5.39 ± 0.89 ^{deB}	5.23 ± 0.76 ^{cdB}
SC13	0.78 ± 0.73 ^{cB}	1.53 ± 0.03 ^{bcAB}	2.41 ± 1.58 ^{efAB}	3.56 ± 0.27 ^{cdA}
SC20	6.22 ± 0.57 ^{bBC}	4.99 ± 0.21 ^{aC}	8.45 ± 1.55 ^{cdAB}	9.85 ± 1.45 ^{bA}

Statistically significant differences among each culture medium fermented by the different bacteria and control were indicated with different lower-case letters (columns), differences between different culture medium fermented by the same bacteria and control were indicated with different capital letters (rows) ($p < 0.05$). Quantification below the level of detection were marked as nd. Culture mediums studied were a) MRS-B, b) W1 (whey), c) W2 (whey complemented with nutrients and salts) and d) W3 (diluted whey complemented with nutrients and salts). The bacteria used were *L. plantarum* SC2 (SC2), *L. plantarum* SC3 (SC3), *L. plantarum* SC6 (SC6), *L. plantarum* SC7 (SC7), *L. plantarum* SC9 (SC9), *L. plantarum* SC13 (SC13) and *L. plantarum* SC20 (SC20).

The quantification of the compounds showed that DL-3-phenyllactic acid was the most abundant, followed by 3-4-dihydroxyhydrocinnamic and benzoic acid in all studied fermented CFS (Table 2). The culture mediums MRS-B and W2 exhibited the highest concentration of these compounds after the fermentation, while the culture medium W1 showed the lowest metabolization of phenolic compounds during the LAB fermentation. The presence of DL-3-phenyllactic acid by all bacteria (excluding the results from the strain *L. plantarum* SC13 which produced all metabolites in low concentrations) ranged from 19.86 to 30.00 mg/L in the MRS-B medium and 15.85–29.35 mg/L in the W2 medium. These two media showed similar concentrations of the phenolic compounds. The strains *L. plantarum* SC7 and *L. plantarum* SC20 producing significantly higher amounts in the W2 medium than in the MRS-B medium. No presence of the studied compounds was observed in the non-fermented controls. Therefore, it can be assumed that the medium W2 was efficient for the metabolization of phenolic compounds with antifungal properties and with the right bacterial strain, it can even exceed the production compared to the MRS-B.

The low presence of antifungal compounds in the culture medium W1 present in the **Fig. 1** can be also observed in the **Table 2**. It could be attributed to the absence of a richer mix of nutrients (the other mediums had nutrients from the addition glucose, yeast extract, tryptone and different salts). When comparing the production of phenolic compounds in the MRS-B and the culture medium W2 similar results were observed. The biggest difference among both culture mediums is the source of proteins, in the MRS-B come from meat and in the W2 medium from whey, primarily soluble proteins from milk (Tomar et al., 2022). It appears that the substitution of the protein source was effective for the production of this metabolites, an interesting result since meat sources are expensive and produce a high ecological impact to be obtained (Kurek et al., 2022). There are several reports in the bibliography that link the presence of DL-3-phenyllactic acid in different fermented matrix used for biopreservation against the fungal growth of many *Aspergillus*, *Fusarium* and *Penicillium* species (Dieuleveux et al., 1998; Ruggirello et al., 2019; J. J. Xu et al., 2021). And a similar tendency can be seen with the benzoic acid and 3-4-dihydroxyhydrocinnamic acid (Broberg et al., 2007; Peyer et al., 2016). Use of the fermentation for the production of metabolites of interest is a common technique in other areas. For example, there are reports in the bibliography of the use of whey and other industrial wastes which though a fermentation can be revalorized for the production of specific compounds such as aminobutyric acid for clinic purposes (Alizadeh Behbahani et al., 2020; Falah et al., 2022). Therefore, managing to imitate the production of antifungal compounds evidenced in MRS-B is an interesting result from this study.

3.2. Antifungal activity assays by qualitative method

The agar diffusion assays performed with a solution of 400 g/L from the fermented CFS are exposed in the **Table 3**. The culture medium W2 evidenced the highest antifungal activity followed by the medium W1, W3 and MRS-B. There was not any inhibition halo superior to 1 cm in the trials with the MRS-B CFS. Nevertheless, all three whey-based mediums evidenced halos superior to 1 cm against different fungal species from *Botrytis*, *Alternaria*, *Aspergillus*, *Fusarium* and *Penicillium* genera. The strain *L. plantarum* SC20 evidenced a good potential for the fungal growth inhibition though the fermentation of whey by exhibiting halo of more than 1 cm against all studied fungal genera. The fungal species *A. Alternata*, *P. verrucosum* and *P. commune* evidenced a low resistant against the fermented whey. In the **Fig. 2** it can be appreciated the assay of the fermented CFS against *P. expansum*. None of the control samples evidenced antifungal activity, therefore, the antifungal properties of the studied CFS were acquired during the LAB fermentation and they were not naturally occurring from the whey.

Table 3. Results from the study of the antifungal activity evaluated by qualitative method agar diffusion assay. Results were expressed as "+++" when the inhibition halo reached more than 1 cm of diameter, as "++" when the inhibition halo was inferior to 0.6 cm, as "+" when the inhibition halo was inferior to 0.2 cm and "-" when no inhibition halo was observed.

a) MRS-B								
Fungi	**Control**	**SC2**	**SC3**	**SC6**	**SC7**	**SC9**	**SC13**	**SC20**
B. cinerea	-	+	+	+	++	++	++	++
A. Alternata	-	++	+	++	++	++	++	++
A. flavus	-	-	+	+	+	+	+	+
A. carbonarius	-	+	+	+	+	+	+	+
F. graminearum	-	+	++	++	++	++	++	++
P. verrucosum	-	++	++	++	++	++	++	++
P. expansum	-	+	++	-	++	++	+	++
P. digitatum	-	+	+	+	++	++	+	+
P. commune	-	++	++	++	++	++	++	++

b) W1

Fungi	Control	SC2	SC3	SC6	SC7	SC9	SC13	SC20
B. cinerea	-	+	+	++	+	+	+++	+++
A. Alternata	-	++	+++	+++	+++	+++	+++	+++
A. flavus	-	+	+	+	+	+	+++	+
A. carbonarius	-	+	+	+	+	+	+	+
F. graminearum	-	++	+++	+	+++	+	+	+++
P. verrucosum	-	+++	+++	+++	+++	+++	+++	+++
P. expansum	-	+++	++	+++	+++	+++	+++	+++
P. digitatum	-	+	+	++	+	+	+	+++
P. commune	-	+++	+++	+++	+++	+++	+++	+++

c) W2

Fungi	Control	SC2	SC3	SC6	SC7	SC9	SC13	SC20
B. cinerea	-	++	+++	++	++	++	++	+++
A. Alternata	-	+++	+++	+++	+++	+++	+++	+++
A. flavus	-	+	++	++	++	++	++	+++
A. carbonarius	-	+	+	+	+	+	+	++
F. graminearum	-	++	+++	++	+++	+++	+++	+++
P. verrucosum	-	+++	+++	+++	+++	+++	+++	+++
P. expansum	-	++	+++	+++	+++	+++	+++	+++
P. digitatum	-	+	++	+	++	++	+	++
P. commune	-	++	+++	+++	+++	+++	+++	+++

d) W3

Fungi	Control	SC2	SC3	SC6	SC7	SC9	SC13	SC20
B. cinerea	-	+	+++	++	+++	++	++	+++
A. Alternata	-	+++	+++	+++	+++	+++	+++	+++
A. flavus	-	-	++	+	+	+	+	++
A. carbonarius	-	+	+	+	+	+	+	+
F. graminearum	-	+	+++	++	+++	++	+++	+++
P. verrucosum	-	+++	+++	+++	+++	+++	+++	+++
P. expansum	-	+	+++	++	+++	+++	+++	+++
P. digitatum	-	+	++	+	+	+	+	++
P. commune	-	++	+++	+++	+++	+++	+++	+++

Culture mediums studied were a) MRS-B, b) W1 (whey), c) W2 (whey complemented with nutrients and salts) and d) W3 (diluted whey complemented with nutrients and salts). The bacteria used were *L. plantarum* SC2 (SC2), *L. plantarum* SC3 (SC3), *L. plantarum* SC6 (SC6), *L. plantarum* SC7 (SC7), *L. plantarum* SC9 (SC9), *L. plantarum* SC13 (SC13) and *L. plantarum* SC20 (SC20).

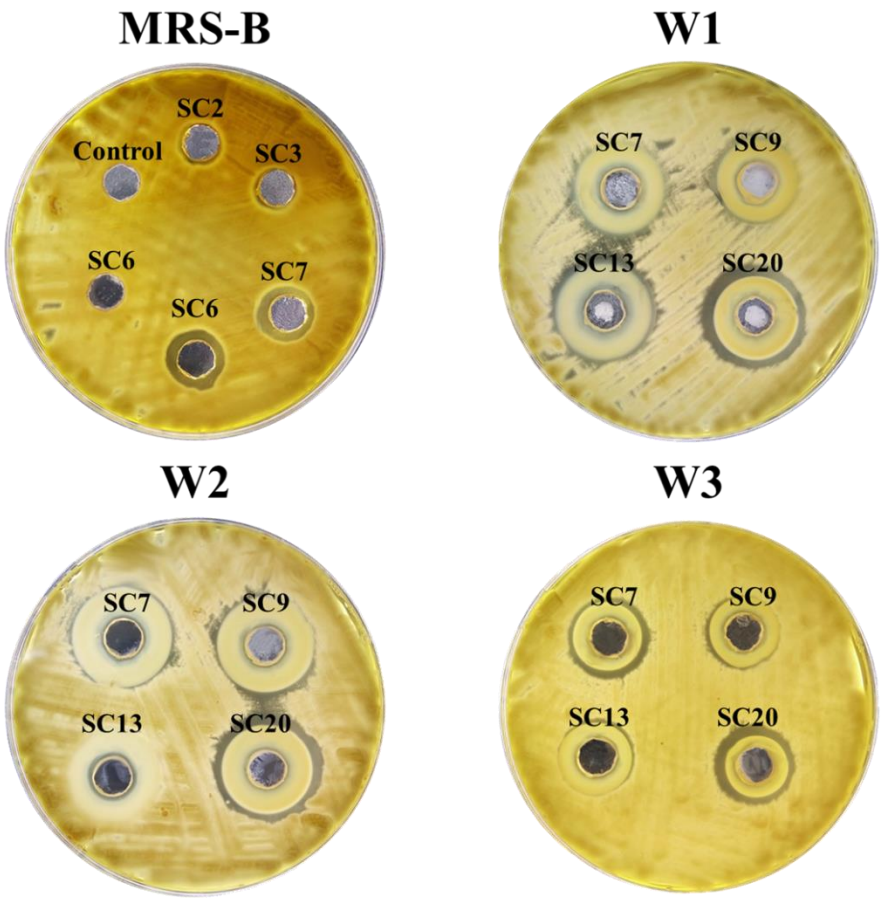

Fig. 2. Antifungal effect of the different CFS from the MRS-B, W1 (whey), W2 (whey complemented with nutrients and salts) and W3 (diluted whey complemented with nutrients and salts) fermented by the lactic acid bacteria against a strain of *P. expansum*.

In the bibliography the report usually evidences low inhibition halos from CFS produced by LAB fermentation of MRS-B. It can be seen in Dopazo et al. (2021) where the CFS extracted after the fermentation only surpassed the 0.8 cm against some fungi. But usually, those inhibition halos only are seen with extracts that are not allowed to use in the food industry (Mohammadi et al., 2021; Rizzello et al., 2011). Or in other cases compounds like essential oils that are quite expensive to obtain and leave undesired flavour in the foods (Li et al., 2020; Xing et al., 2019).

3.3. Antifungal activity assays by quantitative method

The results of the study from the minimal inhibitory concertation and minimal antifungal concentration (MIC-MFC) can be appreciated in the **Table 4**. These assays were performed focusing on the future application of the fermented whey for cheese biopreservation. Therefore,

only the fungal species related to cheese contamination present in the agar diffusion test were chosen. (Casquete et al., 2017). In this assay it can be observed a tendency similar to the one evidenced in the previous test. The fermentation of the culture mediums with whey evidenced more antifungal activity in comparation to the MRS-B CFS. The MRS-B CFS only evidenced antifungal activity after thought all LAB fermentations against *P. verrucosum*. The fermented whey evidenced antifungal activities against *A. flavus* in concentrations ranging from 25 to 200 g/L, *P. expansum* from 13 to 50 g/L, *P. commune* from 25 to 100 g/L and for *P. verrucosum* 13–50 g/L. The *P. digitatum* was the specie with the highest resistance to presenting MFC above the ones evaluated. Among all the fermented whey mediums the lowest MFC were appreciated in in the culture medium W2.

Table 4. Results from the study of the antifungal activity evaluated by quantitative method minimum inhibitory concentration minimum fungicidal concertation assay (MIC-MFC). Results were expressed and g/L of the CFS needed to achieve the inhibition (MIC) or fungicidal activity (MFC).

a) MRS

Fungi	SC2		SC3		SC6		SC7		SC9		SC13		SC20	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
A. flavus	50	>200	100	200	100	>200	100	200	100	200	100	200	100	200
P. expansum	50	200	50	200	50	200	50	100	50	100	50	200	50	200
P. digitatum	50	>200	50	>200	100	>200	100	>200	100	>200	100	>200	100	>200
P. commune	50	>200	50	>200	50	>200	50	200	50	200	50	>200	50	>200
P. verrucosum	25	50	25	25	13	50	13	25	25	25	25	50	25	50

b) W1

Fungi	SC2		SC3		SC6		SC7		SC9		SC13		SC20	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
A. flavus	13	50	25	50	25	200	50	200	25	50	25	50	13	25
P. expansum	6	13	6	25	6	25	13	25	13	25	13	25	13	50
P. digitatum	25	>200	50	>200	25	200	25	>200	25	>200	50	200	6	200
P. commune	3	100	3	50	3	50	3	50	6	100	6	100	2	50
P. verrucosum	25	25	25	25	13	13	13	25	25	50	13	50	3	13

c) W2

Fungi	SC2		SC3		SC6		SC7		SC9		SC13		SC20	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
A. flavus	25	200	25	100	25	200	25	50	12.5	50	25	50	12.5	50
P. expansum	13	50	13	25	13	50	13	25	6	25	50	100	13	25
P. digitatum	13	>200	25	200	25	200	13	200	6.25	>200	12.5	200	25	>200
P. commune	2	50	6	50	13	200	3	100	6	50	6	100	3	50
P. verrucosum	6	13	25	25	13	13	6	13	6	13	13	25	13	13

d) W3

Fungi	SC2		SC3		SC6		SC7		SC9		SC13		SC20	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
A. flavus	6	25	6	50	25	200	25	200	13	25	25	50	13	100
P. expansum	6	13	13	25	7	13	13	25	6	25	6	25	13	25
P. digitatum	13	>200	6	>200	13	>200	25	>200	12.5	>200	100	>200	13	>200
P. commune	3	25	3	25	6	200	13	25	3	25	3	25	2	50
P. verrucosum	6	13	13	13	6	13	6	13	13	25	13	13	13	13

Culture mediums studied were a) MRS-B, b) W1 (whey), c) W2 (whey complemented with nutrients and salts) and d) W3 (diluted whey complemented with nutrients and salts). The bacteria used were *L. plantarum* SC2 (SC2), *L. plantarum* SC3 (SC3), *L. plantarum* SC6 (SC6), *L. plantarum* SC7 (SC7), *L. plantarum* SC9 (SC9), *L. plantarum* SC13 (SC13) and *L. plantarum* SC20 (SC20).

All these results evidence a clear incrementation of the antifungal activity of the CFS W2 in comparation to the CFS from the MRS. Nevertheless, back in the analysis from the main antifungal compounds present in all CFS the MRS was the medium in which there was the greatest occurrence of lactic acid, 3-4-dihydroxyhydrocinnamic, benzoic acid and DL-3-phenyllactic acid. Therefore, two hypotheses can be evidenced from these results. First, there is more important antifungal compounds yet to be detected in the fermented whey which are causing the increase in the antifungal activity. Like the proteinaceous compounds described by Muhialdin et al. (2011). The, hydrocinnamic acid and other phenolic compounds different to the ones detected in the studied CFS in this work are also related to the inhibition of the fungal growth (Lynch et al., 2014). Or the reuterin used for the biopreservation of different foods (Bianchini & Bullerman, 2009). The antifungal activity from these compounds is usually related to a synergistic effect of the different metabolites produced by the bacteria (Nasrollahzadeh et al., 2022). Usually none of the antimicrobial components produced reaches concentrations with antimicrobial activities. The study described in Xing et al. (2019) proved that the MFC of the compounds are far higher than the ones occurring in CFS with lower concentration but with a wide variety of compounds. Secondly, after the fermentation the excess of nutrients present in the MRS can be used by the fungi to grow even in stressed condition against the CFS. As described by Pitt and Hocking (2009) fungi can use a wide spectrum of compounds as matrix for his growth. Even accepting both

hypothesis as true, the goat fermented whey by LAB has proved to be more efficient than the MRS for antifungal control purposes. Whey is a by-product from the cheesemaking industry the price for the formulation of this culture medium should be really low. In addition, this helps solve one of the biggest problems of the dairy industry by starting a new demand from a product that in any other scenario they have to expend money into treat as a residue (Cosentino et al., 2018).

3.4. Qualitative characterization of the antifungal activity and metabolite production in the CFS by principal component analysis

To seek for a better understanding of how the different antifungal metabolites produced during the fermentation helped in the antifungal activity from the CFS. To achieve this objective a multivariate dataset consisting of the antifungal activity (calculated by the average MFC obtained in the previous assay) and the lactic acid, DL-3-phenyllactic acid, 3-4-dihydroxyhydrocinnamic and benzoic acid was analysed by PCA analysis. The objective was to determine if there was a clear difference of the groups (the four studied culture mediums) with parameters used.

In the **Fig. 3**, **Fig. 4** it the principal component analysis of the data obtained against *A. flavus* and *Penicillium* spp., respectively. More than 3 principal components (PC) were obtained from each analysis. In both cases the accumulate variability from the PC1 and the PC2 was above the 90%, therefore, the PC3 was not used for the evaluation. The PC1 explained 82% and the PC2 12% of the variance in the *Aspergillus* analysis (**Fig. 3a**), while the PC1 explained 82% and the PC2 12% of the variance for the *Penicillium* (**Fig. 4a**) The results of both analysis did not evidence differences among the fermented culture mediums (**Fig. 3**, **Fig. 4a**). A total of 4 clusters were detected in both PCA, but none of the clusters were separated by the PCA analysis. It could be considered that the production of the quantified metabolites did not explain the detected antifungal activity. As the fermented CFS clearly evidence lower antifungal concentrations this could mean that other non-quantified molecules are also contributing to antifungal potential of the CFS. It is widely described in the bibliography that in the CFS from LAB fermentation the natural occurrence of each antifungal metabolite is always lower to the minimal antifungal concentration detected by *in vitro* test. It is the pool of different antifungal compounds at low concentrations the key factor producing this antimicrobial activity against molds (Dieuleveux et al., 1998). The biplot analysis obtained from the *Aspergillus* (**Fig. 3b**) and the *Penicillium* (**Fig. 4b**) demonstrated that among the metabolites studied all three quantified phenolic compounds (3-4-dihydroxyhydrocinnamic, benzoic acid and DL-3-phenyllactic acid) were the highly correlated to the antifungal activity against both fungal species, as the loading plots vectors evidenced a negative correlation. Hence, it can be assumed that a bigger production of this LAB metabolites

is related to lower minimum antifungal concentrations of the CFS. Besides, lactic acid also shows a positive correlation to lower MFC, but the concentration of this compound is not that determinant in the antifungal potential of the CFS.

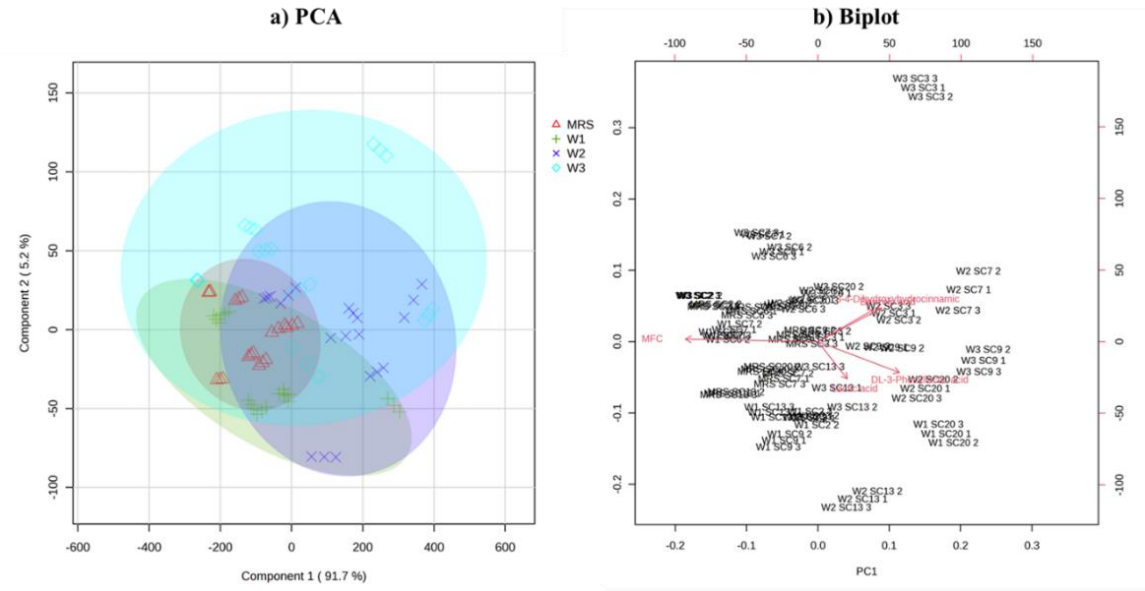

Fig. 3. Principal component analysis (PCA) was performed with the mean of the MFC values of the *Aspergillus flavus* MFC and the different concentrations of the metabolites present in the fermented mediums. The PCA analysis of the data (a) and the biplot analysis (b). The different groups described in the PCA are the fermented MRS (MRS), the fermented whey (W1), the fermented complemented whey (W2) and the fermented diluted whey complemented (W3). Each sample was plotted as the medium of the fermentation, the bacteria used for the fermentation: *L. plantarum* SC2 (SC2), *L. plantarum* SC3 (SC3), *L. plantarum* SC6 (SC6), *L. plantarum* SC7 (SC7), *L. plantarum* SC9 (SC9), *L. plantarum* SC13 (SC13) and *L. plantarum* SC20 (SC20), the replicas were marked as 1, 2 and 3.

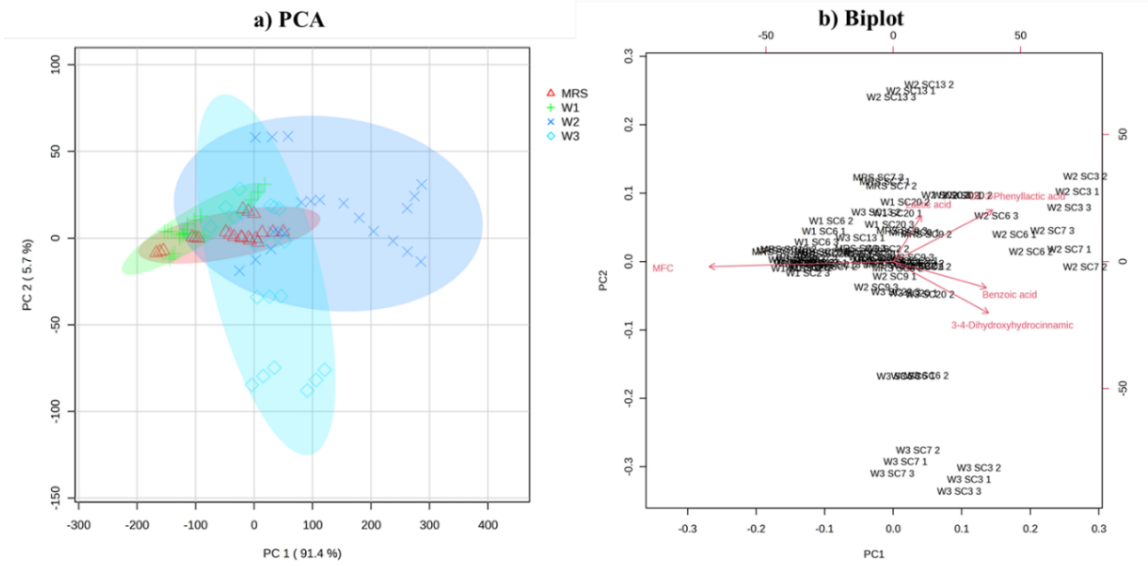

Fig. 4. Principal component analysis (PCA) was performed with the mean of the MFC values of the *Penicilliums* spp. MFC and the different concentrations of the metabolites present in the fermented mediums. The PCA analysis of the data (a) and the biplot analysis (b). The different groups described in the PCA are the fermented MRS (MRS), the fermented whey (W1), the fermented complemented whey (W2) and the fermented diluted whey complemented (W3). Each sample was plotted as the medium of the fermentation, the bacteria used for the fermentation: *L. plantarum SC2* (SC2), *L. plantarum* SC3 (SC3), *L. plantarum* SC6 (SC6), *L. plantarum* SC7 (SC7), *L. plantarum* SC9 (SC9), *L. plantarum* SC13 (SC13) and *L. plantarum* SC20 (SC20), the replicas were marked as 1, 2 and 3.

Table 5. Correlation between the average MFC values obtained for *Aspergillus* and *Penicillium* compared to the concentrations of each antifungal metabolite quantified in this study: lactic acid, DL-3-phenyllactic acid, 3-4-dihydroxyhydrocinnamic and benzoic acid. Results were expressed as the correlation value between each compound and the average MFC and the p-value obtained for each assay.

MFC (correlation coefficient / *p*-value)	***Aspergillus***	***Penicillium***
Lactic acid	- 0.57 / <0.001	- 0.42 / <0.001
DL-3-phenyllactic acid	- 0.93 / <0.001	- 0.85 / <0.001
3-4-dihydroxyhydrocinnamic	- 0.82 / <0.001	- 0.84 / <0.001
Benzoic acid	- 0.88 / <0.001	- 0.90 / <0.001

This result obtained in the biplot were confirmed numerically by a study of the correlation between the MFC values and the metabolite concentrations with a Pearson r test (**Table 5**). Only values lower than - 0.80 and with a p-value below 0.01 were interpreted as a strong correlation. This correlations are negative as higher concentrations of the antifungal compounds tend to reduce the minimal antifungal concentration appreciated in the samples. If the correlation coefficient was superior to 0.8 it will mean that the compound is actually reducing the antifungal activity of the CFS and between −0.8 and 0.8 it will be considered that no correlation was observed (Liang et al., 2022). A significative correlation of all metabolites was achieved wis the data evaluation ($p <$ 0.01). The metabolites DL-3-phenyllactic acid, 3-4-dihydroxyhydrocinnamic and benzoic acid were decreasing the MFC against both fungal species. This negative correlation was - 0.93, - 0.83 and - 0.88 against *Aspergillus* spp. and - 0.85, - 0.84 and - 0.90 for *Penicillium* spp., respectively. All these values were considered to significantly reduce the concentration of CFS need id to achieve antifungal activity as the correlation coefficient was bellow - 0.80. nevertheless, this facto on the lactic acid only achieved a correlation of - 0.57 and - 0.42 against *Aspergillus* spp. and *Penicillium* spp., respectively, therefore, it cannot be considered to affect in the antifungal potential from the CFS.

This data obtained from the multivariate analyst evidence similar results to the ones described in other articles. Lactic acid is the principal antifungal metabolite produced by LAB. It is the principal source of the antifungal activity that these group of microorganisms. This acid acidifies the cytoplasm of other microorganism and caused a disequilibrium in the electrochemical gradient of the cellular membrane, which help the introduction of other antifungal compounds and can cause cellular death (Schnürer & Magnusson, 2005). But this acidification of the environment is not enough to induce the cellular death of the regular fungal species that contaminate food. This decrease in the pH only delays the fungal sporulation as most of the fungal species that are a problem for the industry can grow pH even near to 4 (Pitt & Hocking, 2009). It can be assumed that after a certain concentration of lactic acid the increase of the antifungal effect will be not affected. The key factor in the activity is the other antifungal compound which enter the membrane after the impermeabilization with the lactic acid (Alakomi et al., 2000). That is the reason the analysis determined that DL-3-phenyllactic acid, 3-4-dihydroxyhydrocinnamic and benzoic acid were the metabolites producing the activity. These metabolites are already linked by the bibliography to help in the antifungal potential of the LAB produced CFS (Cortés-Zavaleta et al., 2014; Illueca et al., 2021; Ogunremi et al., 2022).

4. CONCLUSION

Medium composition affected the production of antifungal metabolites in the fermented mediums. The whey complemented with nutrients (W2) evidenced significantly similar production of compounds typically identified as antifungal metabolites to the ones detected in the fermentation of MRS-B. The *in vitro* antifungal assays determined that the W2 medium evidenced higher antifungal activities than the MRS-B fermentation. The fermented W2 medium evidenced an average antifungal activity with concentrations of 100 g/L against *Aspergillus* genera and 118 g/L in the assays against *Penicillium* spp. While in the assays against with MRS-B most of the fermented CFS did not evidence antifungal activity. Finally, the statistical test used for the analysis of the antifungal compounds detected in comparation to the antifungal activity determined that no correlation was observed. Some other antifungal agents produced in the fermentation yet to be determined are also increasing the antifungal potential of the whey based CFS.

This ingredient has the potential to be used in foods for the reduction of fungal species. Further investigations will be necessary to determine completely the pool of metabolites producing the activity. Nevertheless, the application of goat fermented whey as an antifungal agent in the food industry is really promising and can be applied in different bakery, dairy and meat products (such as bread, cheese and sausages). In addition, this use reduces the problematics associated to the decontamination of goat whey from the cheese-making industry.

LWT, 174, 114427 (2023)

3.3. Evaluation of shelf life and technological properties of bread elaborated with lactic acid bacteria fermented whey as a bio-preservation ingredient

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1. INTRODUCTION

Approximately around a 25% of the food worldwide is contaminated with filamentous fungi and the toxins produced by this microorganism (H. Cao, Jiang, et al., 2021). As a result of this fact, fungal and mycotoxigenic contamination have become a severe problem which cost food industry million euros per year and threatens the life of the consumers with their toxigenic effects (Russo et al., 2017; Zain, 2011). Among all the food-contaminant species of moulds, bakery products are especially susceptible to contamination from the *Aspergillus*, *Penicillium*, *Fusarium* and *Rhizopus* (Debonne et al., 2021). *Aspergillus* and *Penicillium* genera are considered the most harmful to human health. This fungal species are the main producers of aflatoxins (AF) and ochratoxin (OTA) in cereal and different cereal-based foods (Bryła et al., 2021). AF ingestion is related to hepatotoxic, mutagenic and carcinogenic activities in animals, and is classified as Group 2B by the International Agency for Research on Cancer (IARC) (Bennett & Klich, 2003; Gupta, 1994). OTA is also related to nephrotoxic, teratogenic, embryotoxic, genotoxic, neurotoxic suppression of the immune system effects (Belkacem-Hanfi et al., 2014).

In the production of bread, the temperatures reached during the baking stage destroy the fungal vegetative forms, but some of the spores are resistant to this treatment and grow in the final product. In addition, post preparation practices lead to an increasing contamination of the bread (Cizeikiene et al., 2013; Jideani & Vogt, 2016). It is interesting to find effective fungicides resistant to high temperatures to prevent the growth of moulds and mycotoxin production after the bakery stage. Chemical preservatives, such as calcium propionate and calcium acetate are commonly added to bread in order to prevent fungal and further toxin contamination. (Perincherry et al., 2019). Nevertheless, there is an increasing demand from the consumer for “natural” and “healthier” food by the reduction of the concentration from these chemical preservatives present in food (Sadiq et al., 2019). As a result, novel technological approaches are being studied to reduce fungal contamination with safer techniques. Use of lactic acid bacteria (LAB) as bio-protective agents postulates as a great alternative to the synthetic origin preservatives.

Many reports in the bibliography show evidence of the antifungal effect of this microorganism (Chen et al., 2021; Illueca et al., 2021). The use of these bacteria as bio-preservative agents has already been proved. Álvarez et al. (2021) used edible coatings incorporating LAB to prolong the shelf life of cherry tomatoes against *Fusarium* sp. and *Rhizopus* sp. Also, Omedi et al. (2021) evidence the bio-preservation potential of bread contaminated by *Cladosporium*, *Aspergillus* and *Penicillium* species using a LAB fermented ingredient. Among all the different uses of the LAB as bio-preservatives the application of a fermented matrix as the antifungal agent is one of the most studied techniques (Bulgasem et al., 2016; Li et al., 2020). In these studies, a nutrient-rich medium is used as a substrate for the bacterial metabolization of different antifungal compounds.

Whey from the cheese preparation is the principal residue produced by dairy industry (Verma & Subudhi, 2021). For each 10 kg of cheese up to 9 kg of whey are produced (Prazeres et al., 2012). This bio-residue is usually used for fertilization, livestock feed and human consumption. Nevertheless, most of this dairy by-product must be processed as residual water (Verma & Subudhi, 2021). When this by-product is not correctly treated is a mayor contaminant agent and regulations over this waste have become more rigorous during last years (Koutinas et al., 2009; Smithers, 2008). This cheese effluent contains a diverse pool of proteins, sugars, salts and fatty acids, which postulates this matrix as an ideal medium for LAB growth (Gregg et al., 2020). The goals agreed by the World Health Organization (WHO) set for 2030 for sustainable development indicate that new methods of waste reduction should be studied (United Nations, 2015). In addition, the ideal method for food conservation ought to be economical and non-complicated to use (Leroy & De Vuyst, 2004). The preparation of a food ingredient which only needs a LAB fermentation of a residue and a future addition to a food preparation accomplishes this description of ideal method.

The objective of this study was to produce an ingredient made of LAB fermented whey for the bio-preservation of bread against toxigenic fungal contamination. To accomplish this goal a) the modifications of the technological properties from the dough and bread were assessed; b) different antifungal compounds present in the dough and bread were quantified; c) the antifungal potential of the LAB fermented whey ingredient was studied in bread contaminated with *Penicillium* spp. and *Aspergillus* spp.

2. MATERIALS AND METHODS

2.1. Materials and microorganism

Media cultures used for the assays: Man Rogosa and Sharpe broth (MRS-B), plate count agar (PCA), Rose Bengal Chloramphenicol agar (RBA) and potato dextrose agar (PDA) were purchased to Liofilchem (Teramo, Italy). The double deionized water from a water purification system (Millipore Corp., Massachusetts, United States). Goat whey was provided by the Quesería Dehesa Dos Hermanas company, S.L (Santa Barbara de Casa, Spain).

Acetic acid glacial (99%), acetonitrile (99.9%), ethyl acetate (99.9%), formic acid (99%), d-glucose anhydrous and methanol (99.9%) were obtained from VWR Chemicals (Radnor, United States). C18, magnesium sulphate, sodium chloride, DL-3-phenyllactic acid (97%), potassium phosphate dibasic, ammonium citrate dibasic, sodium acetate, manganese (II), sulphate monohydrate magnesium sulphate and DL-lactic acid (90%) were acquired from Sigma–Aldrich (St Louis, United States). Tryptone from Liofilchem (Teramo, Italy). Yeast extract from Oxoid Holdings ltd. (Hampshire, United Kingdom).

The wheat flour, salt and sugar were Hacendado, bottled water was from Fuente primavera, and yeast was from Levitan, used in the bread preparation were all brought in Mercadona (Valencia, Spain).

The *Lactiplantibacillus plantarum* 5L1 studied in this paper was previously isolated and identified in previous investigations by the *Colección Española de Cultivos Tipo* (CECT) in Valencia, Spain. Fungi used were *Aspergillus flavus* ISPA 8111, from *Istituto di Scienze delle Produzioni Alimentari* (Bari, Italy) and *Penicillium verrucosum* VTT 01847, from the VTT Culture Collection (Espoo, Finland).

2.2. Preparation and fermentation of the cell free supernatant (CFS)

The studied culture medium was whey complemented with 10 g/L of glucose, 10 g/L of tryptone, 5 g/L of sodium acetate, 4 g/L of yeast extract, 2 g/L of dipotassium phosphate, 2 g/L of ammonium ferric citrate, 0.2 g/L of magnesium sulphate and 0.05 g/L of manganese sulphate.

To produce the cell free supernatant (CFS) first the LAB was incubated in MRS-B for 24 h at 37 °C. Then the pre-inoculum was added to the whey culture medium with a 5% (v/v) proportion and incubated for 72 h at 37 °C. Afterwards, the medium was centrifuged at 3000×g, 4 °C for 15 min. Finally, the CFS was frozen at −20 °C and lyophilised for further uses as ingredient. This CFS was proved in previous investigations pendent to be published to have antifungal activity against the tested fungi.

2.3. Bread baking with whey as ingredients

Dough was prepared as it follows: 300 g of wheat flour was mixed with 6.5 g of salt and a suspension of 165 mL bottled water at 37 °C with 20 g of yeast and 10 g of sugar. Then the dough was kneaded in a breadmaking machine SilverCrest SBB 850 A1 (SilverCrest, Bochum, Germany), for 10 min. Split in parts of 100 g and fermented for 1 h at room temperature. Finally, loaves were baked in a Memmert UNB 100 Oven (Gemini b.v., Apeldoorn, Netherlands) at 200 °C for 45 min and left at room temperature for 20 min. A total of three controls were prepared: a bread replacing salt with flour (non-salt control), a bread (salt control) and a bread with 3000 mg/kg of calcium propionate (salt propionate control). The studied ingredient, the fermented whey, was added at different concentrations: 5 g (1% fermented whey), 25 g (5% fermented whey), and 50 g (10% fermented whey). Flour was replaced with whey for the treatment addition. Salt and sodium propionate concentrations used were the highest permitted by the European Union (EFSA, 2016; European Commission, 2012).

2.4. Effect of the fermented whey on the dough

2.4.1. Effect on the expansion volume

The expansion volume was studied following the steps described in Sang et al. (2020) with a few modifications. Five grams of dough were placed at the bottom of a sterilized graduated cylinder (50 mL). After a fermentation of 1 h at room temperature, the increase in dough volume (cm) was calculated as dough expansion. All the measurements were performed in triplicates.

2.4.1. Effect on the pH and titratable acidity

Before the fermentation of the dough and after the fermentation of the dough the subsequent parameters were measured. The pH from the samples was measured before the fermentation and after the fermentation using a pH-meter XS pH 7 Vio (XS Instruments, Carpi, Italy) equipped with an electrochemical sensor 2 Pore Steel T (XS Instruments, Carpi, Italy). The titratable acidity was determined as mL of NaOH 0.1 M needed to raise the pH of a 10 g sample to pH 8.5 (Hugo et al., 2003). The result were expressed as equivalents of grams of lactic acid per 100 g dough. The equation used to quantify the titratable acidity was:

$$g/100g = (M\ NaOH * V\ NaOH)/Mw\ lactic\ acid) *10$$

Where: g/100 mL are the g of lactic acid for each 100 g of dough, M NaOH is the molar concentration of the NaOH used as titrant, V NaOH the volume of NaOH used as titrant and Mw lactic acid the molecular weight of lactic acid (90).

2.4.3. Effect on the microbial population

The microbial population of the samples was measured as described in Lan et al. (2012) with a few modifications. Serial dilutions of 10 g from the sample were homogenised with a stomacher Masticator Classic 400 mL 220/240 V 50/60 Hz (IUL S.A., Barcelona, Spain) saw in PCA (to determine the total microbial population) and RBA (to determine the total fungal population) then incubated at 25 °C 48 h.

2.5. Impact of the fermented whey on the bread

2.5.1. Impact on the pH and titratable acidity and microbial population

The measure of the pH, titratable acidity, total microbial population and total microbial population were performed on the bread following the steep performed to the dough.

2.5.2. Impact on the specific volume

The specific volume of the bread was studied as described in Mannuramath et al. (2015). Bread were weight then the volume was measured using the millet seed displacement method. A beaker with a calibrated mark was filled with millet grains until it reached the mark, then the bread was put into the baker and filled the millet grains. The displaced volume of seeds by the bread was measured. This measure was performed by triplicate. Specific volume was calculated as the ratio between volume and weight (cm3/g).

2.5.3. Impact on the water activity

The water activity (Aw) was measured using a Humimeter RH2 water activity meter (Max-Schaller-Straße, Styria, Austria) from 3 bread slices (Sadeghian Motahar et al., 2021).

2.5.4. Impact on the objective color values from the crust

Colour changes were measured using a ColorQuest® XE (HunterLab, Leicestershire, United Kingdom) to measure various locations from the crust of the bread (Aldughpassi et al., 2021). Results were expressed as luminosity (L*), the range from 0 (black) to 100 (white), redness (a*) the range from + a* (red) to -a* (green), and yellowness (b*), the range from + b* (yellow) to -b* (blue). Each sample was measured nine times. With these values the browning index (BI) was calculated as described in Kowalski et al. (2022), where:

$$BI = (100*(X-0.31)/ 0.17)$$

where:

$$X = (a^* + 1.75\ L^*)/(5.645\ L^* + a^* - 3.012\ b^*)$$

2.5.5. Impact on the alveolar structure from the crumb

The study of the alveolar structure from the crumb was performed using the steep reported in Genevois and de Escalada Pla (2021) with a few modifications. Bread slices of 10 mm thickness were photographed, then with the software ImageJ V1.52 (NIH, Maryland, United States) the central alveolar characterization was measured (Schneider et al., 2012). Results were expressed as the alveolar percentage on the total area of the bread. Each measure was performed per triplicate. The processes can be observed in **Fig. 1**.

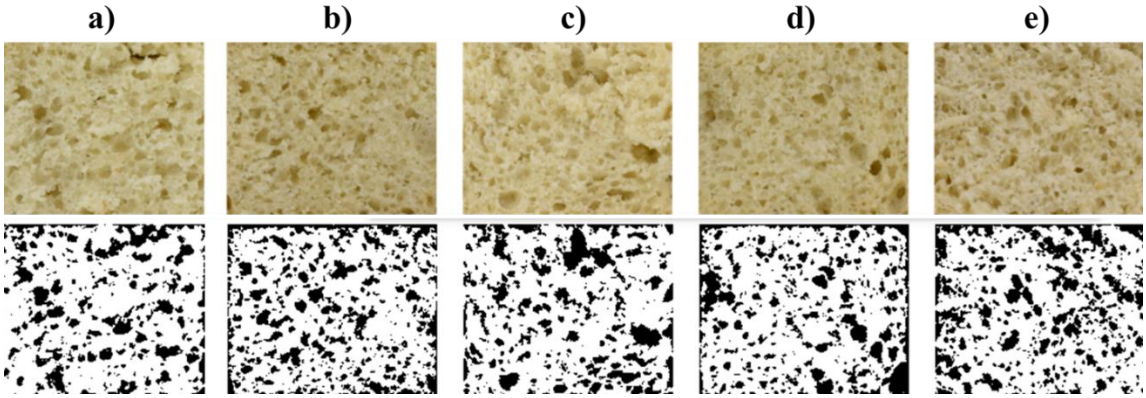

Fig. 1. Alveolar structure from the crumb before and after processed with the software ImageJ V1.52. Non-salt control (a), salt control (b), salt propionate control (c), 1% fermented whey (d) and 5% fermented whey (e).

2.6. Antifungal metabolites present in bread and dough

2.6.1. Study of the occurrence of organic acids in bread and dough

The identification and quantification of the main present organic acids and phenolic compounds from the dough and the bread were performed as described in Dopazo et al. (2021) with a few modifications. The organic acid identification used 5 g of the samples which were diluted in 20 mL doble deionized water and homogenised with an Ultra Ika T18 basic Ultraturrax (IKA®-Werke GmbH & Co., Staufen, Germany) for 1 min. Then centrifuged at 14000×g for 5 min and filtered thought a 0.22 µm pore. For the analysis, a high-performance liquid

chromatography (HPLC) Agilent 1100 Series (Agilent, California, United States) equipped with a quaternary pump, a 20 μL sample injection loop, a Rezex ROA-Organic Acid (150 × 7.8 mm) reverse phase column (Phenomenex Inc, California, United States) and a MD 4015 PDA diode array detector was used. Mobile phase was a solution of water with sulfuric acid at 0.1% at a flow rate of 0.8 mL/min. The assays were replicated 3 times per sample.

2.6.2. Study of the occurrence of phenolic compounds in bread and dough

For the study of the phenolic compounds present in the samples 5 g were diluted in 20 mL doble deionized water and homogenised Ultraturrax for 1 min. Then a QuEChERS extraction was performed of 10 mL of the homogenised mix (Brosnan et al., 2014). In this extraction 10 mL the sample were mixed with 10 mL of ethyl acetate, 4 g de magnesium sulphate and 1 g sodium chloride and vortexed for 1 min. Subsequently, samples were centrifuged at 3000×g for 10 min at 4 °C. The ethyl acetate supernatant was extracted and mixed with 0.15 g C18, 0.9 g magnesium sulphate and vortexed for 1 min. The extract was centrifuged under same conditions. The ethyl acetate phase was transferred to 15 mL tubes and dried under nitrogen flow with a TurboVap (Caliper Life Sciences, California, United States). Before the injection, the dry samples were suspended in 1 mL of a water-acetonitrile 90:10 (v/v) solution. An Agilent 1200 (Agilent, California, United States) was used as chromatograph for the assay. The chromatograph was equipped with a vacuum degasser, an autosampler, a binary pump and a column Gemini C18 (Phenomenex Inc., California, United States). The mobile phase was formic acid 0.1% (v/v) in water as solvent A and 0.1% formic acid in acetonitrile as solvent B. The elution rate was: 0 min, 5% B; 30 min, 95% B; 35 min, 5% B. Volume injected was 20 μL per sample. The run time selected was 37 min and a flow rate of 0.3 mL/min. The mass spectrophotometry (MS) assay was performed with a Q-TOF-MS 6540 Agilent Ultra High-Definition Accurate Mass (Agilent Technologies, California, United States) with an Agilent Dual Jet Stream electrospray ionisation (Dual AJS ESI) operating in the negative ion mode. The analysis was set at: capillary voltage 3.5 kV, drying gas flow (N2) 8.0 L/min, nebuliser pressure 30 psig, temperature of 350 °C and fragmentor voltage of 175 V. Targeted MS/MS study was performed using collision energy of 10, 20 and 40 eV. Calibration curves were conducted using concentrations ranging from of 0.01–1 mg/L. Software used for the data integration was MassHunter Qualitative Analysis Software B.08.00 (Agilent, California, United States). Each sample was injected 3 times (Dopazo et al., 2021).

2.7. Protection of bread from fungal spoilage with fermented whey as ingredient

Adapting the steps from Luz et al. (2020) with a few modifications after the bread preparation under sterile conditions in a Telstar MH 100 laminar flow hood (Telstar, Terrassa, Spain) 4 loafs of bread were contaminated in 4 spots with 10 μL of a fungal suspension 2.5 * 106 spores/mL, reaching a final concentration of 104 spores/g of bread. Then the bread were incubated at room temperature for 7 days. Each day fungal growth was studied by observation on each spot. Finally, 3 replicas of each bread were mixed at random with distilled peptone 0.1% (w/v) water in a 1/10 ratio (w/v). Afterwards, serial dilutions were performed of the samples and sow in PDA plates. After an incubation of 48 h at 25 °C the colony forming unis (CFU) were studied. Results were expressed in spores per gram of bread and the presence or absence of fungal growth per day.

2.8. Statistical analysis

To evaluate significant differences among each sample a multiple comparation analysis was performed with the software InfoStat 2019 using a one-way analysis of variance (ANOVA) test followed by Tukey's HSD post-test for the identification of significant differences (Universidad Nacional de Córdoba, Córdoba, Argentina). Differences between means were determined at $p < 0.01$.

3. RESULTS AND DISCUSSION

3.1. Effect of the fermented whey on the dough

The values of the pH and titratable acidity of the dough before fermentation and after fermentation can be seen in **Table 1** and **Table 2**. The result of the pH measure evidenced that an addition of 1%, 5% and 10% of LAB fermented whey before fermentation significantly decreased the pH of the samples in comparation to the control. Nevertheless, after fermentation only the addition of 10% LAB fermented whey managed to reach a similar reduction. The titratable acidity increased significantly in all doughs that included the fermented whey as ingredient before fermentation and after fermentation in comparation to the control. The reduction of the pH and rise of titratable acidity is generally related to a higher resistance to microbial contamination (Pitt & Hocking, 2009). Besides, low concentrations of this acidic compounds increase the acceptability of the product as they reproduce the flavor of sourdough bread (Bartkiene et al., 2018; Robert et al., 2009).

Table 1. Parameters of the doughs before the fermentation: volume expansion (cm), pH, titratable acidity (lactic acid g/100 g), total microbial population (Log_{10} CFU/g) and total fungal population (Log_{10} CFU/g).

Bread	Volume expansion	pH	Titratable acidity	Total microbial population	Total fungal population
Non-salt control	1.63 ± 0.06^{ab}	5.2 ± 0.1^{c}	4.2 ± 0.2^{c}	8.6 ± 0.2^{a}	8.7 ± 0.1^{a}
Salt Control	1.37 ± 0.12^{b}	5.2 ± 0.1^{c}	4.8 ± 0.4^{c}	8.5 ± 0.1^{a}	8.5 ± 0.1^{a}
Salt propionate control	1.47 ± 0.12^{b}	5.5 ± 0.1^{c}	4.7 ± 0.3^{c}	8.6 ± 0.1^{a}	8.5 ± 0.1^{a}
1% Fermented whey	1.80 ± 0.10^{a}	4.7 ± 0.1^{b}	7.9 ± 0.1^{b}	8.6 ± 0.1^{a}	8.5 ± 0.1^{a}
5% Fermented whey	1.47 ± 0.06^{b}	4.7 ± 0.1^{b}	8.2 ± 0.1^{b}	8.7 ± 0.1^{a}	8.6 ± 0.1^{a}
10 % fermented whey	nd	4.6 ± 0.1^{a}	20.3 ± 0.6^{a}	8.6 ± 0.1^{a}	8.5 ± 0.1^{a}

Data were presented in mean ± SD. Significative differences among samples (columns) were marked different letters ($p < 0.01$). Data under the limit of detection was marked as nd.

Table 2. Parameters of the doughs after the fermentation: pH, titratable acidity (lactic acid g/100 g), total microbial population (Log_{10} CFU/g) and total fungal population (Log_{10} CFU/g).

Bread	pH	Titratable acidity	Total microbial population	Total fungal population
Non-salt control	5.3 ± 0.1^{a}	0.3 ± 0.0^{c}	8.4 ± 0.1^{a}	8.5 ± 0.3^{a}
Salt Control	5.2 ± 0.1^{a}	0.4 ± 0.0^{c}	8.7 ± 0.1^{a}	8.6 ± 0.1^{a}
Salt propionate control	5.7 ± 0.1^{a}	0.3 ± 0.0^{c}	8.6 ± 0.1^{a}	8.6 ± 0.1^{a}
1% Fermented whey	5.3 ± 0.1^{a}	0.7 ± 0.0^{b}	8.7 ± 0.1^{a}	8.6 ± 0.1^{a}
5% Fermented whey	5.3 ± 0.1^{a}	0.8 ± 0.0^{b}	8.4 ± 0.1^{a}	8.4 ± 0.1^{a}
10 % fermented whey	4.5 ± 0.4^{b}	1.8 ± 0.1^{a}	6.2 ± 0.3^{b}	6.5 ± 0.2^{b}

Data were presented in mean ± SD. Significative differences among samples (columns) were marked different letters ($p < 0.01$).

The study of the dough expansion during the 1 h fermentation is shown in **Table 2**. The addition of fermented whey at 1% to the bread showed a significant increase of the expansion volume compared to the control bread. The addition of the nutrients from the fermented whey may increase CO_2 yeast production. Moreover, the decrease of the pH helps to strength gluten networks which rises the alveolar CO_2 retention (Correa et al., 2021; Schober et al., 2003). The 5% addition of fermented whey to the dough gave statistically similar results of the expansion volume compared to the control. Finally, there was no volume increase in the dough with a 10% fermented whey. In these two samples the nutrient and pH phenomena described before is not happening probably due to the substitution of wheat flour with the fermented whey, as the concentration of wheat flour decreases the quantity of gluten and then the alveolar CO_2 retention also drops (Tomar et al., 2022). To increase the expansion volume surfactants are usually added

as ingredient: egg yolk, soy lecithin and glucose oxidase (Y. Cao, Jiang, et al., 2021; Sang et al., 2020). Some other studies that substitute flour in the breadmaking with different residues from the food industry did not show this great results. This evidence can be shown in da Cunha et al. (2021) were this replacement of wheat flour by flour and pulp of *Caryocar brasiliense* lead to a reduction of the dough expansion during the fermentation.

The study of the total microbial population (TMP) and total fungal population (TFP) did not show any significant changes between samples and control before fermentation, as it can be appreciated in **Table 1**, **Table 2**. Dough before fermentation reached concentrations among 8.5 to 8.7 Log10 of colony forming units (CFU)/gram of TMP and TFP. After the 1 h fermentation the TMP and TFP was steady around a similar concentration of Log10 of CFU/gram in the 1% bread, 5% bread and all 3 controls. Nevertheless, the dough with a 10% LAB fermented whey showed a significative a reduction of 2.0 Log10 of CFU/gram of dough compared to the other doughs. Probably as a result of the antimicrobial activity from the compounds present in the fermented whey such as lactic acid (Cizeikiene et al., 2013). This reduction of the microbial population in the 10% bread might be another of the reasons leading to the decrease on the dough expansion, as the yeast are the CO_2 producers in the dough (Tomar et al., 2022). Finally, by the results it can be appreciated that yeast were the main microorganisms found in the doughs and bread. Results from the TMP and TFP evidence similar microbial concentrations in all the samples. This is because Rose-Bengal agar is a selective medium with chloramphenicol, a compound that inhibits bacteria growth (Pilote-Fortin et al., 2021). Non-sourdough bread tent to have microbial population primarily consisting of yeast from *Saccharomyces* spp. (Madden et al., 2022).

3.2. Effect of the fermented whey on the bread

The pH and titratable acidity of the bread can be seen in **Table 3**. The pH was significative lower in the samples with a 5% and 10% fermented whey compared to the control. Similar results can be observed for the titratable acidity where samples also evidenced higher titratable acidity compared to the control bread. In the study of the TMP and TFP no CFU/gram of bread were detected in the in any of the samples (**Table 3**). As is described by Cauvain and Young (2007) at high temperatures like the ones used for the bake (200 °C for 45 min) most the microorganism present in the bread are inactivated.

Table 3. Parameters of the bread were presented in two tables. Table 3a (a): pH, titratable acidity (lactic acid g/100 g), total microbial population (Log_{10} CFU/g), total fungal population (Log_{10} CFU/g), specific volume (cm^3/g). Table 3b (b) luminosity (L*), redness (a*), yellowness (b*), BI (browning index) and porosity (% of alveolar surface in a slice of bread).

a)

Bread	pH	Titratable acidity	Total microbial population	Total fungal population	Specific volume	Aw
Non-salt control	5.5 ± 0.1^{ab}	0.3 ± 0.0^{d}	nd	nd	3.25 ± 0.08^{a}	0.72 ± 0.01^{a}
Salt Control	5.6 ± 0.1^{a}	0.3 ± 0.0^{d}	nd	nd	2.64 ± 0.09^{b}	0.72 ± 0.01^{a}
Salt propionate control	5.4 ± 0.2^{ab}	0.3 ± 0.0^{d}	nd	nd	2.57 ± 0.13^{b}	0.75 ± 0.02^{a}
1% Fermented whey	5.1 ± 0.1^{bc}	0.5 ± 0.0^{c}	nd	nd	2.86 ± 0.23^{ab}	0.77 ± 0.01^{a}
5% Fermented whey	4.8 ± 0.1^{c}	0.6 ± 0.0^{b}	nd	nd	2.61 ± 0.22^{b}	0.78 ± 0.02^{a}
10 % fermented whey	4.3 ± 0.1^{d}	1.4 ± 0.1^{a}	nd	nd	1.59 ± 0.07^{c}	nd

b)

Bread	L*	a*	b*	BI	Porosity
Non-salt control	84.85 ± 0.30^{a}	0.35 ± 0.14^{a}	1.40 ± 0.33^{a}	1.93 ± 0.49^{a}	25.9 ± 1.2^{ab}
Salt Control	84.75 ± 0.42^{a}	0.40 ± 0.22^{a}	1.31 ± 0.41^{a}	1.86 ± 0.58^{a}	23.7 ± 0.7^{ab}
Salt propionate control	85.11 ± 0.23^{a}	0.57 ± 0.11^{a}	1.64 ± 0.21^{a}	2.38 ± 0.26^{a}	22.7 ± 0.6^{b}
1% Fermented whey	84.56 ± 0.43^{a}	0.46 ± 0.22^{a}	0.84 ± 0.44^{a}	1.36 ± 0.68^{a}	26.1 ± 1.0^{ab}
5% Fermented whey	84.56 ± 1.20^{a}	0.46 ± 0.20^{a}	0.84 ± 1.16^{a}	1.36 ± 1.30^{a}	26.4 ± 1.2^{a}
10 % fermented whey	84.44 ± 0.32^{a}	0.41 ± 0.17^{a}	0.95 ± 0.36^{a}	1.46 ± 0.52^{a}	nd

Data were presented in mean ± SD. Significative differences among samples (columns) were marked different letters ($p < 0.01$). Data under the limit of detection was marked as nd.

Specific volume is one of the results (Table 3) described a similar tendency to the evidenced in the in the expansion volume assay. Non-salt control evidenced a significant increase of the cm3/g of bread in comparation to the other bread, except the bread 1%. The specific volume of bread 1% was slightly greater to the salt-control and the salt-propionate-control. Nevertheless, no significative differences were observed among the bread 1%, the bread 5%, the salt-control and the salt-propionate-control. The only a reduction of the specific volume was observed in the bread 10% in comparation to the control bread. Usually, treatments like the one used in this study lead to a reduction of the wheat flour content of bread with no addition of proteases decrease the specific volume of bread (Honda et al., 2021; Sadeghian Motahar et al., 2022). The addition of other ingredients with high sugar concentrations, like red beetroot powder and raw fermented maize, in quantities around 5–10% usually leads to this reduction (Cui et al., 2022; Sun et al., 2022). Water activity values of all bread (**Table 3**) did not evidence any relevant differences. They average a 0.72 to a 0.78 Aw which is below the ideal Aw for the fungal growth (Pitt & Hocking, 2009).

Regarding the colorimetric parameters of the LAB fermented whey bread the luminosity did not show any significative differences compared to the samples. The redness and the yellowness evidenced a similar trend (**Table 3**). Usually, the addition of new ingredients to the bread caused changes in the color. Among many other authors, Cirlincione et al. (2022) reports a reduction in the L*, a* and b* parameter with the *Pleurotus eryngii* powder to the recipe (Angioloni & Collar, 2012; Cirlincione et al., 2022). No significative differences were manifested browning index among the bread. The addition of reductor sugars and proteins increase Maillard reaction during the bakery of the product (Kowalski et al., 2022; Sadeghian Motahar et al., 2022). Nevertheless, the lactose and proteins present in the whey did not alter the color of the bread.

Finally, the study of the alveolar surface evidenced a significative increase of the bread with a 5% fermented whey in comparation to the bread control with propionate and salt (**Table 3**). No significative differences were observed in comparation with the other samples. This alveolar surface is related to the yeast CO_2 production. Higher concentrations of sugars like the lactose present in the whey help to improve this CO_2 production (Zhou et al., 2021). Specific volume, color and porosity of the bread are the attributes that most affect consumer opinion. Higher similarities of these parameters between a modified bread to a regular bread are related to an increase of the acceptance of these products by the consumer (Morais et al., 2014).

3.3. Antifungal metabolites present in bread and dough

After the determination and quantification of the different antifungal metabolites occurrence four different compounds were present in detectable quantities. This observed compounds in the dough and bread are shown in **Table 4**. This phenolic and organic acids were lactic acid, DL-3-phenyllactic acid (PLA), benzoic acid and ferulic acid. Lactic acid was only found in the dough and bread containing the LAB fermented whey. The concentration of this metabolite slightly decreased during process. This compound is the mayor metabolite produced by the LAB fermentation (Carr et al., 2002). Therefore, as it was expected the quantity of this compound was significantly higher when more fermented whey was added to the bread recipe. The concentration of this acid during the bread production. reached concentrations of 1.04 – 1.40 g/kg in the dough and bread with 1% fermented whey, from 5.52 to 6.56 g/kg in the ones with 5% and from 23.96 to 27.16 g/kg in the with a 10%. Among the phenolic compounds only the PLA was present in significative greater concentrations in the 5% and 10% fermented whey bread compared to the control. During the fermentation steep this acid significantly decreased its concentrations in all dough. Nevertheless, PLA concentrations kept stable during the bake steep. The concentration of this compound before the fermentation was 7.86 and 12.78 mg/kg in the 5% fermented whey sample and 10% fermented whey samples, respectively. Then after the

fermentation and in the bread the concentration of PLA decreased to values between 3.49 and 3.65 mg/kg in the 5% fermented whey bread and from 7.28 to 6.24 in the 10% fermented whey sample. Benzoic acid and ferulic acid were found at lower concentrations.

Table 4. Results of the quantification of organic acids (g/kg), lactic acid (a) and phenolic acids (mg/kg), DL-3-phenyllactic acid (b), benzoic acid (c) and ferulic acid (d) present in the dough and bread.

a) Lactic acid

Samples	**Non-salt control**	**Salt Control**	**Salt propionate control**
Before fermentation	nd	nd	nd
After fermentation	nd	nd	nd
After bake	nd	nd	nd
Samples	**1% Fermented whey**	**5% Fermented whey**	**10% fermented whey**
Before fermentation	1.40 ± 0.04^{cB}	6.56 ± 0.1^{bA}	27.16 ± 0.04^{aA}
After fermentation	1.68 ± 0.03^{cA}	5.52 ± 0.04^{bB}	24.44 ± 0.04^{aB}
After bake	1.04 ± 0.03^{cC}	5.64 ± 0.11^{bB}	23.96 ± 0.18^{aC}

b) DL-3-phenyllactic acid

Samples	**Non-salt control**	**Salt Control**	**Salt propionate control**
Before fermentation	0.14 ± 0.00^{cC}	0.24 ± 0.02^{cA}	0.50 ± 0.26^{cA}
After fermentation	0.67 ± 0.00^{cA}	0.25 ± 0.02^{cA}	0.43 ± 0.04^{cA}
After bake	0.24 ± 0.02^{cdB}	0.18 ± 0.02^{cA}	0.15 ± 0.01^{cA}
Samples	**1% Fermented whey**	**5% Fermented whey**	**10% fermented whey**
Before fermentation	1.54 ± 0.15^{cA}	7.86 ± 0.74^{bA}	14.78 ± 1.35^{aA}
After fermentation	1.21 ± 0.08^{cA}	3.49 ± 0.50^{bB}	7.28 ± 0.05^{aB}
After bake	1.84 ± 0.14^{cA}	3.65 ± 0.49^{bB}	6.24 ± 0.02^{aB}

c) Benzoic acid

Samples	Non-salt control	Salt Control	Salt propionate control
Before fermentation	0.19 ± 0.01^{aC}	0.31 ± 0.01^{aC}	0.27 ± 0.06^{aB}
After fermentation	0.50 ± 0.01^{bB}	0.51 ± 0.00^{bB}	0.37 ± 0.02^{cB}
After bake	1.94 ± 0.01^{aA}	1.61 ± 0.01^{bA}	1.33 ± 0.08^{bcA}
Samples	**1% Fermented whey**	**5% Fermented whey**	**10% fermented whey**
Before fermentation	nd	0.75 ± 0.05^{aA}	2.13 ± 0.17^{aA}
After fermentation	0.70 ± 0.02^{aB}	nd	0.40 ± 0.02^{cC}
After bake	1.22 ± 0.07^{cA}	0.80 ± 0.02^{dA}	1.32 ± 0.12^{bcB}

d) Ferulic acid

Samples	Non-salt control	Salt Control	Salt propionate control
Before fermentation	0.24 ± 0.04^{c}	0.70 ± 0.03^{aA}	nd
After fermentation	nd	0.42 ± 0.02^{bB}	0.75 ± 0.12^{a}
After bake	nd	nd	nd
Samples	**1% Fermented whey**	**5% Fermented whey**	**10% fermented whey**
Before fermentation	nd	0.38 ± 0.00^{bA}	nd
After fermentation	0.28 ± 0.04^{bB}	0.30 ± 0.07^{bA}	0.46 ± 0.11^{bA}
After bake	1.03 ± 0.02^{aA}	0.14 ± 0.01^{cB}	0.22 ± 0.05^{bA}

Data were presented in mean ± SD. Values that were significantly different among the stage of the bread preparation between samples (rows) were marked with lower case letters, significative differences during the bread preparation in the same sample (columns) were marked with different capital letters ($p < 0.01$). Data under the limit of detection was marked as nd.

Many authors report the wide antifungal spectrum of the detected metabolites (Axel et al., 2016; Corsetti et al., 1998; Illueca et al., 2021; Magnusson et al., 2003). Lactic acid is a commonly used by the industry as a food preservative. Use of this compound is even approved by Asia and the European Union as it is known to be safe for the consumer and have antimicrobial properties (ANNEX 1: ASEAN MAXIMUM USE LEVELS OF FOOD ADDITIVES, 2021; European Parliment, 2010). PLA is another naturally occurring compounds produced during LAB fermentations (Xu et al., 2021). This compound is known to have antifungal activity against many fungal species by targeting and disaggregating fungal cell membranes (Dieuleveux et al., 1998). Therefore, presence of this compound have been linked to the growth inhibition of many fungal species (Lipinska-Zubrycka et al., 2020; Russo et al., 2017; Schnürer & Magnusson, 2005).

3.4. Bio-preservation of bread

The results of the shelf life of bread monotonized though the 7-day incubation are displayed in **Table 5**. As it can be appreciated the bread with a 10% concentration of fermented whey was not included in this study as a result of not reaching the ideal properties expected from a bread. Focusing on shelf live, both studied bread (the 1% and 5% fermented whey) extended the shelf life of this product compared to the controls. In the bread contaminated with *A. flavus* the shelf life was extended to the fifth day of incubation, while on the control bread the growth of mold was observable after only 3 days. In the bread inoculated with *P. verrucosum* the fungi growth after 4 days in all 3 control bread, after 5 days in the bread with a 1% fermented whey and finally, the bread with a 5% fermented whey the mold only was visible after 7 days incubation. Therefore, the addition of a 5% fermented whey managed to extend the shelf life of the bread 2 days against *A. flavus* and for 3 days against *P. verrucosum* in comparation to the control bread. In **Fig. 2** it can be appreciated the state of the bread after the third and seven days of incubation.

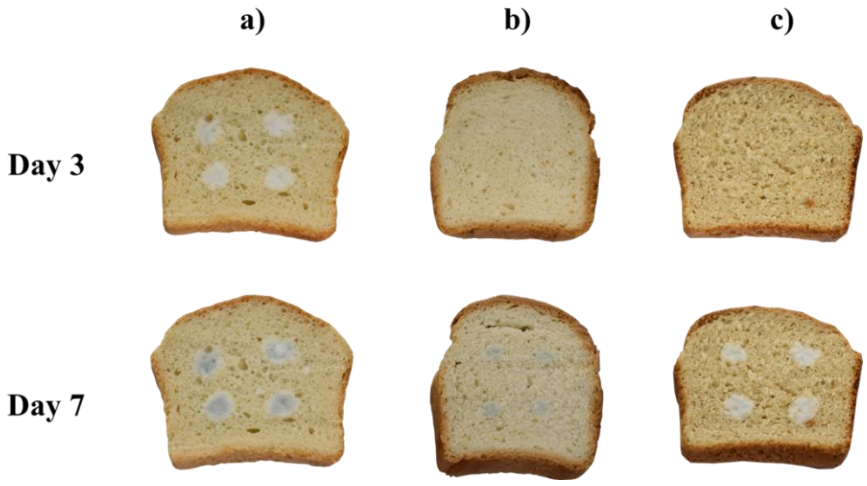

Fig. 2. Loaves of bread inoculated with *P. verrucosum*. Salt Control bread (a) and 1% fermented whey bread (b) and 5% fermented whey bread (c) after 3 and 7 day incubation.

Table 5. Shelf life of the bread loaves contaminated with *A. flavus* (a) and *P. verrucosum* (b) monitored in days. Presence of fungal growth in any of the sports among the 4 replica was indicated as "+".

a) *Aspergillus flavus*

Day	Non-salt control	Salt Control	Salt propionate control	1% Fermented whey	5% Fermented whey
1	-	-	-	-	-
2	-	-	-	-	-
3	-	-	-	-	-
4	+	+	+	-	-
5	+	+	+	-	-
6	+	+	+	+	-
7	+	+	+	+	+

b) *Penicillium verrucosum*

Day	Non-salt control	Salt Control	Salt propionate control	1% Fermented whey	5% Fermented whey
1	-	-	-	-	-
2	-	-	-	-	-
3	-	-	-	-	-
4	+	+	+	-	-
5	+	+	+	+	-
6	+	+	+	+	-
7	+	+	+	+	+

The treatment proved to increase the shelf life of the bread. The microbiological study of the bread (**Fig. 3**) proved the previous results. Bread with a 5% reduced the presence of fungi in a 2.4 LOG10 spores/g of bread compared to the control bread. Other bread contaminated by these fungi did not show any significative differences between them. It should be pointed that the control bread with calcium propionate, the most common antifungal product used in the bakery industry, did not managed to extend the shelf life of the bread more than the studied treatment (EFSA, 2016). On the other hand, the treatment against *A. flavus* did not evidence a reduction of the fungal population after the 7 days incubation.

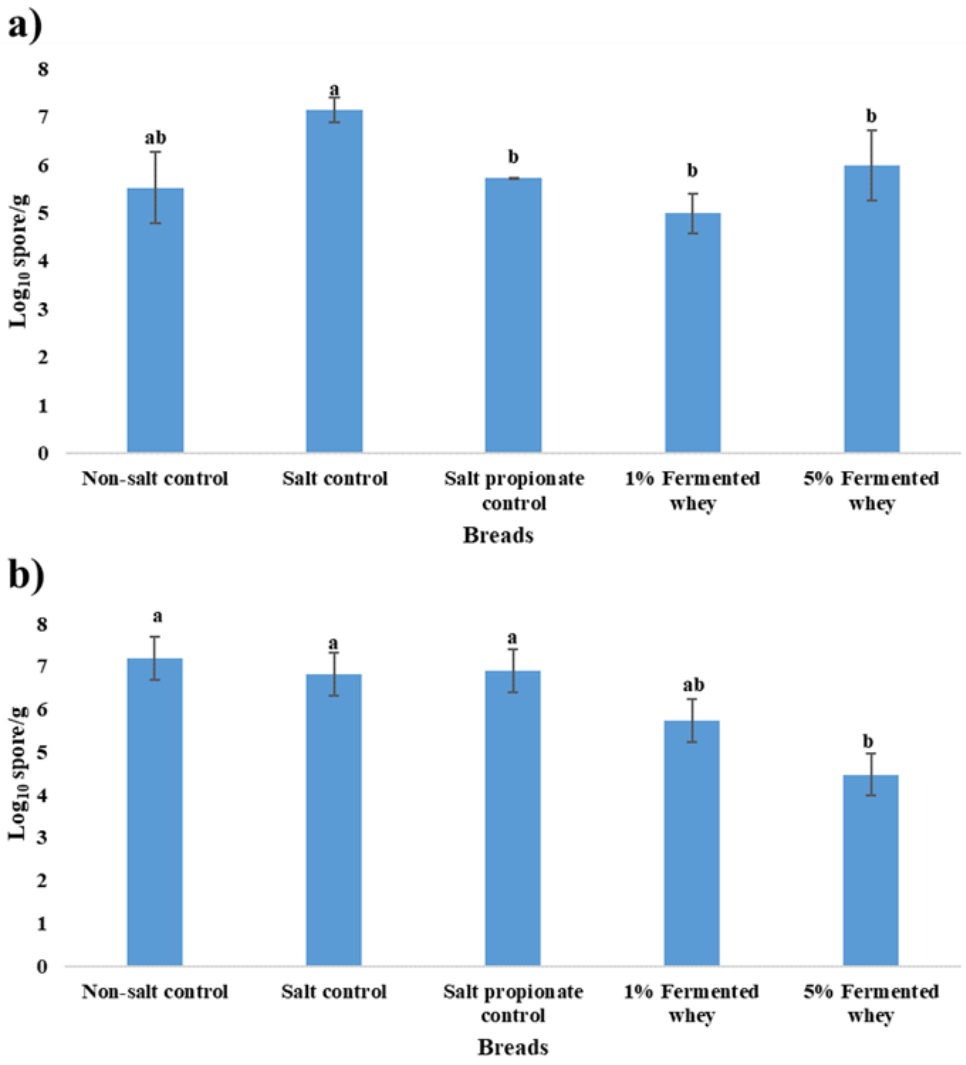

Fig. 3. Results of determination of the fungal colony forming units in bread contaminated with *A. flavus* (a) and *P. verrucosum* (b) after a 7 day incubation. The difference of letters indicates significant differences ($p < 0.01$).

The use from the LAB fermented whey as a bio-preservative agent of bread is really interesting for many reasons, especially two. First, in Spain and other countries the addition of milk derivates to bread is legal and allowed by the legislation (Real Decreto 308/2019). Secondly, lactic acid bacteria have the consideration as Qualified Presumption of Safety (QPS) by the European Union and Generally-regarded-as-safe (GRAS) by the Food and Drug Administration of the United States of America, this means that the addition of this microorganisms in permitted in foods among these regions. In addition, the other advantages exposed in the introduction postulates the use of this antifungal agent as an interesting alternative or complementary product

to the commonly used in the industry, like the calcium propionate, propionic acids, or sodium benzoate (Mandal et al., 2013).

The use of LAB fermented whey ingredient is a novel application for the bread bio-preservation. There are very few applications of for a LAB fermented whey in the bibliography. Luz et al. (2021) studies evidence the possible use of fermented whey by *L. plantarum* and *L. ghanensis* species as a substitute of water in the bread production to extend the shelf life of bread for 2 days compared to a control bread with sodium propionate against a *Penicillium* expansum contamination. Some other possible applications of LAB to preserve bread can be found. Gerez et al. (2009) reports the use of sourdough starters with LAB with antifungal properties is *Lactobacillus plantarum*, *Lactobacillus reuteri*, and *Lactobacillus brevis* for the preservation of bread against *A. niger* obtains similar result that a calcium propionate control. Lavermicocca et al. (2000) study evidence the potential in *in vitro* test of many species of *Lactobacillus alimentarius*, *Lactococcus lactis*, *Lactobacillus brevis* and *Lactobacillus plantarum* to inhibit the growth of fungi from the *Aspergillus* and *Penicillium* genera that typically contaminate bread.

4. CONCLUSIONS

This ingredient (LAB fermented whey) has the potential to reducing the use of the chemical additives added by the industry to bread to prevent fungal contamination. The addition of this ingredient to a bread preparation did not greatly alter the physical parameters of the bread. In addition, an increase of the shelf life of the bread was achieved in comparation to a regular bread and a bread with calcium propionate as an additive. This reduction of the fungal contamination was achieved with fungal concentrations far superior to the ones detected naturally. Further studies will be focused on the scalable production and sensory evaluation of the bakery product with the ingredient. Moreover, the production of fermented whey by lactic acid bacteria is really cheap. This subproduct is the principal residue from the cheese making industry and is a great matrix for lactic acid bacterial fermentation. Finally, in addition, the revalorization from the whey is among the 2030 objectives for sustainable development proposed by the WHO.

Food Bioscience, 58, 103588 (2024)

3.4. Revalorization of beer brewing waste as an antifungal ingredient for bread biopreservation

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1. INTRODUCTION

Nowadays, there is an increasing interest in finding alternative uses for the by-products produced by the food industry. With a world growing population that has surpasses seven billion humans in the planet the United Nations is encouraging the investigation to identifying ways for more sustainable use of resources and the revalorization the food wastes (United Nations, 2015). Beer is the most consumed alcoholic drink in globally, among all beverages is the third in consumption after water and tea (Habschied et al., 2020). Beer is mainly produced with a malt in a process known as brewing (Albanese et al., 2017). During the production of beer, the main waste is the brewer's spent grain also called beer bagasse (BB). Approximately 5–6 kg of bagasse are produced for every 100 litters of beer (Del Rio Osorio et al., 2021). This bagasse brewing is a solid residue primarily used for feed stock, as a fertilized in for crops and for biogas production (Kwoczynski & Čmelík, 2021; Loomis et al., 2020). These uses are not enough to significantly reduce the quantities of this waste, therefore, ways for the conversion of the bagasse are being studied (Martínez et al., 2012).

Bagasse is the residue produced from the filtration of the wort during the brewing process. This filtered residue is mainly composed of the malt seeds after the fermentation, therefore, is a matrix rich in nutrients. The composition of malt bagasse after the beer processing contains a 73% of carbohydrates (64% of them fibber), 24% protein content, 4% lipids and 2% ashes (Tassoni et al., 2020). Several studies repot the fermentation of this matrix for the production of compounds with value for the industry. In the investigation Moran-Aguilar et al. (2021) it is reported the production of cellulases and xylanases with an *Aspergillus niger* fermentation. In other examples, by the fermentation with other microorganism this matrix has been used for the production of biofuels, organic acids and biosurfactants among many other products (Khonngam & Salakkam, 2019; Pérez-Bibbins et al., 2015).

There is a growing interest from consumers in the ingredients used in the food industry, which has led to an increased demand for a reduction of the chemical preservatives (Cosentino et al., 2018). One of the alternatives more widely investigated in recent years is the use of lactic acid bacteria (LAB) for the biopreservation of foods (Li et al., 2020; Mcnair et al., 2018). LAB fermentation has been proven to extend the shelf life of foods against fungal contamination. Biopreservation with this microorganism has the potential to partially or totally reduce the use of chemical preservatives (Dopazo, Luz, Mañes, et al., 2021; Lavermicocca et al., 2000). This antifungal activity is induced by a wide spectrum of antifungal compounds metabolized during the fermentation, such as, organic acids, phenolic acids and bioactive peptides (Endo & Okada, 2008; Peyer et al., 2016; Varsha & Nampoothiri, 2016). For this fermentation different matrixed

have been used, even wastes from the industry (Luz, Quiles, et al., 2021). And bagasse is a rich in nutrients matrix that can be used for the cultivation of lactic acid bacteria.

Fungal contamination is a significant challenge faced by the bread industry worldwide. In Europe, annual losses are estimated to surpass 200 million euros, particularly due to contamination from the *Aspergillus* and *Penicillium* genera (Garcia et al., 2019; Legan, 1993). These fungi produce mycotoxins, including the cancerogenic aflatoxin B1 which can severely affect consumers and animal health (Marin et al., 2013). Preventing fungal contamination is of utmost importance to the food industry, this contamination is usually prevented by the use of calcium propionate (Gagiu et al., 2013). However, there is a growing interest in reducing its usage, and researchers are exploring new approaches to address this issue (Gerez et al., 2009; Rizzello et al., 2011). Bread production involves the use of a diverse range of ingredients to achieve desired properties such as texture, reduced microbial contamination and enhanced flavour. Ingredients such as whey, egg yolk, and red beetroot powder have been researched with good results for improvement of this attributes (Cui et al., 2022; Luz, Quiles, et al., 2021; Sang et al., 2020). Furthermore, as described in Sadeghi et al. (2023) use of microorganism in bread have been proved to increase not only the quality but also the security of different bread and bread-like products. Therefore, bread is a suitable matrix for the addition of new ingredient, such as the fermented BB to increase the shelf life of this product.

Following this trend the aim of this work was a) to optimize a culture medium with beer bagasse for LAB fermentation and characterize the antifungal properties of this fermented medium, b) study the modification of the technological properties from the dough and bread when the fermented culture medium was added as an ingredient, c) to investigate the antifungal compounds present in the dough and bread with the fermented medium, d) to determine if the addition of this ingredient reduces the fungal contamination and toxigenic occurrence in the bread.

2. MATERIAL AND METHODS

2.1. Chemicals and microorganisms

BB was provided by TYRIS (Maicerías Españolas S.A., Valencia, Spain). Milli-Q water with a resistivity of <18 Megaohm-cm was obtained from the Milli-Q purification system (Millipore, Bedford, United States). Lactic acid, and phenyllactic acid (PLA) standards were provided by BaChem (Weil am Rhein, Germany). Buffered peptone water, Potato Dextrose Agar (PDA), Man Rogosa Sharpe Agar (MRS Agar), and Man Rogosa Sharpe Broth (MRS Broth) were obtained from Liofilchem (Teramo, Italy). Acetonitrile (ACN), ethyl acetate (EA), and methanol (MeOH), glucose, yeast extract, tryptone, ammonium sulphate, sodium chloride, magnesium

sulphate, potassium phosphate, and manganese sulphate were procured from Sigma-Aldrich (Dublin, Ireland). The wheat flour, sugar, and salt used in the study were Hacendado brands, fresh yeast was obtained from Levitan, and mineral water was from Fuente primavera, all of which were obtained from Mercadona (Valencia, Spain). Aflatoxin B1, B2, G1, and G2 with a purity ≥97% were purchased from Sigma–Aldrich (St. Louis, Missouri, United States).

The antimicrobial potential of LAB strains isolated from breast milk was previously established in (Luz, Quiles, et al., 2021). Both strains were identified as *Lactiplantibacillus plantarum* (L. plantarum) through 16 S rRNA gene sequencing by the Spanish Type Culture Collection (CECT) by the 16 S rRNA gene sequencing method as described by Zeigler (2005) isolates were classified as two different strains: *L. plantarum* H1 (H1) and *L. plantarum* L1 (L1), with accession numbers OR899226 and OR899227, respectively. Fungal species used in this study were *Aspergillus flavus* ITEM 8111 and *Alternaria alternata* ITEM 8123 purchased from the ITEM Collection from *Istituto di Scienze delle Produzioni Alimentari* (ISPA) (Bari, Italy). And *Penicillium commune* CECT 20767 from the CECT located at the University of Valencia (Valencia, Spain).

2.2. Preparation and fermentation of the BB culture mediums

A culture medium based on BB was prepared with different concentrations of the ingredient, including 5% (BB5), 10% (BB10), and 20% (BB20). The medium were supplemented with 10.0 g/L of glucose, 10.0 g/L of yeast extract, 10.0 g/L of tryptone, 6.5 g/L of sodium chloride, 2.5 g/L of ammonium sulphate, 2.5 g/L of dipotassium phosphate, 0.25 g/L of magnesium sulphate, and 62.5 mg/L of manganese sulphate. After complete homogenization through magnetic stirring, the medium were autoclaved at 120 °C for 15 min and inoculated with LAB (from 24-h incubation in MRS), at a concentration of 5% (v/v) previously washed 2 times with a PBS solution 0.1 M. The inoculated mediums were fermented at 37 °C for 72 h and subsequently frozen for further analysis. A control of each fermentation was performed using culture medium with the supplements without BB and fermented with each the bacteria.

2.3. Determination of the antifungal activity from the fermented BB culture mediums

The protocol for assessing the antifungal activity of the fermented culture mediums was adapted from Cortés-Zavaleta et al. (2014). The fermented culture mediums were centrifuged, then the cell free supernatant (CFS) was separated from the pellet and then frozen at −80 °C prior to lyophilization using a FreeZone 2.5 L freeze-drying equipment (Labconco, Missouri, United States). A suspension of 500 g/L of the lyophilized CFS was prepared with distilled water and

added in a 1:10 (v/v) proportion to Potato Dextrose Agar (PDA) that was tempered to 45 °C, resulting in a final concentration of 50 g/L. PDA plates with the CFS-containing were inoculated with 10 µL of a fungal suspension and incubated at 25 °C for 96 h. Controls were prepared using PDA without CFS. Triplicate assays were performed, and the area of the mycelial growth was measured at 48 and 96 h after inoculation for both the samples (S) and control (C). The results were expressed as the mean ± standard deviation of the diameter measured, and the percentage of inhibition (I%) was calculated by comparing the samples with the control, as follows:

$$I\% = (C - S) / C \times 100$$

2.4. Characterization of the antifungal compounds present in the fermented BB culture mediums

For the analysis of the pH from the samples a pH-meter session tm + ph3 (Hach Company, Colorado, United States) equipped with an electrode Sension + CAT 5010 T (Hach Company, Colorado, United States) was used for measuring the samples per triplicate before and after the medium fermentation. The quantification of the lactic acid was performed as described in Dopazo, Luz, Mañes, et al. (2021) from the CFS. Results were expressed as g/L.

The identification and quantification of the PLA was performed as described in Dopazo, Luz, Mañes, et al. (2021) from the CFS. First a QuEChERS extraction of 10 mL from the CFS samples was performed as described in Brosnan et al. (2014). This assay was performed per triplicate. The results were quantified in mg/L. All results were displayed as the mean ± standard deviation.

2.5. Breadmaking procedure with fermented BB medium as an ingredient

The culture medium with a 20% BB fermented by the strain *L. plantarum* L1 were chosen as antifungal ingredient, which was frozen and subsequently lyophilized and grinded into a powder. The bread was prepared following the subsequent steps: 165 g of mineral water, 20 g of fresh yeast, and 10 g of sugar were added to 300 g of wheat flour and 6.5 g of salt. This dough was kneaded for 10 min in a bread-making machine (SilverCrest SBB 850 A1, SilverCrest, Bochum, Germany). After fermentation for 1 h at 30 °C, the dough was covered with foil and baked at 200 °C for 25 min. The baked bread was cooled to 25 °C for further analysis. A total of six samples were prepared, including a control (BC) consisting of the ingredients mentioned above, a control with the addition of 1.5 g of calcium propionate (BCP), which is the maximum allowed by European legislation (EFSA, 2016). Two treatments were prepared by substituting the

fermented BB culture medium powder for flour at 10% (B10%) and 20% (B20%). Finally, two controls were prepared: BC10% and BC20%, which consisted of the addition of the non-fermented BB medium at 10% and 20% (w/w) concentration.

2.6. Effect from the fermented BB medium as an ingredient in the dough and bread

In order to investigate the impact of the fermented BB-medium on the dough, the following experiments were conducted. First, the volume expansion of the dough was measured by placing 5 g of the dough in the bottom of a sterilized graduated tube with a capacity of 50 mL The total microbial counting from the samples was preformed using 10 g of the dough/bread and following the steeps described in Dopazo et al. (2023). After fermentation for 1 h at 30 °C, the volume expansion was determined as the increase in the height of the dough inside the tube (Sang et al., 2020).

Colour changes were measured using a ColorQuest® XE (Hunter Lab, Leicestershire, United Kingdom) to measure various locations from the crust of the bread and calculate the browning index with the method described in Aldughpassi et al. (2021). The water activity (Aw) of the bread loaves was measured using a Humimeter RH2 water activity meter (Max-Schaller-Straße, Styria, Austria) to evaluate the effect of fermented BB medium on the dough. The % of alveolar surface of the crumb was studied following the steps described in Dopazo et al. (2023). The specific volume was determined using the millet seed displacement method (Mannuramath et al., 2015). The bread samples were placed in a beaker filled with chia seeds, and the difference in volume between the same amount of chia seeds in the beaker with and without the bread was used to calculate the specific volume. The bread samples were then weighed, and the specific volume was calculated as the ratio of volume to weight (cm3/g). The moisture content of the bread samples was determined by heating 10 g of sample in an oven at 105 °C for 24 h, and the water concentration was calculated by weighing the samples before and after heating (Guimarães et al., 2020). All experiments were carried out in triplicate, and the results were expressed as the mean ± standard deviation.

2.7. Antifungal metabolite occurrence in the fermented BB powder and the bread with the fermented BB medium powder as ingredient

The pH analysis of the ingredient, dough before and after fermentation, and bread after baking was conducted using an XS pH 7 Vio pH-meter (XS Instruments, Carpi, Italy) fitted with a 2 Pore Steel T electrochemical sensor (XS Instruments, Carpi, Italy). For the quantification of lactic acid and PLA was 10 g of the samples were homogenized with distilled water and centrifuged. The supernatant was used foe the analysis of the lactic acid and PLA following the steps described in "Material and methods 2.4".

2.8. Effect from the fermented BB as an ingredient in the shelf life and fungal growth on bread

The investigation of the extension of shelf life and fungal occurrence in the bread was carried out with modifications to the methodology described by Luz et al. (2019). The species *A. flavus* and *P. commune* are typical contaminants of bread, therefore, these fungi were used in this assay (Quiles et al., 2018). Under sterile conditions, loaves of bread were inoculated with 10 µL of a fungal suspension of 106 spores/mL at four different locations, resulting in a final concentration of 103 spores/g of bread. The drops were dried and then placed in a sterile bag for daily monitoring of fungal growth in the contaminated areas. The results of fungal growth were expressed as the percentage of growth spots per day and presented as the mean ± standard deviation. A total of 5 replicas were used for each trial.

2.9. Metabolite profiling of fungal growth on bread treated with BB ingredient

To investigate the effect of fermented BB medium on the metabolite profiles of *A. flavus* and *P. commune* after a 7-day incubation period, the methods described in (Dopazo, Luz, Mañes, et al., 2021) were adapted for this study. Samples were crushed using an electric coffee grinder (Cecotec, Valencia, Spain), and 5 g of the samples were extracted with 20 mL of methanol using an ultraturrax. After centrifugation at 3000×g for 15 min, the supernatant was dried using a Buchi R-215 Rotavapor system (Hampton, New Hampshire, United States). The samples were suspended in 2 mL of methanol, filtered through a 0.22 µm pore, and analysed using a UHPLC 1290 Infinity II equipped with a quadrupole time of flight mass spectrometer (Agilent 6546 Q-TOF) set in positive ionization mode. For chromatographic analysis, an Agilent Zorbax RRHD SB-C18, 2.1 mm × 50 mm, 1.8 µm column was used with Milli-Q water containing 0.1% formic acid (v/v) as mobile phase A and acetonitrile containing 0.1% formic acid (v/v) as mobile phase B in a gradient elution of: 0 min, 2% B; 22 min, 95% B; 25 min, 5% B at a flow rate of 0.4 mL/min. A total of 5 µL of each sample were analysed in triplicate. The parameters set for the

Dual AJS ESI were: gas temperature: 325 °C; nebulizer pressure: 40 psig; gas flow: 10 L/min; sheath gas flow: 12 L/min; sheath gas temperature: 295 °C; nozzle voltage: 500 V; capillary voltage: 4000 V; skimmer: 70 V; Fragmentor: 120 V; product ion scan range: 100–1500 Da; MS/MS scan rate: 3 spectra/s; maximum precursors per cycle: 2; MS scan rate: 5 spectra/s; and collision energy: 10, 20, 40 eV. Mycotoxins calibration curves were performed at concentrations of 0.01–5 mg/L. Integration, data elaboration, and identification of metabolites were performed using MassHunter Qualitative Analysis software B.08.00 and library PCDL Manager B.08.00.

2.10. Statistical analysis

The one-way ANOVA test, followed by the Tukey HSD post hoc test for multiple comparisons ($p < 0.05$) was used to perform the evaluations. These statistical tests were performed using the software InfoStat 2019 (Universidad Nacional de Córdoba, Córdoba, Argentina).

3. RESULTS AND DISCUSSION

3.1. Determination of the antifungal activity from the fermented BB culture mediums

Table 1 shows the data obtained from the investigation of antifungal activity presented by LAB fermented BB culture mediums. Both fermented mediums with a 20% of BB managed to reduce the growth of *A. flavus* in comparison to the control samples at 48 h. The highest inhibition was achieved by the 20% BB medium fermented by the strain *L. plantarum* L1, reaching an 28% inhibition. However, after 96 h no inhibition was observed by any of the fermented culture mediums. Regarding the experiments performed against *P. commune*, similar results were obtained, only the mediums with a 20% BB concentration significantly reduced the growth of the fungal contaminant compared to the control. The percentage of reduction was 5.9% greater in the medium with a 20% BB fermented by the *L. plantarum* L1 in comparation to the *L. plantarum* H1. After 96 h, not only the 20% BB fermented medium but also the 10% BB fermented medium were able to significantly inhibit the fungal growth compared to the control. In the test against *A. alternaria*, the 20% BB fermented medium showed significantly higher inhibition of mold growth at 48 and 96 h. The fermented 20% BB medium by *L. plantarum* L1 exhibited the highest inhibition rate compared to other LAB strain. Presumably, fermentation by *L. plantarum* L1 in this medium produced a CFS with higher antifungal potential. Moreover, the medium with 20% BB provided a richer environment for the metabolization of antifungal compounds during the LAB fermentation than the mediums with lower quantities of BB.

Table 1. Results from the study from the antifungal activity presented by the fermented beer bagasse mediums fermented by the LAB. Bacterial strain used for the fermentation were *L. plantarum* H1 and *L. plantarum* L1. The different mediums used were PDA (control), PDA with fermented culture mediums with a 5% of beer bagasse (BB 5%), PDA with fermented culture mediums with a 10% of beer bagasse (BB 10%) and PDA with fermented culture mediums with a 20% of beer bagasse (BB 20%). The treatments were evaluated against a) *Aspergillus flavus*, b) *Penicillium commune* and c) *Alternaria alternata*.

a) *A. flavus*

Medium	*L. plantarum* H1				*L. plantarum* L1			
	48 h		96 h		48 h			
	Diameter	% Reduction	Diameter	% Reduction	Diameter	% Reduction	Diameter	% Reduction
Control	2.5 ± 0.1^{b}	-	4.5 ± 0.0^{a}	nd	2.5 ± 0.1^{c}	-	4.5 ± 0.0^{a}	nd
BB 5%	2.3 ± 0.1^{b}	8.0	4.5 ± 0.0^{a}	nd	2.2 ± 0.1^{bc}	12.0	4.5 ± 0.0^{a}	nd
BB 10%	2.0 ± 0.1^{a}	20.0	4.5 ± 0.0^{a}	nd	2.1 ± 0.2^{ab}	16.0	4.5 ± 0.0^{a}	nd
BB 20%	1.9 ± 0.0^{a}	24.0	4.5 ± 0.0^{a}	nd	1.8 ± 0.1^{a}	28.0	4.5 ± 0.0^{a}	nd

b) *P. commune*

Medium	*L. plantarum* H1				*L. plantarum* L1			
	48 h		96 h		48 h		96 h	
	Diameter	% Reduction	Diameter	% Reduction	Diameter	% Reduction	Diameter	% Reduction
Control	1.7 ± 0.2^{b}	_	2.6 ± 0.1^{b}	-	1.7 ± 0.2^{b}	-	2.6 ± 0.1^{b}	-
BB 5%	1.5 ± 0.1^{ab}	11.8	2.1 ± 0.2^{a}	19.2	1.6 ± 0.3^{b}	5.9	2.4 ± 0.2^{b}	7.7
BB 10%	1.3 ± 0.2^{ab}	23.5	2.0 ± 0.2^{a}	23.1	1.2 ± 0.1^{ab}	29.4	1.9 ± 0.2^{a}	26.9
BB 20%	1.1 ± 0.1^{a}	35.3	1.9 ± 0.1^{a}	26.9	1.0 ± 0.1^{a}	41.2	1.6 ± 0.1^{a}	38.5

c) *A. alternata*

Medium	*L. plantarum* H1				*L. plantarum* L1			
	48 h		96 h		48 h		96 h	
	Diameter	% Reduction	Diameter	% Reduction	Diameter	% Reduction	Diameter	% Reduction
Control	1.7 ± 0.1^{b}	_	3.7 ± 0.1^{c}	-	1.7 ± 0.1^{c}	-	3.7 ± 0.1^{c}	-
BB 5%	0.9 ± 0.2^{a}	47.1	2.6 ± 0.1^{b}	29.7	1.3 ± 0.1^{b}	23.5	2.6 ± 0.2^{b}	29.7
BB 10%	0.8 ± 0.1^{a}	52.9	2.3 ± 0.1^{b}	37.8	1.1 ± 0.2^{b}	35.3	2.0 ± 0.1^{a}	45.9
BB 20%	0.6 ± 0.1^{a}	64.7	1.9 ± 0.2^{a}	48.6	0.5 ± 0.1^{a}	70.6	1.7 ± 0.1^{a}	54.1

Data was displayed as mean ± SD or % of reduction. Diameter was measured in cm and % reduction was measured as the average percentage of reduction from the diameter of the samples in comparation to the control. Samples marked as nd when no reduction was detected and "-" for the control reduction. Different letters were used to signal significative differences among the different fermented mediums at a specific time (columns). ($p < 0.05$).

The antifungal potential of LAB fermented CFS is well documented in the literature. First, a screening process for finding the right matrix for a certain strain is necessary to obtain a CFS with antifungal properties. For instance, Maciej Serda et al., (2021) used olive mill wastewater and rice hulls as a fermentation matrix for LAB to different strains of *Fusarium* spp., *Penicillium* spp and *Aspergillus* spp. In another study, Iosca et al. (2023) used a CFS of LAB fermented wheat bread waste and cheese whey for the biopreservation against *Penicillium* spp and *Aspergillus* spp. in the agar diffusion test. This study aimed to imitate the actual conditions in which the treatment will be applied. Instead of an agar diffusion test, as used in the above studies, the CFS was directly added to the agar. Similar methodology has been described in Ogunremi et al. (2022), and the results from the *in vitro* test were consistent when applied to food products.

3.2. Characterization of the antifungal compounds present in the fermented BB culture mediums

Table 2 provides the data from the pH thought the 72-h fermentation. Prior to fermentation, pH values ranging from 6.30 to 5.96 were observed in the culture media. Increased quantities of BB resulted in a significant reduction of the pH in the culture medium. After 72 h of fermentation, all samples showed a decrease in pH to below 4. The pH of the fermented media with BB was significantly lower compared to the control with regular culture medium, indicating a reduction of more than 0.3 pH units. However, no significant differences were observed when comparing the fermented media by the different LAB strains. Lower pH values are often associated with an increased production of antifungal metabolites such as lactic acid or acetic acid, as reported in the literature (Schnürer & Magnusson, 2005). In addition, lower pH are related to the decrease and delay of fungal growth (Pitt & Hocking, 2009).

Table 2. Results from the study of the antifungal metabolites occurrence in the BB mediums fermented by the LAB. Bacterial strain used for the fermentation were *L. plantarum* H1 *and L. plantarum* L1. The different mediums used were culture medium (control), culture medium with a 5% of beer bagasse (BB 5%), culture medium with a 10% of beer bagasse (BB 10%) and culture medium with a 20% beer bagasse (BB 20%). In the *L. plantarum* H1 and *L. plantarum* L1 an inoculation of this microorganism was added before the incubation.

Lactic acid bacteria	Medium	pH 0h	pH 72h	Lactic acid	Phenyllactic acid
L. plantarum H1	Control	6.30 ± 0.01^{d}	3.84 ± 0.01^{d}	12.1 ± 0.09^{b}	24.1 ± 0.09^{b}
	BB 5%	6.09 ± 0.02^{c}	3.55 ± 0.03^{c}	13.10 ± 0.07^{c}	25.70 ± 0.85^{b}
	BB 10%	6.01 ± 0.01^{b}	3.44 ± 0.02^{ab}	15.50 ± 0.01^{e}	27.78 ± 0.38^{c}
	BB 20%	5.96 ± 0.01^{a}	3.40 ± 0.01^{a}	20.26 ± 0.01^{g}	29.92 ± 0.97^{d}
L. plantarum L1	Control	6.30 ± 0.01^{d}	3.88 ± 0.01^{d}	10.04 ± 0.01^{a}	20.7 ± 0.09^{a}
	BB 5%	6.09 ± 0.02^{c}	3.54 ± 0.03^{c}	14.1. ± 0.08^{d}	25.51 ± 1.2^{b}
	BB 10%	6.03 ± 0.01^{b}	3.48 ± 0.02^{bc}	18.48 ± 0.01^{f}	28.80 ± 0.66^{cd}
	BB 20%	5.98 ± 0.01^{a}	3.42 ± 0.03^{a}	21.40 ± 0.10^{h}	30.41 ± 0.23^{d}

Data was displayed as mean ± SD. Results were expressed as pH, g/L of lactic acid and mg/L of phenyllactic acid. Different letters were used to signal significative differences of the studied parameters among the fermented mediums (columns). ($p < 0.05$). Results below the limit of detection were marked as nd.

Table 2 also shows the concentrations of lactic acid and PLA in the fermented media after 72 h. Lactic acid concentrations ranged from 10.04 to 21.40 g/L. Greater concentrations of the BB product in the mediums significantly increased the production of this acid. Mediums with a 5%, 10% and 20% of BB the bacteria produced and average of 13.60, 16.99 and 20.83 g/L, respectively, of lactic acid. In the mediums with BB the strain *L. plantarum* L1 produced significantly higher quantities of this metabolite compared to the other LAB strain. The fermentation of the culture medium with 20% BB by the *L. plantarum* L1 produced the highest concentration of lactic acid at 21.40 g/L. The PLA production was also affected by the selection the concentration of BB in the culture medium. Concentrations of BB superior to a 10% in the culture medium significantly improved the production of this metabolite. While in the control the concentrations averaged the 22.4 mg/L in the fermentation of the 20% BB culture mediums concentrations of PLA reached average values of 30.17 mg/L. No differences were observed among the LAB strains in the production of PLA in the BB-complemented media. Therefore, the strain *L. plantarum* L1 was determined to be more suitable for the fermentation of the BB matrix.

The increment in the production of lactic acid with increasing amounts of BB in the medium was expected, as the metabolization of sugars by LAB is linked to the production of lactic acid (Punia Bangar et al., 2022). BB is a matrix with various sugars that are liberated and metabolized by the bacteria during fermentation (Moran-Aguilar et al., 2021). Several studies link

the occurrence high concentrations of lactic acid to better bio-preservation properties of the bacteria in foods contaminated by fungi after the screening of several LAB isolates (El oirdi et al., 2021). In Ruggirello et al. (2019) higher production of lactic acid was linked to higher antifungal activities after the screening of 200 LAB isolates in *in vitro* assays. The presence of PLA is also a good indicator of the antifungal potential of a LAB-fermented CFS, as observed in studies by Debonne et al. (2020) and Rajanikar et al. (2021) against fungal species belonging to the *Aspergillus*, *Penicillium*, and *Fusarium* genera. The fermented medium with 20% BB and fermented by the strain *L. plantarum* L1 exhibited better antifungal properties and higher concentrations of antifungal metabolites, therefore, it was chosen for the production of the bread ingredient.

3.3. Effect from the fermented BB as an ingredient in the dough and bread

The results from the study of the volume expansion, water activity, moisture, and specific volume values can be observed in **Table 3**. It can be appreciated that the addition of the BB ingredient led to a significantly reduction the volume expansion of the dough in comparation to the control bread. The water activity from the bread with the fermented BB ingredient was lower in comparation to the control bread. The study of the weight loss evidenced values in concordance with the Aw results, the bread with a 10% of the fermented ingredient managed to retain more water during the bake. Moreover, the moisture content was significantly lower in the bread samples containing 10% BB (fermented and non-fermented). But this difference in the water content among all samples was below the 2%, indicating that this parameter was not significantly affected by the addition of BB. The specific volume of the bread samples varied. The lowest values observed in bread with fermented BB ingredient. This outcome was expected since the volume expansion values were lower also in the BB samples. The reduction of the volume expansion and specific volume of the dough and bread was an anticipated consequence of substituting wheat flour with BB ingredient, as this substitution led to a decrease in the concentration of gluten proteins, which play a critical role in retaining CO_2 during bread rise (Genevois & de Escalada Pla, 2021; Hernández-Aguirre et al., 2019). Besides, the decrease in water activity may slow fungal growth and even inhibit the germination of various fungal species (Ibrar et al., 2020; Perrone et al., 2020). Results of the colorimetric study, porosity and total microbial count did not evidence significative differences among the samples. Therefore, the addition of the ingredient did not affect this parameter. The absence of effect in the total counting is an exceptional result as this ingredient does not have antimicrobial activity against the planification yeast. Some LAB extracts may have anti-yeast potential, which can decrease the baking process (Bayrock & Ingledew, 2004).

Table 3. Results of the addition of the LAB fermented BB culture medium as ingredient in the characteristics the dough and the bread.

a)

Sample	Volume expansion	Aw	Moisture	Specific volume	BI
Control	1.8 ± 0.2^{c}	0.98 ± 0.03^{b}	34.0 ± 1.9^{b}	0.48 ± 0.1^{e}	1.51 ± 0.48^{a}
Propionate control	1.5 ± 0.1^{bc}	0.97 ± 0.01^{b}	33.9 ± 0.4^{b}	0.47 ± 0.1^{d}	1.71 ± 0.27^{a}
Control BB medium 5%	1.4 ± 0.1^{b}	0.96 ± 0.08^{b}	32.9 ± 0.1^{ab}	0.49 ± 0.1^{f}	1.92 ± 0.68^{a}
Control BB medium 10%	1.2 ± 0.1^{ab}	0.95 ± 0.02^{b}	32.1 ± 0.7^{a}	0.46 ± 0.1^{c}	2.10 ± 0.43^{a}
BB medium 5%	1.3 ± 0.2^{b}	0.86 ± 0.04^{a}	32.7 ± 0.5^{ab}	0.43 ± 0.1^{b}	1.87 ± 0.70^{a}
BB medium 10%	0.9 ± 0.2^{a}	0.84 ± 0.05^{a}	32.0 ± 0.7^{a}	0.42 ± 0.1^{a}	2.30 ± 0.62^{a}

b)

Sample	Porosity	Weight loss	Total microbial count		
			Non-fermented dough	Fermented dough	Bread
Control	21.3 ± 0.2^{a}	19.3 ± 0.4^{b}	8.4 ± 0.1^{a}	8.2 ± 0.1^{a}	nd
Propionate control	20.7 ± 1.4^{a}	18.2 ± 0.8^{ab}	8.5 ± 0.1^{a}	8.1 ± 0.1^{a}	nd
Control BB medium 5%	21.7 ± 0.6^{a}	17.8 ± 0.6^{ab}	8.5 ± 0.1^{a}	8.2 ± 0.1^{a}	nd
Control BB medium 10%	20.2 ± 1.0^{a}	17.4 ± 0.8^{ab}	8.4 ± 0.1^{a}	8.1 ± 0.1^{a}	nd
BB medium 5%	21.2 ± 1.2^{a}	18.0 ± 0.3^{ab}	8.6 ± 0.1^{a}	8.1 ± 0.1^{a}	nd
BB medium 10%	21.0 ± 0.9^{a}	17.5 ± 0.5^{aa}	8.5 ± 0.1^{a}	8.2 ± 0.1^{a}	nd

Data was displayed as mean ± SD. Results were expressed in the a: as cm (volume expansion of dough during fermentation), Aw, % of water in the bread (moisture), 3 cm3/g (specific volume) and browning index (BI). And in the b: % alveolar surface in a slice of bread (porosity), % of water loss (weight loss after the bake) and Log10 CFU/g of bread (total microbial counting). Different letters were used to signal significative differences of the studied parameters among the fermented mediums (columns). ($p < 0.05$). Results below the limit of detection were marked as nd.

3.4. Antifungal metabolite occurrence in the fermented BB powder and the bread with the fermented BB medium powder as ingredient

The results from the quantification of the pH, lactic acid and PLA present in the ingredient and dough-bread can be observed in **Table 4**, **Table 5**, respectively. In the ingredient the treatment evidenced a pH of 3.8 while in the control BB the pH was 6.1. This result was expected since no LAB fermentation occurred in the control. These values are similar to the ones obtained in the CFS characterization, evidencing that the production of the ingredient did not affect the pH. Likewise, as the no fermentation occurred in the control bread (control, propionate control and BB-control) the presence of lactic acid and PLA was below the limits of detection, while in the fermented ingredient values of lactic acid reached concentrations of 9.1 g/L and values of the PLA

were 135.1 mg/L. The pH of the dough and bread made with the fermented BB ingredient was significantly lower than that of the control bread, and the pH of the samples remained stable during fermentation but increased after baking, likely, due to water loss in the oven. The concentration of lactic acid described a linear tendency in concentration during the whole baking process, probably due to degradation or evaporation in the oven. The concentration of lactic acid was significantly higher in bread made with 10% fermented BB ingredient (average of 8.1 g/kg) than those made with 5% fermented BB ingredient (average of 4.2 g/kg), and the same trend was observed for PLA concentrations. The bread made with 10% BB ingredient had PLA concentrations exceeding 0.20 mg/kg, while those made with 5% BB ingredient had an average concentration of 0.09 mg/kg.

Table 4. Quantification from the pH and the main antifungal metabolites present in the ingredient: lactic acid and the phenyllactic acid.

Ingredient	pH	Lactic acid	PLA
Control BB medium	6.1 ± 0.2^{b}	nd	nd
BB medium	3.8 ± 0.1^{a}	91.0 ± 0.2	135.6 ± 0.5

Data was displayed as mean ± SD. Results were expressed as pH, g/kg of lactic acid and mg/kg of phenyllactic acid. Different letters were used to signal significative differences of the studied parameters among the fermented mediums (columns). ($p < 0.05$). Results below the limit of detection were marked as nd.

Table 5. Quantification from the pH (a) and the main antifungal metabolites present in the dough and the bread: lactic acid (b) and the phenyllactic acid (c).

a) pH			
Sample	**Dough**	**Fermented dough**	**Bread**
Control	5.2 ± 0.0^{b}	5.3 ± 0.1^{c}	5.9 ± 0.0^{d}
Propionate control	5.2 ± 0.2^{b}	5.1 ± 0.1^{bc}	5.9 ± 0.0^{d}
Control BB medium 5%	5.2 ± 0.0^{b}	5.3 ± 0.2^{c}	5.6 ± 0.0^{c}
Control BB medium 10%	5.2 ± 0.1^{b}	4.9 ± 0.1^{b}	5.6 ± 0.1^{c}
BB medium 5%	4.5 ± 0.1^{a}	4.2 ± 0.1^{a}	4.7 ± 0.0^{b}
BB medium 10%	4.3 ± 0.1^{a}	4.1 ± 0.0^{a}	4.3 ± 0.0^{a}

b) Lactic acid (g/Kg)			
Sample	**Dough**	**Fermented dough**	**Bread**
Control	nd	nd	nd
Propionate control	nd	nd	nd
Control BB medium 5%	nd	nd	nd
Control BB medium 10%	nd	nd	nd
BB medium 5%	3.9 ± 0.2^{a}	4.2 ± 0.3^{a}	4.5 ± 0.1^{a}
BB medium 10%	7.9 ± 0.1^{b}	8.1 ± 0.2^{b}	8.3 ± 0.2^{b}

c) PLA (mg/Kg)			
Sample	**Dough**	**Fermented dough**	**Bread**
Control	nd	nd	nd
Propionate control	nd	nd	nd
Control BB medium 5%	nd	nd	nd
Control BB medium 10%	nd	nd	nd
BB medium 5%	0.09 ± 0.02^{a}	0.10 ± 0.03^{a}	0.09 ± 0.02^{a}
BB medium 10%	0.20 ± 0.03^{b}	0.24 ± 0.07^{b}	0.84 ± 0.03^{b}

Data was displayed as mean ± SD. Results were expressed as pH, g/kg of lactic acid and mg/kg of phenyllactic acid. Different letters were used to signal significative differences of the studied parameters among the fermented mediums (columns). ($p < 0.05$). Results below the limit of detection were marked as nd.

The reduction of the pH might be another important factor in the reduction of the Aw in the treatment samples described before, the bibliography clearly explains the relation of those two parameters (Loureiro & Malfeito-Ferreira, 2003). Lower pH and higher acidity values are often associated with desirable flavours in bread, and consumers appreciate new flavours in the market (Gagiu et al., 2013). Additionally, this can also lead to a retardation of fungal growth in food (Marín et al., 2019). Higher concentrations of lactic acid and PLA have been reported to increase the shelf life of food in several studies. Many papers link the production of different antifungal metabolites such as hydroxy fatty acids, organic acids and even peptides by LAB species to a decrease in the fungal occurrence in a certain degree (Black et al., 2013; Ebrahimi et al., 2020; Riolo et al., 2023; Sadeghi et al., 2019). From all of them the presence of lactic acid and PLA are the most determinant in the antifungal activity, according to the literature (Lavermicocca et al., 2000). For example, Russo et al. (2017) linked the presence of lactic acid and PLA to a reduction in fungal presence in cereal-based products. Also, the review by Schnürer & Magnusson (2005) described several examples of fungal reduction in food due to the presence of lactic acid and PLA.

3.5. Study of the antifungal activity from the fermented BB ingredient in contaminated bread

The data obtained from the study of the shelf life of the bread thought the 7-day incubation can be observed in **Table 6**. In trials conducted to assess resistance against *A. flavus* contamination, fungal growth was observed in the control, control BB medium 5%, control BB medium 10%, and bread 5% BB after 4 days of incubation. After 5 days the growth of the fungi was appreciated in the bread 10% and in the propionate control after 6 days. However, on day 4, the presence of fungal spores was 100% in the control bread, while in the Control BB medium 10% and BB medium 5%, the percentage was significantly lower, at 75% and 60%, respectively, indicating a small inhibition of fungal germination in these samples. In samples contaminated with *P. commune*, fungal growth was observed in the control, control BB medium 5%, control BB medium 10%, bread 5% BB, and bread 10% BB after 3 days of incubation. The fungal growth in the propionate control was not observed until the day 5. Even when the fungal growth was visible after the day 3 the percentage of grown spots was significantly lower in the bread 10% BB in comparation to other samples in which fungal presence was observed, only 25% of the sports grew, while in the control this number was 75%. In **Fig. 1** it can be observed how the determination of the fungal growth was detected.

Table 6. Study of the shelf life of the bread slices contaminated with *A. flavus* (a) and *P. commune* (b) monitored in days.

a) *A. flavus*					
Sample	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**
Control	0 ± 0^{a}	100 ± 0^{c}	100 ± 0^{b}	100 ± 0^{b}	100 ± 0^{a}
Propionate control	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	60 ± 21^{a}	100 ± 0^{a}
Control BB medium 5%	0 ± 0^{a}	100 ± 0^{c}	100 ± 0^{b}	100 ± 0^{b}	100 ± 0^{a}
Control BB medium 10%	0 ± 0^{a}	74 ± 11^{b}	100 ± 0^{b}	100 ± 0^{b}	100 ± 0^{a}
BB medium 5%	0 ± 0^{a}	60 ± 14^{b}	100 ± 0^{b}	100 ± 0^{b}	100 ± 0^{a}
BB medium 10%	0 ± 0^{a}	0 ± 0^{a}	100 ± 0^{b}	100 ± 0^{b}	100 ± 0^{a}

b) *P. commune*

Sample	Day 3	Day 4	Day 5	Day 6	Day 7
Control	75 ± 0^{d}	100 ± 0^{b}	100 ± 0^{b}	100 ± 0^{b}	100 ± 0^{a}
Propionate control	0 ± 0^{a}	0 ± 0^{a}	50 ± 0^{a}	75 ± 0^{a}	100 ± 0^{a}
Control BB medium 5%	75 ± 0^{d}	100 ± 0^{b}	100 ± 0^{b}	100 ± 0^{b}	100 ± 0^{a}
Control BB medium 10%	68 ± 21^{cd}	100 ± 0^{b}	100 ± 0^{b}	100 ± 0^{b}	100 ± 0^{a}
BB medium 5%	45 ± 11^{c}	100 ± 0^{b}	100 ± 0^{b}	100 ± 0^{b}	100 ± 0^{a}
BB medium 10%	25 ± 0^{b}	100 ± 0^{b}	100 ± 0^{a}	100 ± 0^{b}	100 ± 0^{a}

Results were expressed as the mean percentage ± standard deviation of the spots in which fungal growth was appreciated each day on the contaminated bread loaves. Different letters were used to signal significative differences among the samples each day (row). ($p < 0.05$). When no fungal growth was detected, samples were marked as nd.

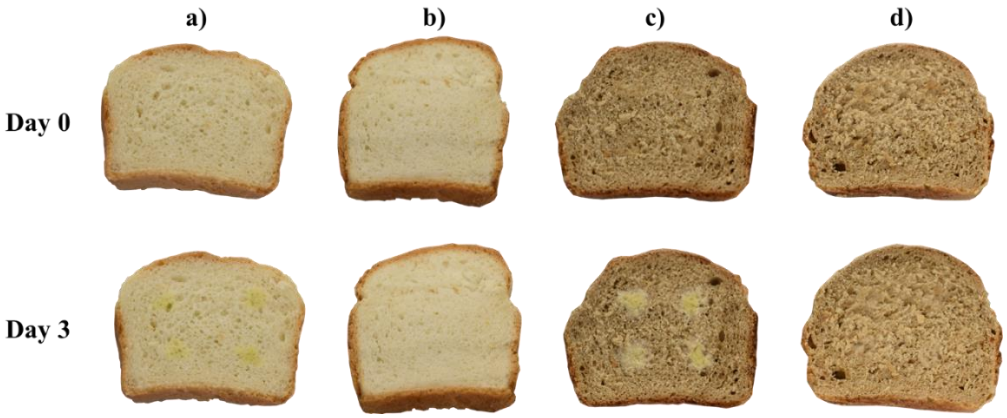

Fig. 1. Slice of bread inoculated with *A. flavus* after 0 and 3 day incubation. It can be observed the control bread (a), propionate control (b), 10% BB control (c) and 10% BB (d).

The use of chemical preservatives has been liked to some unclear biological risks; therefore, food authorities are encouraging the research of new methods for food preservation (Mohammadzadeh-Aghdash et al., 2019). Use of LAB as a bio-preservation agent have been presented as a good alternative to these chemical compounds (Barrios-Roblero et al., 2019; Chen et al., 2021). In addition, these species of bacteria have been considered "Generally Regarded As Safe" (GRAS) by the Food and Drug Administration (FDA) and the "Qualified Presumption of Safety" (QPS), the list for authorized use in the food and feed in the European Union (Saladino et al., 2016). While some articles report the use of fermented waste from other microorganisms for the biopreservation of bread, such as *Rhizopus oryzae* (not considered as in the GRAS and QPS list), this study demonstrates the effectiveness of LAB fermented BB against fungal species in food (Denardi-Souza et al., 2018). Also, BB has also been used as a matrix for solid fermentation by Metarhizium anisopliae for the production of cell wall-degrading enzymes for antimicrobial purposes (Aita et al., 2019).

3.6. Identification and quantification from the metabolite profiling of fungal growth on bread with fermented BB as an ingredient

The fungal metabolite profile of bread contaminated with fungi was examined and the results are presented in **Fig. 2.** No mycotoxigenic production was observed in the bread contaminated by *P. commune*. However, bread contaminated by *A. flavus* showed the presence of aflatoxins B1, B2, G1, and G2, which are frequently monitored mycotoxins in cereals according to European regulations (Romero-Sánchez et al., 2022). Aflatoxin B1 and aflatoxin G2 were detected in all bread samples. The control bread was the only sample in which all four aflatoxins quantified were in detectable concentrations. The bread with a 10% BB sowed an absence of aflatoxin B2 and G1 and a decrease in the concentration of aflatoxin B1 to a significantly similar concentration detected in the propionate control. The propionate bread control, control BB medium 5%, control BB medium 10%, BB medium 5%, and BB medium 10% reduced the total aflatoxin occurrence by over 64% compared to the control. The control propionate bread was the most effective treatment, reducing the concentration of the toxins by 98%, followed by the bread with 10% BB, which reduced the concentration of toxins by 86%. The reduction total aflatoxin presence evidenced by the 10% BB bread was 15% greater than in the 10% BB bread control.

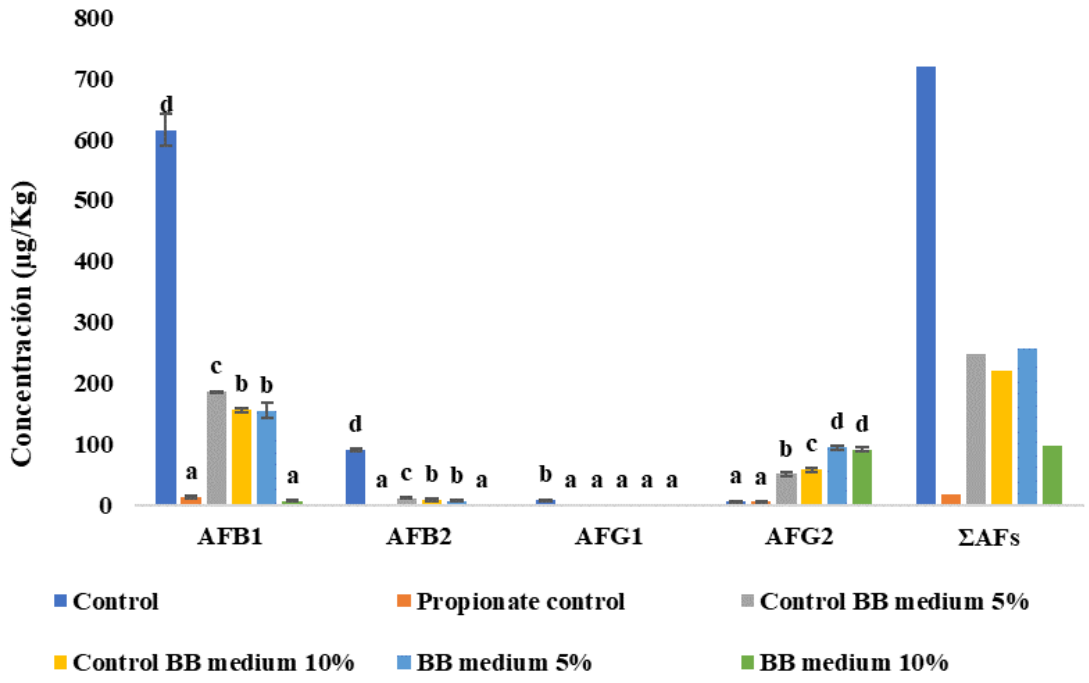

Fig. 2. Results of the fungal metabolites produced by *Aspergillus flavus* in the bread inoculated with the fungi after a 7-day incubation. Metabolites detected were aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1) and aflatoxin G2 (AFG2).

Results were expressed as mg/kg of bread (AFB1, AFB2, AFG1 and AFG2) and the total summation of all mycotoxins in each sample (ΣAF). Metabolites below the level of detection were marked as nd. Different letters were used to signal significative differences of a single mycotoxin concentration among the samples ($p < 0.05$).

These results suggest that fermentation of the BB is not the only aspect that affected mycotoxin occurrence in the samples. All treatments with the (fermented and non-fermented) BB as ingredient reduced the concentration of aflatoxin B1 compared to the control bread. Presumably some of the compounds from the BB may be affecting the aflatoxin production by the fungi. Plants and plant-based product produce some antifungal compounds by their own (Shi et al., 2019). This plant metabolites are usually peptides which affect the fungi in all stages of the contamination, from the germination to the metabolization of compounds, such as mycotoxins (Wang et al., 2009). Furthermore, there was a significative increase of the mycotoxin reduction when comparing the results evidenced in by the fermented to the non-fermented ingredients, suggesting that fermentation is a key factor in aflatoxin reduction. It is also noteworthy that propionate did not reduce the aflatoxin B1 production compared to the 10% BB bread, as both had a similar anti-mycotoxigenic effect.

The literature shows other conservation alternatives without the use of chemical preservatives for the reduction of aflatoxin occurrence in bread. In Pashaei and Hagh Nazari (2019) the combination of a Saccharomyces cerevisiae strain with and infrared treatment reduced the aflatoxin presence in bread prepared with contaminated flour. Other assays like the ones performed in Krusong et al. (2019) evidenced that the use of a vapor-phase ethanol in the bread can inhibit the production of aflatoxin in contaminated bread. Use of methods which use LAB for bioconservation has been also reported (Badji et al., 2023). Ebrahimi et al. (2020) correlates the production of a specific cyclic dipeptide to the reduction of aflatoxin management to reduce the aflatoxin content in an 85% when incorporating Pediococcus pentosaceus strain to a sourdough. After research in the literature this is the first article reporting the use of BB, a waste of the industry for the bioconservation of by LAB against fungal proliferation in bread and toxigenic occurrence.

4. CONCLUSION

The characterization of the BB culture mediums fermented by the LAB evidenced that the culture medium with BB at 20% fermented by the strain *L. plantarum* L1 had the highest antifungal potential and produced the greatest quantity of antifungal compounds from the studied samples. Therefore, this product was used as ingredient to increase the shelf life in the production of bread. The presence of the ingredient raised the concentration of antifungal compounds such as lactic acid and PLA, which led to an increase in the bread shelf life and a reduction in the occurrence of aflatoxin B1 compared to a control bread. Furthermore, when comparing bio-preservation accomplished by the fermented BB ingredient to a propionate control in bread, similar results were obtained. This work achieved the revalorization of a food industry residue, beer bagasse, through LAB fermentation, to produce a bio-preservation agent for bakery products against fungal contamination. Moreover, further investigations can be performed for the application of this agent in new foods.

Food Bioscience, 58, 103703 (2024)

3.5. Revalorization of Rice Bran as a Potential Ingredient for Reducing Fungal Contamination in Bread by Lactic Acid Bacterial Fermentation

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1. INTRODUCTION

Society's challenge is to sustainably feed over seven billion people on a resource-limited planet. Revaluing food industry by-products offers a solution to reduce hunger and optimize resource utilization (United Nations, 2015). Rice, a vital global food staple (Zhuang et al., 2019), generates a by-product known as rice bran (RB) during milling, producing over 63 million tons annually. Typically, RB is used for fuel or livestock feed (Webber et al., 2014). RB is nutrient-rich, containing unsaturated lipids, polysaccharides, and proteins, as well as bioactive compounds like flavonoids, tocopherols, and phenolic acids, which can be harnessed through fermentation (Goufo & Trindade, 2014). Therefore, the potential for RB revalorization holds promise for addressing food security and resource sustainability challenges.

RB is a nutrient and bioactive-rich by-product that presents an ideal substrate for lactic acid bacteria (LAB) fermentation (Alauddin et al., 2019). LAB-mediated biopreservation has gained significant traction as a food preservation technique in recent years (Li et al., 2020; Mcnair et al., 2018). Increasing consumer awareness of food ingredients has driven demand for reduced chemical preservatives (Cosentino et al., 2018).

Lactic acid fermentation has proven effective in extending the shelf life of foods, offering the potential to significantly reduce or eliminate the use of chemical preservatives (Punia Bangar et al., 2022). This antifungal activity arises from a diverse spectrum of antifungal compounds produced during fermentation. Organic acids, bioactive peptides, and phenolic acids represent some of the bioactive compounds identified as effective against contaminating microorganisms during LAB fermentation (Peyer et al., 2016).

The bread serves as a versatile food matrix where numerous ingredients can be incorporated to modify rheology, extend shelf life, and enhance flavour. The literature showcases the utilization of various ingredients, such as whey, egg yolk, and red beetroot powder, to achieve these objectives (Cui et al., 2022). Bread is a common target for fungal contamination, leading to substantial economic losses in the food industry, particularly in regions like Europe, where annual losses are estimated to exceed 200 million euros (Garcia et al., 2019). The primary culprits behind bread contamination are fungal species belonging to the *Aspergillus* and *Penicillium* genera (Garcia et al., 2019). Some strains within these genera can produce mycotoxins, notably aflatoxin B1, linked to carcinogenic effects (Marin et al., 2013). Consequently, preventing fungal and toxigenic contamination in food ranks among the foremost concerns of the food industry.

Currently, calcium propionate stands as the most widely used additive for fungal preservation in bakery products, with a maximum allowable level of 0.3 % in Europe (Gagiu et al., 2013). However, efforts to reduce reliance on this chemical preservative have spurred the exploration of alternative approaches (Rizzello et al., 2011). Following this trend, the aims of this

study were a) to optimize and characterize the antifungal properties of a LAB fermented RB medium to use it as an antifungal bread ingredient, b) study the modification of the technological properties of the dough and bread when the ingredient was added, c) investigate the antifungal compounds present in the dough and bread with the ingredient, and d) determine if the addition of this ingredient reduces fungal contamination and mycotoxin occurrence in the bread.

2. MATERIAL AND METHODS

2.1. Chemicals and microorganisms

Milli-Q water (resistivity <18 MΩ cm) was sourced via a Milli-Q purification system (Millipore, Billerica, MA, USA). Chemicals including acetonitrile (ACN), methanol (MeOH), ethyl acetate (EA), glucose, yeast extract, tryptone, ammonium sulfate, sodium chloride, magnesium sulfate, dipotassium phosphate, and manganese sulfate were procured from Sigma-Aldrich (St. Louis, MO, USA). Lactic acid, phenyllactic acid (PLA), and gallic acid standards were obtained from BaChem (Weil am Rhein, Germany). Buffered peptone water, Potato Dextrose Agar (PDA), Man Rogosa Sharpe Agar (MRS Agar), and Man Rogosa Sharpe Broth (MRS Broth) were acquired from Liofilchem (Teramo, Italy). RB was supplied by DACSA GROUP (Maicerías Españolas S. A., Valencia, Spain). Aflatoxin B1, B2, G1, G2, and ochratoxin were purchased from Sigma–Aldrich with a purity of ≥97 %. Basic ingredients including wheat flour, sugar, salt, yeast, and mineral water were obtained from Mercadona (Valencia, Spain).

The LAB strains utilized in this investigation were isolated from breast milk samples. Both isolates were identified as members of the *L. plantarum* species using the 16S rRNA gene sequencing method outlined by Zeigler (2005). This identification was conducted through the Spanish Type Culture Collection (CECT) housed at the University of Valencia (Valencia, Spain). The isolates were further classified into two distinct strains: *L. plantarum* H1 (H1) and *L. plantarum L1* (L1). The fungal species employed in this study comprised *Aspergillus flavus* ITEM 8111 (A. flavus) and *Alternaria alternata* ITEM 8123 (*A. alternata*), both obtained from the ITEM Collection at the *Istituto di Scienze delle Produzioni Alimentari* (ISPA) in Bari, Italy. Additionally, *Penicillium commune* CECT 20767 (*P. commune*) was sourced from the CECT at the University of Valencia (Valencia, Spain).

2.2. Preparation and fermentation of the RB mediums

The rice bran-based medium was prepared at various concentrations: 5 % (RB5), 10 % (RB10), and 20 % (RB20). The RB was supplied by DACSA GROUP. These media were supplemented with 10.0 g/L of glucose (Sigma-Aldrich), 10.0 g/L of yeast extract (Sigma-Aldrich), 10.0 g/L of tryptone (Sigma-Aldrich), 6.5 g/L of sodium chloride (Sigma-Aldrich), 2.5 g/L of ammonium sulfate (Sigma-Aldrich), 2.5 g/L of dipotassium phosphate (Sigma-Aldrich), 0.25 g/L of magnesium sulfate (Sigma-Aldrich), and 62.5 mg/L of manganese sulfate (Sigma-Aldrich). All components were dissolved in distilled water. These ingredients had previously undergone assessment for their suitability in promoting LAB growth in unpublished studies and are deemed safe for human consumption. After achieving thorough homogenization through magnetic stirring, the media were autoclaved at 120 °C for 15 min and then allowed to cool to 25 °C. Subsequently, the media were inoculated with 5 % (v/v) of a 108 CFU/mL MRS solution (Liofilchem) containing LAB that had been pre-cultured for 24 h at 37 °C. Microbial count of the inoculum was carried out by performing 1/10 serial dilutions in peptone water (Liofilchem) and inoculated on MRS Agar (Liofilchem) in order to check that all inoculums were at the desired concentration. The inoculated media underwent fermentation for 72 h at 37 °C before being frozen for subsequent analysis.

2.3. Determination of the antifungal activity of the fermented RB mediums

The assessment of antifungal activity in the mediums followed the procedure outlined by Cortés-Zavaleta et al. (2014) with minor modifications. Initially, the fermented mediums underwent centrifugation, which resulted in the separation of the cell-free supernatant (CFS) from the pellet. Subsequently, the pellet was filtered through a 0.22 nm sterile filter. The CFS was subsequently frozen at −80 °C and lyophilized using freeze-drying equipment, specifically the FreeZone 2.5 L by Labconco (Kansas, MO, USA). A suspension was prepared using 500 g/L of the dry CFS, mixed with distilled water in a 1/10 (v/v) ratio of PDA pre-templated to 45 °C. The resulting mixture was then plated onto a PDA. Subsequently, the plates were inoculated with 10 µL of a fungal spore suspension and incubated at 25 °C for 96 h. Control plates were prepared using PDA alone.

The mycelial growth area was determined by measuring the growth diameter of the samples (S) and the control (C) after 48 and 96 h. The results were expressed as the mean ± standard deviation of the measured diameters and the percentage of inhibition (I %) from the samples in comparison to the control, calculated as follows:

$$I\,\% = \frac{(C-S)}{C}\ x\ 100$$

2.4. Characterization of Antifungal Compounds in Fermented RB media

To assess the pH of the samples, a pH-meter (Sension + tm + ph3, Hach Company, Loveland, CO, USA) equipped with a Sension + CAT 5010T electrode (Hach Company) was employed for sample measurement. The characterization and quantification of lactic acid and PLA within the CFS were conducted following the procedures outlined in Dopazo et al. (2021). All analyses were performed in triplicate for accuracy and consistency.

2.5. Breadmaking procedure with fermented RB medium as an ingredient

The ingredient studied for the addition to the bread-making procedure was the medium with a 20 % RB fermented by the strain *L. plantarum* H1. The fermented medium was frozen to – 80 °C, lyophilized with freeze-drying equipment, and ground to obtain a powder to use as a bread ingredient.

To prepare the bread, 165 g of mineral water, 20 g of yeast, and 10 g of white sugar were added to 300 g of wheat flour, along with 6.5 g of common fine salt, resulting in a dough of 501.5 g. The mixture was kneaded for 10 min in a SilverCrest SBB 850 A1 bread-making machine (SilverCrest, Bochum, Germany). The resulting dough was divided into three doughs of 167.16 g. The doughs were fermented for 1 h at 30 °C, covered with foil, and baked at 200 °C for 25 min. Finally, the bread loaves were tempered at 25 °C for further analysis. Six samples were prepared, including a control sample (Control bread) using the specified ingredients. Additionally, a control propionate sample (Propionate control bread) was created by incorporating 1.5 g of calcium propionate, adhering to the maximum permissible amount according to European legislation (EC) No. 2016/683 of 2 May 2016 (European Commission, 2016, p. 28). Two treatments involved the use of fermented RB (rice bran) medium powder at concentrations of 10 % (Fermented RB ingredient 10 % bread) and 20 % (Fermented RB ingredient 20 % bread). Lastly, two bread samples were produced by incorporating non-fermented rice bran at concentrations of 10 % (Control RB ingredient 10 % bread) and 20 % (Control RB ingredient 20 % bread) by weight. In this instance, the powder was derived by freeze-drying the rice bran culture medium without bacterial inoculation.

2.6. Effect of fermented RB medium as an ingredient on dough and bread quality

To assess the impact of the RB ingredient on the dough, the following tests were conducted. Initially, volume expansion was measured by placing 5 g of dough in the bottom of a 50 mL sterilized graduated tube. After 1 h of fermentation at 30 °C, the increase in volume expansion (measured in cm grown inside the tube) was calculated as the dough expansion (Sang et al., 2020).

The pH of the samples before and after fermentation was determined using a pH-meter (XS pH 7 Vio, XS Instruments, Carpi, Italy) equipped with an electrochemical sensor (2 Pore Steel T, XS Instruments). Titratable acidity of the dough, both before and after fermentation, was determined as the volume of 0.1 M NaOH needed to raise the pH of 10 g of bread homogenized with 90 mL of distilled water using an ultraturrax Ultra Ika T18 basic (IKA®-Werke GmbH & Co., Staufen, Germany) to a pH of 8.5 (Hugo et al., 2003). The results were expressed as equivalent grams of lactic acid per kg of the sample.

To analyze the effect of the fermented RB ingredient in the bread, the water activity (Aw) of the samples was measured from bread loaves using a Humimeter RH2 water activity meter (Max-Schaller-Straße, Styria, Austria). The specific volume was determined using the millet seed displacement method (Mannuramath et al., 2015). The breads were placed in a beaker filled with chia seeds, and the difference in volume of the same amount of chia seeds in the beaker with and without the bread was used for volume calculation. The breads were then weighed, and the specific volume was calculated as the ratio between volume and weight of the samples (cm3/g). Moisture content in the breads was determined by heating 10 g of sample in an oven at 105 °C for 24 h. Before and after this treatment, samples were weighed to calculate the water concentration (Guimarães et al., 2020).

Finally, pH and titratable acidity measurements were conducted as previously described with the dough. All assays were performed in triplicate, and the results were expressed as the mean ± standard deviation.

2.7. Detection of antifungal metabolites

To quantify lactic acid and PLA, 10 g of each sample was homogenized with water and centrifuged. The resulting supernatant was filtered through a 0.22 μm pore filter and analyzed using Agilent 1100 series HPLC (Agilent, Santa Clara, CA, USA) with a DAD detector (Agilent 1100 Series G1315B) for lactic acid. PLA content was determined by purifying the supernatant using a QuEChERS method and analyzing it with an Agilent 1290 Infinity II LC UHPLC System coupled with Q-TOF-MS detector (Agilent 6546 Q-TOF; Agilent Technologies, Waldbronn, Germany), following the same procedures as described in “Section 2.4 Characterization of

Antifungal Compounds in Fermented RB Media" This enabled the assessment of antifungal metabolites in both the fermented RB powder and bread with the fermented RB ingredient.

2.8. Effect of fermented RB on bread shelf life

To assess the influence of the fermented RB ingredient on bread shelf life, we adapted the method from Luz et al. (2019). Bread loaves were aseptically inoculated with 10 µL of a fungal suspension (106 spores/mL) at four spots, resulting in a concentration of 103 spores/g of bread. After drying, loaves were placed in sterile bags for daily observation of fungal growth in the contaminated areas.

Fungal growth results were expressed as the percentage of growth spots per day and presented as the mean ± standard deviation. Each trial included 5 replicates.

2.9. Assessment of fermented RB as an Ingredient's impact on fungal growth on bread

To quantify fungal growth following a 7-day incubation, contaminated bread samples were homogenized within sterile bags containing 0.1 % (w/v) peptone water using a stomacher masticator (IUL instruments, Barcelona, Spain) for 30 s. Subsequently, serial dilutions were prepared from the samples and plated on PDA plates, which were then incubated at 25 °C for 48 h. After incubation, the colony-forming units (CFU) of the fungi were quantified, and the results were expressed as Log_{10} UFC/g of bread.

2.10. Mycotoxin profiling of fungal growth on RB-treated bread

To investigate the impact of the fermented RB ingredient on the metabolite profiling of *A. flavus* ITEM 8111 and *P. commune* CECT 20767 after a 7-day incubation, adapting the procedures outlined in Dopazo et al. (2021). Samples were processed by crushing them with an electric coffee grinder (Cecotec, Valencia, Spain). Subsequently, 5 g of the samples were extracted by adding 20 mL of methanol and then homogenized using an ultraturrax. After centrifugation at 3000×g for 15 min, the supernatant was dried using a Buchi R-215 Rotavapor system (BUCHI Labortechnik GmbH, Essen, Germany). The resulting samples were suspended in 2 mL of methanol, filtered through a 0.22 µm pore filter, and analyzed using an Agilent 1290 Infinity II LC UHPLC System equipped with a quadrupole time-of-flight mass spectrometer (Agilent 6546 Q-TOF) set in positive ionization mode.

For the chromatographic analysis, an Agilent Zorbax RRHD SB-C18, 2.1 mm × 50 mm, 1.8 μm column was used, with Milli-Q water containing 0.1 % formic acid (v/v) as mobile phase A and acetonitrile with 0.1 % formic acid (v/v) as mobile phase B in a gradient elution program: 0 min, 2 % B; 22 min, 95 % B; 25 min, 5 % B, at a flow rate of 0.4 mL/min. A total of 5 μL of each sample were analyzed in triplicate. The parameters set for the Dual AJS ESI were as follows: gas temperature: 325 °C; nebulizer pressure: 40 psig; gas flow: 10 L/min; sheath gas flow: 12 L/min; sheath gas temperature: 295 °C; nozzle voltage: 500 V; capillary voltage: 4000 V; skimmer: 70 V; Fragmentor: 120 V; product ion scan range: 100–1500 Da; MS/MS scan rate: 3 spectra/s; maximum precursors per cycle: 2; MS scan rate: 5 spectra/s; and collision energy: 10, 20, 40 eV. Calibration curves for mycotoxins were prepared at concentrations ranging from 0.01 to 5 mg/L. Integration, data processing, and metabolite identification were performed using MassHunter Qualitative Analysis Software Version B.08.00 and library PCDL Manager Version B.08.00 for Windows 7 (Agilent Technologies).

2.11. Statistical analysis

To evaluate significant differences among each sample a multiple comparison analysis was performed with the software InfoStat 2019 (Universidad Nacional de Córdoba, Córdoba, Argentina). The differences between the samples were analyzed by one-way ANOVA, followed by the Tukey HSD post hoc test for multiple comparisons ($p \leq 0.01$).

3. RESULTS AND DISCUSSION

3.1. Effect of antifungal activity of fermented RB media

The results of antifungal activity from different LAB-fermented RB media were summarized in **Table S1**. All fermented media significantly reduced the diameter of *A. flavus* ITEM 8111 growth compared to control samples at 48 h ($p \leq 0.01$). The medium fermented with *L. plantarum* H1 achieved the highest inhibition, reaching 32 %. However, after 96 h of incubation, no inhibition was observed compared to the control.

Table S1. Antifungal activity of rice bran mediums fermented by *L. plantarum* H1 *and L. plantarum* L1 against *Aspergillus flavus* (a), *Penicillium commune* (b), and *Alternaria alternata* (c).

a) *A. flavus*

Medium	*L. plantarum* H1				*L. plantarum* L1			
	48 h		96 h		48 h		96 h	
	Diameter	% Reduction	Diameter	% Reduction	Diameter	% Reduction	Diameter	% Reduction
Control	2.5 ± 0.1^{d}	-	4.5 ± 0.0^{a}	nd	2.5 ± 0.1^{d}	-	4.5 ± 0.0^{a}	nd
RB 5%	2.1 ± 0.1^{bc}	16.0	4.5 ± 0.0^{a}	nd	2.2 ± 0.1^{c}	12.0	4.5 ± 0.0^{a}	nd
RB 10%	1.9 ± 0.0^{ab}	24.0	4.5 ± 0.0^{a}	nd	1.8 ± 0.2^{a}	28.0	4.5 ± 0.0^{a}	nd
RB 20%	1.7 ± 0.0^{a}	32.0	4.5 ± 0.0^{a}	nd	1.8 ± 0.1^{a}	28.0	4.5 ± 0.0^{a}	nd

b) *P. commune*

Medium	*L. plantarum* H1				*L. plantarum* L1			
	48 h		96 h		48 h		96 h	
	Diameter	% Reduction	Diameter	% Reduction	Diameter	% Reduction	Diameter	% Reduction
Control	1.7 ± 0.2^{b}	_	2.7 ± 0.1^{c}	-	1.7 ± 0.2^{b}	-	2.7 ± 0.1^{c}	-
RB 5%	1.4 ± 0.1^{ab}	17.6	2.3 ± 0.3^{bc}	14.8	1.4 ± 0.2^{ab}	17.6	2.3 ± 0.3^{bc}	14.8
RB 10%	1.3 ± 0.2^{ab}	23.5	2.2 ± 0.2^{abc}	18.5	1.3 ± 0.2^{ab}	23.5	2.1 ± 0.1^{ab}	22.2
RB 20%	1.0 ± 0.1^{a}	41.2	1.7 ± 0.1^{a}	37	1.1 ± 0.1^{a}	35.3	1.9 ± 0.1^{ab}	29.6

c) *A. alternata*

Medium	*L. plantarum* H1				*L. plantarum* L1			
	48 h		96 h		48 h		96 h	
	Diameter	% Reduction	Diameter	% Reduction	Diameter	% Reduction	Diameter	% Reduction
Control	1.7 ± 0.1^{e}	_	3.7 ± 0.0^{d}	-	1.7 ± 0.1^{e}	-	3.7 ± 0.2^{d}	-
RB 5%	1.0 ± 0.1^{bc}	41.2	3.3 ± 0.1^{bc}	10.8	1.4 ± 0.1^{bc}	17.6	3.4 ± 0.1^{cd}	8.1
RB 10%	0.9 ± 0.1^{bc}	47.1	3.0 ± 0.2^{ab}	18.9	1.1 ± 0.1^{c}	35.3	3.0 ± 0.1^{ab}	18.9
RB 20%	0.5 ± 0.1^{a}	70.6	2.9 ± 0.1^{a}	21.6	0.8 ± 0.1^{b}	52.9	2.9 ± 0.2^{a}	21.6

Control was PDA; RB 5% (PDA enriched with a 5% concentration of fermented rice bran medium), RB 10% (PDA enriched with a 10% concentration of fermented rice bran medium), and RB 20% (PDA enriched with a 20% concentration of fermented rice bran medium). Data was displayed as mean ± SD or % of reduction. % reduction was measured as the average percentage of reduction from the diameter of the samples in comparison to the control. Samples were marked as nd when no reduction was detected and "–" for the control reduction. Different letters were used to signal significative differences among the different fermented mediums at a specific time (columns) ($p \leq 0.01$).

In assays against *P. commune* CECT 20767, only the medium with 20 % RB fermented by LAB significantly reduced mold growth compared to the control at 48 h ($p \leq 0.01$). The reduction was 5.9 % greater in the medium with 20 % RB fermented by *L. plantarum* H1 compared *to L. plantarum* L1. After 96 h, the results for *P. commune* CECT 20767 followed a similar pattern as at 48 h. Only the medium with 20 % RB fermented was able to reduce fungal growth ($p \leq 0.01$), with a 7.4 % greater inhibition in the *L. plantarum* H1 fermented medium compared to the other LAB strain.

In trials against *A. alternata* ITEM 8123, the highest inhibition of mold growth was achieved by the 20 % RB fermented medium by *L. plantarum* H1 ($p \leq 0.01$), reaching a 70 % reduction. After 96 h, both bacteria-fermented media achieved a 21.6 % reduction.

It can be concluded that fermentation by *L. plantarum* H1 produced CFS with higher antifungal activity, and the medium with 20 % RB provided a richer matrix for antifungal metabolite production during LAB fermentation.

The antifungal properties of LAB-fermented CFS have been well-documented in the literature. Once the right LAB strain is identified for fermentation of a selected matrix, the resulting CFS may have the potential as an antifungal agent. In Dopazo et al. (2021), a CFS fermented by LAB demonstrated antifungal activity against various *Aspergillus* and *Alternaria* species. Omedi et al. (2021) used pitaya fruits as a matrix for CFS production, inhibiting the growth of *Penicillium* spp. and *Aspergillus* spp. This study aimed to replicate real application conditions, using direct incorporation of CFS into agar instead of agar diffusion tests. Ogunremi et al. (2022) followed a similar methodology, with consistent results when applied to bakery products.

While there were few articles discussing the use of rice bran for antifungal purposes, Christ-Ribeiro et al. (2019) reported the use of a phenolic extract from RB and spirulina fermented by *Rhizopus oryzae*. The results showed antifungal activity against *Penicillium* verrucosum. De Souza et al. (2010) employed a similar approach, using a phenolic extract of RB fermented by *Chlorella phyrenoidosa* to combat fungal contaminants. The advantage of the antifungal treatment studied in this work, compared to those in the literature, was its suitability for use in food, as it eliminated the need for solvents used in phenolic extraction.

3.2. Characterization of the antifungal compounds present in the fermented RB mediums

Table S2 showed the results of pH evolution and the quantification of lactic acid and PLA in the fermented RB mediums. Initially, the mediums had a pH range of 6.04–6.22, with increasing RB concentration slightly elevating the pH, particularly between 5 % and 20 % RB. After 72 h of fermentation, the control samples maintained a constant pH, while the fermented samples exhibited a pH decrease to an average of 3.54–3.68 ($p \leq 0.01$). These results suggested that RB concentration did not significantly influence pH reduction during fermentation. Lower pH values were often associated with increased production of antifungal metabolites such as lactic acid or acetic acid, known to inhibit fungal growth (Schnürer & Magnusson, 2005).

Table S2. Quantification of antifungal metabolites in rice bran mediums fermented by *L. plantarum* H1 and *L. plantarum* L1.

Lactic acid bacteria	Medium	pH 0h	pH 72h	Lactic acid	Phenyllactic acid
Control	RB 5%	6.04 ± 0.01^{a}	6.02 ± 0.02^{c}	nd	nd
	RB 10%	6.10 ± 0.01^{bc}	6.05 ± 0.02^{cd}	nd	nd
	RB 20%	6.19 ± 0.01^{d}	6.16 ± 0.01^{d}	nd	nd
L. plantarum H1	RB 5%	6.08 ± 0.02^{b}	3.55 ± 0.03^{a}	18.10 ± 0.09^{b}	29.80 ± 0.85^{ab}
	RB 10%	6.11 ± 0.01^{c}	3.60 ± 0.02^{ab}	22.50 ± 0.01^{d}	31.08 ± 0.38^{ab}
	RB 20%	6.22 ± 0.01^{e}	3.59 ± 0.01^{ab}	32.60 ± 0.01^{f}	32.36 ± 0.97^{b}
L. plantarum L1	RB 5%	6.09 ± 0.02^{bc}	3.54 ± 0.03^{a}	17.60 ± 0.08^{a}	30.51 ± 1.2^{ab}
	RB 10%	6.11 ± 0.01^{c}	3.68 ± 0.02^{b}	20.40 ± 0.01^{c}	29.00 ± 1.66^{a}
	RB 20%	6.18 ± 0.00^{d}	3.57 ± 0.03^{a}	22.80 ± 0.10^{e}	30.37 ± 1.23^{ab}

RB 5% (Medium enriched with a 5% concentration of rice bran), RB 10% (Medium enriched with a 10% concentration of rice bran), and RB 20% (Medium enriched with a 20% concentration of rice bran). Data was displayed as mean ± SD. Different letters were used to signal significative differences in the studied parameters among the fermented mediums (columns) ($p \leq 0.01$). Results below the limit of detection were marked as nd.

Table S2 also showed the quantification of lactic acid and PLA in the mediums. Lactic acid concentrations ranged from 18.10 to 32.60 g/L, and the addition of increasing amounts of RB significantly increased lactic acid production ($p \leq 0.01$). The average lactic acid concentrations in the 5 %, 10 %, and 20 % RB media were 17.85, 21.45, and 27.70 g/L, respectively. The strain *L. plantarum* H1 consistently produced significantly higher lactic acid concentrations compared to the other strain ($p \leq 0.01$). In contrast, RB concentrations did not significantly affect PLA production, with most samples having an average concentration of 30.52 mg/L. Notably, lactic acid and phenyllactic acid were not detected in the control samples.

The production of lactic acid was linked to sugar metabolization by LAB, and the increase in RB concentration expectedly led to higher lactic acid production due to the complex matrix of RB, including sugars (Alauddin et al., 2019). Higher lactic acid production was often associated with increased antifungal activity of the bacterial strain. PLA presence was a strong indicator of effective antifungal potential in fermented mediums. Studies have associated PLA with antifungal activities against fungal species like *Aspergillus*, *Penicillium*, and *Fusarium*. Overall, the fermented medium with 20 % RB fermented by *L. plantarum* H1 exhibited superior antifungal results and higher concentrations of antifungal metabolites, thus it was selected as a bread ingredient. Several studies have highlighted the relationship between lactic acid production and the biopreservation properties of LAB against fungal contaminants. El Oirdi et al. (2021) found that higher lactic acid production in LAB-fermented mediums correlated with increased biopreservation properties, particularly in fruits contaminated by fungi.

Research by Peyer et al. (2016) have shown that PLA is associated with antifungal activities against fungal species belonging to genera such as *Aspergillus*, *Penicillium*, and *Fusarium*. In our study, the fermented medium containing 20 % RB, fermented by the strain *L. plantarum* H1, demonstrated superior antifungal results and higher concentrations of these antifungal metabolites. These results rendered it a suitable option as an ingredient for bread production, indicating its potential to extend the shelf life of bread and inhibit fungal contamination.

3.3. Influence of the fermented RB as an ingredient in the dough and bread quality evaluation

The analysis of the dough and bread characteristics revealed notable differences between the control bread and those with a 20 % fermented RB ingredient (**Table S3**).

Table S3. Effects of *L. plantarum* H1-fermented rice bran medium as an ingredient on pH, titratable acidity, volume expansion, water activity, moisture content, and specific volume of the dough and bread.

a)

Sample	pH			Titratable acidity		
	Dough	Fermented dough	Bread	Dough	Fermented dough	Bread
Control	5.00 ± 0.20^{b}	5.23± 0.01^{bcd}	5.60 ± 0.01^{def}	8.7 ± 0.6^{a}	6.1 ± 0.7^{a}	3.2 ± 0.1^{a}
Propionate control	5.20 ± 0.04^{bcd}	5.20 ± 0.10^{bc}	5.30 ± 0.02^{bc}	13.0 ± 1.9^{ab}	9.9 ± 2.0^{ab}	3.9 ± 0.2^{b}
Control RB medium 10%	5.40 ± 0.30^{bcde}	5.60 ± 0.06^{cdef}	5.70 ± 0.01^{ef}	13.4 ± 1.9^{ab}	12.0 ± 1.2^{bc}	6.5 ± 0.3^{c}
Control RB medium 20%	5.90 ± 0.10^{f}	5.60 ± 0.30^{cdef}	5.90 ± 0.04^{f}	14.4 ± 1.9^{b}	14.5 ± 2.7^{bc}	9.5 ± 0.0^{d}
RB medium 10%	4.40 ± 0.01^{a}	4.30 ± 0.20^{a}	4.50 ± 0.10^{a}	14.4 ± 0.5^{b}	17.0 ± 1.1^{c}	11.7 ± 0.2^{e}
RB medium 20%	4.10 ± 0.02^{a}	4.10 ± 0.02^{a}	4.20 ± 0.01^{a}	27.5 ± 1.4^{c}	26.5 ± 1.0^{d}	20.3 ± 0.1^{f}

b)

Sample	Volume expansion	Aw	Moisture	Specific volume
Control	1.80 ± 0.20^{b}	0.98 ± 0.03^{b}	31.1 ± 0.7^{a}	0.48 ± 0.10^{b}
Propionate control	1.50 ± 0.06^{ab}	0.96 ± 0.01^{b}	31.0 ± 0.7^{a}	0.47 ± 0.21^{b}
Control RB medium 10%	1.30 ± 0.06^{a}	0.97 ± 0.08^{b}	32.9 ± 0.4^{b}	0.46 ± 0.20^{b}
Control RB medium 20%	1.20 ± 0.15^{a}	0.95 ± 0.02^{b}	31.9 ± 0.1^{ab}	0.47 ± 0.15^{b}
RB medium 10%	1.60 ± 0.30^{ab}	0.83 ± 0.04^{a}	33.0 ± 1.9^{b}	0.45 ± 0.12^{b}
RB medium 20%	1.20 ± 0.10^{a}	0.82 ± 0.05^{a}	31.7 ± 0.5^{ab}	0.41 ± 0.10^{a}

Control did not receive any treatment; Propionate control (Dough treated with propionate); Control RB ingredient 10% (Dough enriched with a 10% concentration of non-fermented rice bran ingredient); Control RB ingredient 20% (Dough enriched with a 20% concentration of non-fermented rice bran ingredient); Fermented RB ingredient 10% (Dough enriched with a 10% concentration of fermented rice bran ingredient); and Fermented RB ingredient 20% (Dough enriched with a 20% concentration of fermented rice bran ingredient). Data was displayed as mean ± SD. Different letters were used to signal significative differences among the bread formulations (columns) ($p \leq 0.01$).

The pH values demonstrated a distinct trend. Control bread, including the control, control with propionate, and control with non-fermented RB, maintained pH values above 5.00 throughout the bread production process. In contrast, breads containing the 10 % RB fermented ingredient had a consistently lower pH, dropping below 4.50 ($p \leq 0.01$). This pH reduction was observed in both the dough and the final bread, and there were no significant changes during fermentation or baking.

Titratable acidity exhibited interesting variations as well. The control bread had lower titratable acidity compared to the other bread variants. Notably, the bread with a 20 % RB fermented ingredient displayed significantly higher acidity than the others ($p \leq 0.01$). In the dough, it reached an average of 27.0 g/kg, while in the bread itself, it averaged 20.3 g/kg. Titratable acidity decreased at each stage of the bread-making process.

The decrease in titratable acidity observed at each stage of the bread-making process was noteworthy. Lower pH and higher acidity levels were typically associated with the development of desirable aromas in bread, as indicated by Gagiu et al. (2013). These newly fermented flavors were often appreciated by consumers in the market, as reported by Omedi et al. (2019). Furthermore, this adjustment in acidity contributed to the inhibition of fungal formation in food, as highlighted by Marín et al. (2019). It aligned with the broader goal of extending the shelf life of bread by creating an environment less conducive to fungal growth.

Volume expansion was another parameter of interest. Breads containing the 20 % fermented RB ingredient showed reduced volume expansion compared to regular bread (control bread) ($p \leq 0.01$). This reduction was expected because lower concentrations of wheat flour result in decreased bread expansion. The substitution of RB for wheat flour diminished the concentration of gluten proteins, which play a crucial role in retaining CO_2 and inflating the bread (Honda et al., 2021).

Water activity (Aw) levels in breads with the fermented RB ingredient were consistently lower than those in all control breads ($p \leq 0.01$). Lower Aw values were associated with reduced fungal growth and could even inhibit the germination of several fungal species. Moisture content showed slight variations, with only the breads containing a 10 % RB control ingredient and a 10 % RB fermented ingredient exhibiting elevated moisture levels compared to the control samples ($p \leq 0.01$). Finally, the specific volume of the samples was slightly lower in the bread with a 20 % RB fermented ingredient ($p \leq 0.01$). However, this reduction was not visually noticeable.

In summary, the addition of a 20 % fermented RB ingredient had a significant impact on the bread's characteristics. It lowered the pH, increased titratable acidity, reduced volume expansion, lowered water activity, and had subtle effects on moisture content and specific volume (Loureiro & Malfeito-Ferreira, 2003). These changes not only altered the bread's properties but also contributed to its extended shelf life by creating an environment less conducive to fungal growth (Casquete et al., 2017). These modifications aligned with consumer preferences for unique flavors and improved preservation in the market.

3.4. Antifungal metabolite occurrence in the fermented RB powder and the breads with the fermented RB ingredient powder as an ingredient

In the context of antifungal metabolite occurrence in both fermented RB powder and breads containing the fermented RB ingredient powder, we conducted analyses on pH, lactic acid, and PLA levels. These results were presented in **Table 1**. As anticipated, the pH of the control ingredient was measured at 6.2, while that of the fermented ingredient was 3.7 ($p \leq 0.01$), consistent with the findings obtained from the characterization of the fermented RB mediums (**Table S2**). Notably, no acids were detected in the control samples, whereas the fermented RB samples exhibited concentrations of 13.4 g/kg of lactic acid and 182.3 mg/kg of PLA. Consequently, breads containing significant levels of lactic acid and PLA were those incorporating the fermented RB ingredient, as detailed in **Table 2**. Specifically, the bread with 20 % of the RB fermented ingredient exhibited notably higher concentrations of lactic acid and PLA compared to other fermented treatments ($p \leq 0.01$). Following the baking process, these acids were found to maintain slightly higher concentrations than in the dough, owing to the loss of water content. This suggests that these acids were resistant to degradation during baking. On average, the concentrations of lactic acid and PLA in the bread with 20 % of the RB fermented ingredient were 16.0 g/kg and 1.0 mg/kg, respectively.

Table 1. Quantification from the main antifungal metabolites present in the rice bran medium: pH, lactic acid, and phenyllactic acid.

Ingredient	pH	Lactic acid	PLA
Control RB medium	6.2 ± 0.2^{b}	nd	nd
RB medium	3.7 ± 0.1^{a}	103.4 ± 0.2	182.3 ± 0.6

Data was displayed as mean ± SD. Different letters were used to signal significative differences in the studied parameters among the fermented mediums (columns) ($p \leq 0.01$). Results below the limit of detection were marked as nd.

Table 2. Quantification from the main antifungal metabolites present in the dough and the bread: lactic acid (a) and phenyllactic acid (b).

a)

Sample	Dough	Fermented dough	Bread
Control	nd	nd	nd
Propionate control	nd	nd	nd
Control RB medium 10%	nd	nd	nd
Control RB medium 20%	nd	nd	nd
RB medium 10%	10.4 ± 1.5[a]	10.1 ± 1.2[a]	9.3 ± 0.4[a]
RB medium 20%	15.0 ± 0.3[b]	15.9 ± 1.0[b]	17.0 ± 0.5[b]

b)

Sample	Dough	Fermented dough	Bread
Control	nd	nd	nd
Propionate control	nd	nd	nd
Control RB medium 10%	nd	nd	nd
Control RB medium 20%	nd	nd	nd
RB medium 10%	0.28 ± 0.14[a]	0.23 ± 0.04[a]	0.35 ± 0.02[a]
RB medium 20%	0.82 ± 0.02[b]	0.84 ± 0.17[b]	1.32 ± 0.15[b]

Data was displayed as mean ± SD. Different letters were used to signal significative differences in the studied parameters among the fermented mediums (columns) ($p \leq 0.01$). Results below the limit of detection were marked as nd.

Higher concentrations of PLA and lactic acid have been consistently associated with an extended shelf life of food products, as documented in various studies. Russo, Arena, et al. (2017) established a connection between the presence of lactic acid and PLA and a reduction in fungal occurrences in cereal-based products. Similarly, Schnürer and Magnusson (2005) provided several instances in their review where lactic acid and PLA effectively reduced fungal occurrences in food items. Additionally, lactic acid was accepted as a food preservative within the global food industry. Its safety for consumers and its regulatory approval in foods were well-established and recognized by ASEAN (2021) and the European Parliament regulation (EC) No 1333/2008 (European Parliament, 2008).

3.5. Study of the antifungal activity of the fermented RB ingredient in contaminated bread

Table 3 presented the results of the study examining the shelf life of bread over a 7-day incubation period. When assessing the trials against *A. flavus* ITEM 8111 contamination, fungal growth was evident after 4 days of incubation in the control, control RB ingredient 10 %, and control RB ingredient 20 %. By day 5, fungal growth had occurred in both 10 % and 20 % fermented RB ingredients, and by day 6, it had begun in the propionate control.

Table 3. Study of the shelf life of the bread slices contaminated with *A. flavus* (a) and *P. commune* (b) monitored each day.

a) *A. flavus*					
Sample	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**
Control	0 ± 0^{a}	90 ± 14^{b}	100 ± 0^{c}	100 ± 0^{b}	100 ± 0^{a}
Propionate control	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	75 ± 0^{a}	100 ± 0^{a}
Control RB medium 10%	0 ± 0^{a}	90 ± 14^{b}	100 ± 0^{c}	100 ± 0^{b}	100 ± 0^{a}
Control RB medium 20%	0 ± 0^{a}	10 ± 14^{b}	90 ± 22^{c}	100 ± 0^{b}	100 ± 0^{a}
RB medium 10%	0 ± 0^{a}	0 ± 0^{a}	95 ± 11^{c}	100 ± 0^{b}	100 ± 0^{a}
RB medium 20%	0 ± 0^{a}	0 ± 0^{a}	45 ± 21^{b}	100 ± 0^{b}	100 ± 0^{a}

b) *P. nordicum*					
Sample	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**
Control	95 ± 11^{c}	100 ± 0^{b}	100 ± 0^{b}	100 ± 0^{a}	100 ± 0^{a}
Propionate control	0 ± 0^{a}	0 ± 0^{a}	45 ± 21^{a}	90 ± 22^{a}	100 ± 0^{a}
Control RB medium 10%	50 ± 25^{b}	100 ± 0^{b}	100 ± 0^{b}	100 ± 0^{a}	100 ± 0^{a}
Control RB medium 20%	0 ± 0^{a}	100 ± 0^{b}	100 ± 0^{b}	100 ± 0^{a}	100 ± 0^{a}
RB medium 10%	20 ± 32^{ab}	100 ± 0^{b}	100 ± 0^{b}	100 ± 0^{a}	100 ± 0^{a}
RB medium 20%	0 ± 0^{a}	0 ± 0^{a}	90 ± 22^{b}	100 ± 0^{a}	100 ± 0^{a}

Data was displayed as mean ± SD. Different letters were used to signal significative differences in the studied parameters among the bread samples (columns) ($p \leq 0.01$).

Particularly, the bread containing 20 % of the RB fermented ingredient exhibited a reduction in fungal occurrence at day 5 compared to the control and the 20 % RB control ($p \leq 0.01$). In this treatment, less than 50 % of the spots showed fungal growth, while in the controls, fungal growth exceeded 90 % of the spots.

In samples contaminated with *P. commune* CECT 20767, fungal growth was observed after 3 days in the control bread, control RB ingredient 10 %, and fermented RB ingredient 10 %. After 4 days, fungal occurrence became evident in the control RB ingredient 20 %, and by day 5 of the incubation, it had started to grow in the propionate control bread and the bread with a 20 % RB

fermented ingredient. Notably, the 20 % RB fermented ingredient treatment achieved comparable results in the shelf life analysis when compared to the use of propionate as a preservative against this fungus. A visual representation of the fungal growth determination was plotted in **Fig. 1**.

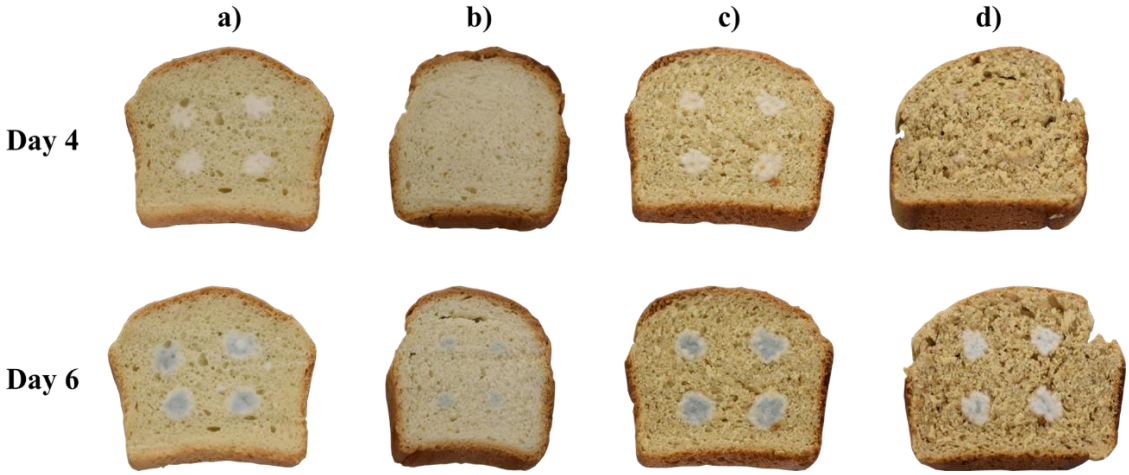

Fig. 1. Slice of bread inoculated with *P. commune* after 4 and 6 day incubation. It can be observed the control bread (a), propionate control (b), 10 % RB control (c) and 20 % RB (d).

For a comprehensive analysis of fungal growth in various samples, we quantified fungal spores/gram of bread after a 7-day incubation period (Table 4). The samples that significantly reduced fungal growth compared to others were the propionate control and the bread with 20 % RB fermented ingredient ($p \leq 0.01$), with no discernible differences in fungal population between these two samples. The 20 % RB fermented ingredient treatment effectively reduced counts to 4.7 Log CFU/g against *A. flavus* ITEM 8111 and 4.4 Log spores/g against *P. commune* CECT 20767. This reduction was significant when compared to the control bread ($p \leq 0.01$), which had spore counts of 5.4 and 5.2 Log spores/g for *A. flavus* ITEM 8111 and *P. commune* CECT 20767, respectively. These results aligned with the findings of the shelf life study, indicating that bread containing a 20 % RB fermented ingredient exhibits antifungal properties similar to those of propionate.

Table 4. Results of the determination of the fungal population in the bread after a 7-day incubation.

Sample	*A. flavus*	*P. commune*
Control	5.4 ± 0.1^{c}	5.2 ± 0.1^{bc}
Propionate control	4.7 ± 0.0^{a}	4.6 ± 0.1^{a}
Control RB medium 10%	5.3 ± 0.1^{c}	5.3 ± 0.0^{c}
Control RB medium 20%	5.1 ± 0.1^{b}	5.1 ± 0.0^{b}
RB medium 10%	5.0 ± 0.0^{b}	5.3 ± 0.1^{c}
RB medium 20%	4.7 ± 0.1^{a}	4.4 ± 0.1^{a}

Data was displayed as mean ± SD. Different letters were used to signal significative differences in the studied parameters among the fermented mediums (columns) ($p \leq 0.01$).

The pursuit of novel methods for substituting or reducing chemical preservatives was strongly endorsed by food authorities and regulatory bodies (Schmidt et al., 2019). Notably, LAB have gained recognition as safe additives in food products, as affirmed by both the EFSA and the European Union. Therefore, the utilization of LAB as a biopreservative agent held significant promise for the food industry (Nasrollahzadeh et al., 2022).

In a recent study conducted by Russo, Fares, et al. (2017), the authors' focus was on the potential protective qualities of *L. plantarum* UFG 121. Impressively, this strain completely suppressed the growth of *F. culmorum* after just one week of storage. Moreover, this LAB demonstrated the ability to modulate the growth of molds in samples contaminated with *A. flavus*, *P. chrysogenum*, and *P. expansum*. However, no antagonistic effects were observed against *A. niger* and *P. roqueforti*. These findings highlighted the potential of *L. plantarum* UFG 121 as a highly effective biocontrol agent in bread production, revealing the varying responses of different mold species to this biological control approach.

While certain articles have explored the use of fermented rice bran for antifungal purposes, it was crucial to note that these studies often involved other microorganisms that were not considered as safe as LAB according to regulatory authorities. For instance, *Rhizopus oryzae* has been employed to extend the shelf life of bakery products (Denardi-Souza et al., 2018). Additionally, rice bran fermented with *Rhizosphere streptomyces* has been employed for the biopreservation of soybeans (Sari et al., 2021). Nevertheless, our study represented a pioneering effort as it was the first report of rice bran fermentation by LAB effectively combating fungal species in food products.

3.6. Identification and quantification of mycotoxins in bread

Table 5 showed the outcomes of the fungal metabolite profile in bread contaminated by fungi. Notably, no mycotoxin production was observed in the breads contaminated by *P. commune* CECT 20767. In contrast, breads contaminated by *A. flavus* ITEM 8111 revealed the presence of mycotoxins commonly monitored by European Regulations in cereals, specifically aflatoxins B1, B2, G1, and G2 (Romero-Sánchez et al., 2022).

Table 5. Results of the fungal metabolites produced by *Aspergillus flavus* in the bread inoculated with the fungi after a 7-day incubation. Metabolites detected were AFB1, AFB2, AFG1 and AFG2.

Sample	**AFB1**	**AFB2**	**AFG1**
Control	615.7 ± 26.5^{c}	92.0 ± 1.0^{b}	8.0 ± 0.5
Propionate control	12.3 ± 2.5^{a}	nd	nd
Control RB medium 10%	157.1 ± 4.0^{b}	13.8 ± 1.5^{a}	nd
Control RB medium 20%	12.3 ± 1.5^{a}	nd	nd
RB medium 10%	10.0 ± 0.5^{a}	nd	nd
RB medium 20%	8.4 ± 1.3^{a}	nd	nd
Sample	**AFG2**	**ΣAF_s**	**% reduction**
Control	90.0 ± 2.0^{b}	719.7	-
Propionate control	nd	17.4	97.6
Control RB medium 10%	10.2 ± 1.5^{a}	189.5	73.7
Control RB medium 20%	nd	46.7	93.5
RB medium 10%	nd	30.4	95.8
RB medium 20%	nd	24.3	96.6

Metabolites detected were AFB1 (aflatoxin B1), AFB2 (aflatoxin B2), AFG1 (aflatoxin 12) and AFG2 (aflatoxin G2). Data was displayed as mean ± SD and % of reduction. Different letters were used to signal significative differences in the studied parameters among the fermented mediums (columns) ($p \leq 0.01$).

Aflatoxin B1 was detected in all bread samples, with concentrations reaching 615 μg/kg in the control bread, 12 μg/kg in the bread with 20 % control RB ingredient, and in the propionate control bread, and 8 μg/kg in the bread with 20 % fermented RB ingredient. AFB2, the second most abundant mycotoxin, was only detected in the control bread, with concentrations of 92 μg/kg, and in the bread with 10 % control RB ingredient, with concentrations of 13.8 μg/kg. The control bread was the sole sample in which all four quantified aflatoxins were detected.

Comparatively, the propionate bread control, 20 % control RB ingredient, 10 % fermented RB ingredient, and bread with 20 % fermented RB ingredient exhibited a reduction in total aflatoxin levels of more than 93 % when compared to the control bread ($p \leq 0.01$). Notably, no significant differences were observed in the production of these fungal metabolites among these samples.

Based on these results, it is evident that fermentation was not the sole factor influencing the reduction of mycotoxins in the bread samples. Even the bread containing 10 % control RB ingredient exhibited a significant reduction in the concentration of all aflatoxins compared to the control bread. This suggested that specific compounds present in the rice bran may have interfered with the metabolic pathway of these fungal secondary compounds. Existing literature indicated that various factors contributed to the antifungal and antitoxigenic activity within a matrix, encompassing not only the metabolites produced by bacteria during fermentation but also other components from the fermentation substrate, such as antifungal peptides or molecules (Le Lay et al., 2016).

Furthermore, the addition of a 10 % fermented RB ingredient produced by the strain *L. plantarum* H1 resulted in a decrease in the occurrence of fungal metabolites compared to the 10 % RB control ingredient, highlighting the significance of fermentation in aflatoxin reduction. It was noteworthy that propionate did not exhibit a substantial reduction in fungal metabolite production compared to the other treatments; all three demonstrated a similar antimycotoxigenic effect.

Alternative techniques for reducing aflatoxin occurrence have been explored. Quiles et al. (2019) reported the use of volatile compounds from mustard, achieving a 70 % reduction in aflatoxin B1. Other authors have reported the antifungal and anti-toxigenic activities of LAB as biocontrol agents. Mohammadi et al. (2021) conducted *in vitro* assays using LAB, resulting in a remarkable 94 % reduction in aflatoxin occurrence in PDA plates against *A. flavus*. Rämö et al. (2022) achieved similar results with a commercial strain of *L. plantarum*, reducing aflatoxin B1 occurrence by 90 % in proteinaceous plant-based foods. Moreover, the presence of certain LAB-produced compounds, such as PLA, has been linked to a reduction in aflatoxin occurrence *in vitro* (Guimarães et al., 2018).

Despite the limited number of articles reporting on the reduction of aflatoxins in bread through LAB bioconservation, Illueca et al. (2023) demonstrated that sourdough bread made exclusively with a LAB inoculum significantly reduced the occurrence of aflatoxins B1, B2, and G1 when compared to regular breads.

4. CONCLUSION

The *in vitro* assays conclusively determined that the medium containing 20 % RB, fermented by the strain *L. plantarum* H1, exhibited significant antifungal activity and yielded the highest quantity of antifungal compounds among the tested samples. Consequently, this medium was incorporated as an ingredient in bread production to enhance the product's shelf life.

The analyses of the bread samples revealed that the addition of this ingredient did not result in significant alterations to critical bread parameters, including moisture content and specific volume. Instead, the presence of this ingredient notably increased the concentration of antifungal compounds such as lactic acid and PLA, leading to an extension of the bread's shelf life and a reduction in aflatoxin occurrence compared to the control bread. Remarkably, when these results were compared with those obtained from bread containing sodium propionate, similar outcomes were observed.

This work introduces a novel and economically viable application of a by-product from the rice industry, namely RB, as a biopreservative agent in bakery products to combat fungal contamination. Furthermore, these promising findings open the door for further investigations into the potential application of this agent in a broader range of food products.

4 GENERAL DISCUSSION

DISCUSIÓN GENERAL

4. DISCUSIÓN GENERAL

El trabajo de investigación realizado a lo largo de esta tesis doctoral ha buscado estudiar el aprovechamiento de residuos de la industria alimentaria para el desarrollo de nuevos agentes antifúngicos y antitoxigénicos mediante la fermentación con bacterias ácido lácticas. A lo largo de estos ensayos se han estudiado los metabolitos implicados en la actividad contra hongos y diversas formas de aplicación de los productos en alimentos, para así reducir la aparición de contaminaciones de hongos y sus micotoxinas.

Inicialmente, se procedió a la selección de las bacterias que mejor actividad antifúngica presentaban tras la fermentación de los diferentes medios de cultivo preparados con los distintos residuos alimentarios (suero lácteo, bagazo de cerveza y salvado de arroz) y medio de cultivo MRS. Las bacterias empleadas en estos primeros análisis han sido aisladas e identificadas a nivel de cepa por la Colección Española de Cultivos Tipo (CECT) de diferentes matrices para anteriores estudios y forman parte de la colección de nuestro grupo de investigación. Los distintos tratamientos fermentados se analizaron mediante pruebas *in vitro* para poder determinar su capacidad antifúngica contra distintas especies de hongos pertenecientes a los géneros *Alternaria*, *Aspergillus*, *Botrytis*, *Fusarium* y *Penicillium*, de forma cualitativa y cuantitativa.

Tras la determinación de la actividad antifúngica *in vitro*, se realizó un estudio de los diferentes metabolitos implicados en la actividad antifúngica observada en los anteriores ensayos para comprender mejor los procesos que explican su potencial contra el crecimiento de hongos. Los diferentes metabolitos se analizaron mediante el empleo de equipos HPLC-DAD, HPLC-Q-TOF-MS.

Con el objetivo de probar la capacidad antifúngica de los residuos fermentados por las BAL se han desarrollado dos estrategias distintas para probar su actividad en el control del crecimiento de hongos contaminantes en alimentos y la producción de toxinas en los mismos.

La primera de ellas, en colaboración con el centro AIMPLAS Instituto Tecnológico del Plástico, se desarrolló un separador de lonchas de queso que incorporaba suero lácteo fermentado por BAL con el objetivo de alargar la vida útil de quesos contaminados por *Penicillium commune*, *Penicillium verrucosum* y *Penicillium solitum*.

En segundo lugar, se evaluó el potencial de los diferentes residuos (suero lácteo, bagazo de cerveza y salvado de arroz) fermentados por las BAL de alargar la vida útil de panes contaminados por *Aspergillus* spp. y *Penicillium* spp. al ser añadidos como ingrediente antifúngico. Con el fin de asegurar que el ingrediente no alteraba de forma considerable las cualidades físico-quimias y organolépticas del pan, también fueron evaluadas en comparación a panes sin el ingrediente. Finalmente se evaluó como los ingredientes producidos con bagazo de cerveza y salvado de arroz

reducían la aparición de aflatoxinas en los panes donde se estudió la vida útil ante la contaminación de *A. flavus*.

4.1. Evaluación de la actividad antifúngica "in vitro"

4.1.1. Evaluación de la actividad antifúngica del MRS fermentado por las BAL

La evaluación de la actividad antifúngica de un extracto centrifugado libre de bacterias (CFS) de medio de cultivo MRS se analizó mediante técnicas *in vitro* cualitativas para hacer una primera caracterización y cuantitativas para determinar la concentración a la cual se apreciaba una actividad inhibitoria del crecimiento de hongos y antifúngica. El ensayo cualitativo se realizó mediante el método de difusión en agar. Los resultados se pueden observar el **Punto 3.2. Table 3**. Todos los CFS bacterianos evidenciaron halos de inhibición del crecimiento de los hongos contra *B. cinérea*, *A. alternata*, *A. carbonarius*, *F. graminearum*, *P. verrucosum*, *P. expansum*, *P. digitatum* y *P. commune*. La ausencia de inhibición por este tratamiento solo se observó contra *A. flavus* y *P. expansum* por alguna de las cepas (*L. plantarum* SC2 y *L. plantarum* SC6). En general, en este tratamiento se manifestaron unos halos de inhibición inferiores a 0.6 cm en hongos de especies más susceptibles a estos tratamientos como las pertenecientes a los géneros *Botrytis* spp., *Alternaria* spp. y *Fusarium* spp. Mientras que en otras especies más resistentes (*Aspergillus* spp. y *Penicillium* spp.), se apreciaron también halos de menor tamaño, inferiores a 0.2 cm o incluso ausencia de halo.

La cuantificación de la actividad antifúngica se determinó mediante el ensayo de determinación de la mínima concentración inhibitoria y mínima concentración antifúngica (MIC-MFC). Para este trabajo, se buscaba aplicar estos tratamientos sobre queso y, por lo tanto, se redujeron el número de hongos estudiados a solo los que tenían capacidad de contaminar este alimento (**Punto 3.2. Table 4**). El CFS del MRS logró inhibir el crecimiento de los hongos en concentraciones entre 13 y 25 g/L contra *P. verrucosum*, 50 y 100 contra *P. expansum*, *P. digitatum* y *P. commune*, y contra *A. flavus* de entre 100 y 200 g/L. Solo se observó actividad antifúngica contra *P. verrucosum* en intervalos de concentraciones entre los 25 y 50 g/L, y contra *P. expansum* de 200 g/L. El resto de las especies fúngicas pudo resistir concentraciones mayores a 200 g/L contra la mayoría de BAL.

El MRS es un medio de cultivo preparado para el crecimiento óptimo de las BAL y en este medio de cultivo son capaces de metabolizar gran parte de los metabolitos antifúngicos típicos en su producción. El MRS no es apto para su aplicación en alimentos. No obstante, un primer estudio de la actividad del MRS fermentado por las BAL es útil como indicador de la potencial antifúngico que puede demostrar una BAL en otros medios de cultivo. Investigaciones anteriores

han demostrado la capacidad fungistática de otras cepas BAL. Extractos de MRS fermentado por una serie de cepas de *L. plantarum* concentrados 20/1 lograron inhibir el crecimiento de *A. parasiticus* y *P. expansum* produciendo halos de inhibición menores a 10 mm (Saladino et al., 2016). De igual modo en De Muynck et al. (2004) se concentra el MRS fermentado por las BAL en discos de papel y tras la aplicación de estos discos a placas de PDA sembradas con hongos se produjo una inhibición del crecimiento de los hongos.

4.1.2. Evaluación de la actividad antifúngica del suero de leche fermentado por las BAL

La actividad antifúngica del suero de leche fermentado por las BAL fue analizada en dos de los artículos tratados en esta tesis doctoral. Los datos se pueden apreciar en el **Punto 3.1. Table 2** y en el **Punto 3.2. Table 3**. Para investigar esta actividad, se procedió a la realización de un estudio del suero de leche dulce fermentado de forma directa. Paralelamente se desarrollaron 2 medios de cultivo a base de suero lácteo enriquecidos, con dos objetivos, primero, entender que nutrientes aprovechaban más eficientemente las BAL y, segundo, para alcanzar una mejor comprensión de como el enriquecimiento con otros ingredientes podía afectar al crecimiento y producción de metabolitos de las BAL. Finalmente, se estudiaron 3 medios de cultivo diferentes a base de suero lácteo: Suero (W1), suero complementado con nutrientes (W2) y suero diluido al 20% y complementado con nutrientes (W3).

La determinación de la actividad antifúngica cualitativa *in vitro* presentó generalmente una gran acción contra todos los hongos con los que se trabajó pertenecientes a los géneros *Aspergillus* spp., *Botrytis* spp., *Alternaria* spp., *Penicillium* spp. y *Fusarium* spp. Las especies *A. flavus*, *A. carbonarius*, *P. digitatum* y *P. solitum* evidenciaron la mayor resistencia contra este tipo de tratamiento. En este último, la mayoría de aislados no lograba inhibir su crecimiento, mientras que para el resto sí que se observaba una ligera inhibición superior al milímetro. En contraposición, *A. alternata*, *P. expansum*, *P. commune* y *P. verrucosum* evidenciaron una gran susceptibilidad a este tratamiento. Este resultado fue altamente positivo ya que estas dos últimas especies de hongos suelen ser típicos contaminantes de queso, que es la matriz alimentaria sobre la que se pretendía aplicar inicialmente este agente antifúngico (Pitt & Hocking, 2022). Se observaron halos de inhibición con este medio de cultivo superiores a los 0.2 cm y mayores a 1 cm contra los hongos más sensibles como *P. commune* y *P. verrucosum*.

Como cabía esperar, los resultados de la evaluación cuantitativa de la actividad antifúngica de los sueros fermentados evidenciaron unos resultados semejantes a los obtenidos por las mismas muestras en las pruebas cualitativas (**Punto 3.1. Table 3** y **Punto 3.2. Table 4**). El medio de cultivo W1 alcanzó MIC 2 a 50 g/L contra los hongos pertenecientes a *Penicillium* spp., y de 13 a 50 g/L contra *A. flavus*. Los medios de cultivo a con suero enriquecido con nutrientes lograron

una actividad inhibitoria semejante. En el análisis de la MFC es donde se apreciaban mayores diferencias. Las especies *P. expansum, P. camemberti* y *P. digitatum* fueron las únicas donde alguno de los tratamientos no logró inhibir su crecimiento a la concentración probada más alta (200 g/L). Respecto al resto de especies de hongos estudiadas en estos ensayos, los medios de cultivo con suero lácteo evidenciaron MFC en un rango entre los 13 g/L a los 200 g/L. El hongo más susceptible al tratamiento fue el *P. verrucosum* y el *P. commune*, para los cuales se apreciaron rangos de MFC entre 13 a 50 y 25 a 100 g/L, respectivamente. Cabe destacar que estos medios fermentados lograban tener actividades antifúngicas contra *A. flavus* a concentraciones de 25 g/L y que estas se daban con el medio fermentado W1. Los resultados mostraron que, contra los diversos *Penicillium* spp. evaluados, los medios de cultivo enriquecidos evidenciaban unas medias de MFC levemente menores, mientras que contra *A. flavus* el suero lácteo W1 fue el más eficaz.

En la comparación de estos resultados no se encontraron grandes diferencias entre el enriquecimiento del medio de cultivo a base de suero cuando fue enriquecido o no. Este resultado muestra una buena versatilidad por parte de las BAL en cuanto a los nutrientes básicos que se pueden encontrar en algunos desechos alimentarios. Se observó que eran capaces de aprovechar diversos monosacáridos como la glucosa y la lactosa, y fuentes de nitrógeno, como las proteínas del suero, triptona y la provenientes del extracto de levadura. En la comparación de estos resultados con otros extractos fermentados por BAL, se puede apreciar el alto potencial de emplear el suero fermentado como agente antifúngico. Otros autores como Izzo et al. (2020) han descrito datos semejantes a los evidenciados en estas pruebas utilizado suero de búfala fermentado por BAL. Pero cuando la comparación se realiza con otros CFS se puede apreciar el potencial del suero. Autores como Sung et al. (2023) y Yépez et al. (2017) han probado la actividad del CFS de MRS fermentado y, en ambos estudios, los valores de MFC obtenidos contra hongos pertenecientes a los mismos géneros que los utilizados en esta tesis superaban los obtenidos con el suero lácteo. O en este propio trabajo, los valores obtenidos con el MRS fermentado por las bacterias, que se pueden contemplar en el subapartado anterior son menos activos contra el crecimiento de hongos en comparación al suero. Al equiparar estos estudios con otros realizados con extractos puros, se observan unos datos semejantes, dato de interés debido a que la utilización del MRS en este estudio era meramente comparativa para evaluar el suero lácteo. Normalmente los extractos y purificados son más eficaces que un fermentado, pero mucho más difíciles de obtener debido a su purificación (Ogunremi et al., 2022). Extractos de fermentados de BAL han mostrado MFC entre 0.3 a 12.5 g/L con diversos ácidos puros (ácido láctico, ácido acético, entre otros. O autores como Xing et al. (2019) describen MIC contra *Aspergillus neoniger* y *Penicillium roqueforti* de 50 g/L con aceites esenciales extraídos de plantas.

4.1.3. Evaluación de la actividad antifúngica del bagazo de cerveza fermentado por las BAL

Para la evaluación de la actividad antifúngica de los medios de cultivo con bagazo de cerveza (BB) fermentados se realizó una prueba que incorporaba directamente el tratamiento en el PDA donde se realizaron los ensayos *in vitro*. Así pues, se prepararon placas de medio de cultivo para hongos, PDA, a las que se incorporó el tratamiento a diferentes concentraciones y se cultivó el hongo en estas placas y una placa de PDA para que actuara como control del crecimiento del hongo. De esta manera se podía observar cómo se reducía el crecimiento del hongo en contacto directo con el tratamiento, así se conseguía simular unas condiciones más semejantes a la realidad cuando se pretendía aplicar el tratamiento. Esta prueba se realizó contra *A. flavus*, *P. commune* y *A. alternata*. Se inocularon las placas objeto de estudio y se evaluó la actividad inhibitoria a las 48 y 96 h de crecimiento (**Punto 3.4. Table 1**).

En la evaluación de la actividad antifúngica contra *A. flavus* a las 48 h de incubación en medio PDB con un 10% y 20% evidenció una reducción significativa del crecimiento del hongo, logrando una reducción de hasta un 28% con respecto al control. Tras 96 h ninguno de los tratamientos evaluados consiguió reducir el crecimiento del *A. flavus*. Respecto a los datos obtenidos en el estudio contra *P. commune*, solo los tratamientos al 20% de bagazo de cerveza fermentado conseguían reducir de forma significativa el crecimiento del hongo a las 48 h, no obstante, esta reducción fue mucho mayor y alcanzando de media un 38% respecto al control de PDA. En la segunda medida a 96 h se podía seguir apreciando una reducción significativa del crecimiento del hongo tanto en el tratamiento a 10% como a 20%, alcanzado una reducción del 39% en el tratamiento más eficaz. Finalmente, en el estudio realizado contra *A. alternata* se contemplaban los mejores resultados con el agente antifúngico a base de BB. Solo un 5% del tratamiento conseguía reducir el diámetro de crecimiento del hongo de forma significativa, a concentraciones más altas esta reducción superaba el 60%. Tras 96 h, esta reducción se continuaba observado en todas las concentraciones de tratamiento estudiado, siendo de media en la concentración más alta superior al 50% de inhibición del crecimiento. Entre las dos bacterias con las que se trabajó, la que mayor efectividad presentó contra el crecimiento de los hongos fue el aislado *L. plantarum* L1.

En la bibliografía no existen precedentes del uso de BB fermentado por BAL como agente antifúngico. En el proceso de producir un agente antifúngico mediante la fermentación por BAL, el primer paso es encontrar la bacteria correcta para la fermentación de la matriz. Estudios como Iosca et al. (2023) hacen una primera caracterización de diferentes BAL de una forma semejante a este trabajo, es decir, para determinar la cepa más efectiva para su empleo en la fermentación de residuos panarios y, de esta forma, lograron inhibir el crecimiento de *Penicillium* spp. y *Aspergillus* spp. en ensayos de difusión en agar. También, otros estudios han utilizado residuos de

cultivos fermentados por BAL para reducir el crecimiento de *F. graminearum* en ensayos *in vitro*. Respecto a la metodología empleada en este ensayo autores como Ogunremi et al. (2022) describen una alta consistencia entre los resultados de este método *in vitro* y la aplicación como agente antifúngico en alimentos.

4.1.4. Evaluación de la actividad antifúngica del salvado de arroz fermentado por las BAL

Para la evaluación de la actividad antifúngica del salvado de arroz (RB) fermentado por las BAL, se procedió a la realización de un estudio semejante al descrito anteriormente con el bagazo de cerveza. Los resultados de este ensayo se pueden observar en el **Punto 3.5. Table S1**. En los ensayos con *A. flavus*, el tratamiento fermentado a todas las concentraciones con las que se trabajó fue capaz de inhibir de forma significativa el crecimiento del hongo después de 48 h de incubación. No obstante, tras 92 h no se aprecia una reducción en los tratamientos respecto al control. Al comparar ambos fermentados por las BAL, se observaba un incremento de la actividad inhibitoria del 4% al comparar la cepa *L. plantarum* H1 con *L. plantarum* L1. En los ensayos contra *P. commune* solo el tratamiento con RB al 20% lograba disminuir el crecimiento del moho de forma significativa respecto a control. Después de 92 h de incubación, los resultados se asemejaban a los observados a las 48 h. Solo la concentración del tratamiento a 20% conseguía reducir significativamente el crecimiento del hongo. De forma general, el aislado *L. plantarum* H1 mostró unos porcentajes de reducción mayores durante todo el ensayo contra este hongo. Finalmente, en el último de los ensayos contra *A. alternata*, todos los tratamientos reducían el crecimiento del hongo a las 48 h y 92 h. De nuevo, el RB fermentado por la BAL *L. plantarum* H1 evidenció una mayor eficacia en su actividad antifúngica mostrando a las 48 h un porcentaje de reducción un 20% mayor que el otro tratamiento estudiado.

Igual que con la anterior matriz no existen reportes previos de la utilización del RB con este fin. Otros autores por su parte sí que han empleado otros residuos que han mostrado en resultados similares, como el empleo de residuos de la producción de pitaya que lograban frenar el crecimiento de *Penicillium* spp. y *Aspergillus* spp. en pruebas *in vitro* (Omedi et al., 2021). En la búsqueda de otros agentes de bioconservación preparados con residuos del arroz, se pueden encontrar ejemplos de distintos extractos fenólicos. Se ha reportado actividad antifúngica de estos extractos obtenidos tanto de RB, un fermentado por *Rhizopus oryzae*, como de *Chlorella phyrenoidosa*, que evidenciaban una alta actividad contra una gran gama de hongos contaminantes de alimentos (Christ-Ribeiro et al., 2019; de Souza et al., 2010). Se ha de tener en cuenta en esta comparación la gran ventaja que presenta el tratamiento aquí estudiado respecto a los extractos. Primero, su manejo requiere una técnica más simple y segundo, no se emplean los disolventes necesarios para la extracción de los compuestos fenólicos.

4.2. Caracterización de los compuestos antifúngicos presentes en los residuos alimentos fermentados

4.2.1. Caracterización de los compuestos antifúngicos presentes en el suero lácteo fermentado

4.2.1.1. Estudio del pH del suero lácteo fermentado

Una parte importante de los metabolitos producidos por las BAL son ácidos como el ácido láctico y ácido acético, además, la actividad antimicrobiana que presentan contra otros microorganismos esta intrínsecamente relacionada con la bajada del pH, ya que facilita la acción de los diversos metabolitos antimicrobianos metabolizados por las BAL (Schnürer & Magnusson, 2005). A consecuencia de este factor, es de interés la medida del pH como indicativo, primero, de un correcto crecimiento de la BAL, y segundo, como indicador de si la matriz fermentada tendrá buena actividad evitando el crecimiento de otros microorganismos como los hongos contaminantes (Muñoz et al., 2011; Schillinger et al., 2006).

Sabiendo esto, se realizó la medida del pH de las diferentes muestras de suero fermentado que se pretendían analizar para la producción del separador de quesos con actividad antifúngica (**Punto 3.1. Table 1**). Se observó una clara reducción del pH en todos los tratamientos respecto al control. En los tratamientos se apreciaban valores de pH entre 3.18 a 4.21, mientras que en el control se mantenía en un pH típico del suero de leche dulce 6.19.

4.2.1.2. Estudio de los ácidos orgánicos en el suero lácteo fermentado

Entre todos los metabolitos producidos por las BAL es ácido láctico el que metabolizan en mayor abundancia. En el artículo donde se desarrolla un separador de queso las BAL estudiadas evidenciaron una producción de ácido láctico en cantidades que oscilaban entre los 4.01 a 6.98 g/L, siendo el medio fermentado por la *L. plantarum* BN17 la mayor productora de este metabolito. Todos los tratamientos evidenciaban concentraciones significativamente superiores de ácido láctico con respecto al control, del cual se encontraba a una concentración de 0.27 g/L (**Punto 3.1. Table 4**). La presencia del láctico es muy común en el suero de leche incluso de forma previa a la fermentación realizada en esta tesis, ya que es un residuo obtenido bajo una técnica que no requiere de esterilidad en su proceso, por ende, en el periodo de tiempo que dura el desuerado, refrigeración y transporte se puede encontrar ácido láctico debido a la contaminación natural (Prazeres et al., 2012).

En el estudio comparativo entre el MRS y el suero lácteo fermentado se observó una mayor producción de este metabolito en el MRS en comparación a los sueros de leche fermentados W1, W2 y W3 (**Punto 3.2. Table 1**). Las concentraciones en el MRS oscilaban entre 15.83 a 18.02

g/L, mientras que en suero lácteo fermentado W1 eran entre 4.89 a 6.54 g/L, en el suero enriquecido con nutrientes W2, eran entre 7.91 a 11.70 g/L y en el diluido enriquecido W3, entre 3.14 a 4.80 g/L. Cabe puntualizar que los controles presentaban inicialmente concentraciones altas de láctico, entre 2.27 a 2.31 g/L, que probablemente venían de una fermentación natural. No obstante, el estudio estadístico evidenciaba que todos los controles presentaban una concentración inferior a los tratamientos. Los resultados más significativos en esta comparación fueron los siguientes. Primero, pese a tener una mayor tasa de producción de láctico en el medio de cultivo MRS, esto no se tradujo en una mayor actividad contra el crecimiento de los hongos, como se ha visto en los resultados del anterior punto. Esto se evidencia también al estudiar los controles de suero W1 y W2, que no presentaban concentraciones muy alejadas de ácido láctico respecto al tratamiento, pero no tenían actividad contra los hongos. Segundo, la producción del suero lácteo es mayor con lactosa y otros azucares minoritarios propios de la leche que con glucosa ya que pese a tener una misma concentración de glucosa en el medio de cultivo W2 y W3 la producción de ácido láctico bajaba de forma drástica en el W3 cuando se comparaba la fermentación hecha por la misma bacteria en cada medio de cultivo.

La bibliografía reconoce al ácido láctico como el principal metabolito en la actividad antifúngica y fungistática producida por las BAL. Se ha sugerido tras numerosos estudios de caracterización que este ácido funciona en sinergia con el resto de los metabolitos antifúngicos (Schnürer & Magnusson, 2005). Altas concentraciones de ácido láctico pueden inducir a la parada del crecimiento celular e incluso la muerte celular, aunque, a unos niveles muy superiores a los encontrados en las matrices fermentadas por BAL (Dalié et al., 2010). Cuando se compara con otros trabajos se puede apreciar que la producción del ácido láctico en medios de cultivo similares a los empleados en este estudio suele tener valores similares (Luz et al., 2020; Luz, Quiles, et al., 2021).

4.2.1.3. Estudio de la actividad antioxidante y fenoles totales en el suero lácteo fermentado

La evaluación de la actividad antioxidante y de los compuestos fenólicos totales en el suero lácteo se realizó como caracterización previa a la producción del separador de quesos antifúngico. Los resultados pueden apreciarse en el **Punto 3.1. Table 5**. Se seleccionó el suero fermentado por la bacteria *L. plantarum* BN17. Como se pudo apreciar en los resultados, en el estudio de la actividad antioxidante, mediante la técnica de estabilidad del radical libre del $DPPH^{+}$, la fermentación del suero lácteo incrementaba la capacidad antioxidante un 20% respecto al control. De forma semejante, la determinación de los compuestos fenólicos totales mediante el método Folin–Ciocalteu reveló que la fermentación incrementaba la presencia de fenoles en el medio de

unos 3.31 mg equivalentes de ácido gálico por litro en el control a unos 15.48 mg/L en el tratamiento.

Las BAL producen un gran espectro de compuestos antioxidantes durante el proceso fermentativo como respuesta directa y forma de protección contra el estrés oxidativo (Qi et al., 2021). Esta actividad es provista por la producción de una serie de enzimas como la lactasa oxidasa, superóxido dismutasa, exopolisacáridos y otros componentes producidos por las propias BAL (Jeong et al., 2021). Estudios con otras BAL empleando el mismo método de evaluación dan actividades antioxidantes relativas similares a las obtenidas con el suero fermentado en esta tesis. Como por ejemplo en Jeong et al. (2021) donde la fermentación de la matriz con varias cepas de *L. plantarum* incrementó de media la actividad antioxidante relativa del medio de cultivo MRS en un 20%.

La fermentación ácido láctica por las BAL también está altamente relacionada con el incremento de los compuestos fenólicos presentes en la matriz antes, en comparación de forma previa a la fermentación. En la literatura se han realizado estudios similares sobre distintos alimentos como zumos donde tras una fermentación de 24 h con una cepa de *L. plantarum* incrementó la concentración de estos compuestos de 100 mg equivalentes de ácido gálico por litro, en el control, a unos 150 mg/L, en el tratamiento. Entre todos los compuestos fenólicos que producen las BAL se encuentran gran parte de los metabolitos que intervienen en la actividad antifúngica de las bacterias, por lo tanto, un incremento de la presencia de estos suele ser un buen indicador de actividad fungistática (Pacheco-Ordaz et al., 2018).

4.2.1.4. Estudio de los compuestos fenólicos en el suero lácteo fermentado

En el estudio comparativo de los medios de cultivo a base de suero de leche en comparación con el MRS se analizó la abundancia relativa de un total de 19 compuestos fenólicos (**Punto 3.2. Fig 1**). Los compuestos que se apreciaron en mayor cantidad en todas las fermentaciones fueron el 3-4-dihidroxihidrocinámico, ácido benzoico y ácido DL-3-feniláctico (PLA), los resultados de la cuantificación se pueden observar en el **Punto 3.2. Table 2**. En general, todas las bacterias fueron capaces de producir estos metabolitos en cantidades detectables, menos la cepa *L. plantarum* SC13. La mayor producción de estos metabolitos fue detectada en el medio de cultivo MRS. Cabe recordar que es un medio ideal para el crecimiento de las BAL, seguido por el medio de cultivo a base de suero de leche y enriquecido (W2), el suero diluido y enriquecido (W3) y, finalmente, la matriz compuesta solo por suero (W1), en esta última no se apreciaba casi la presencia de ningún metabolito. Ninguno de los controles evidenció la presencia relativa de los 19 compuestos estudiados.

Tras el análisis de estos resultados se procedió a la cuantificación con el uso de patrones de los compuestos fenólicos más abundantes. Se determinó que el PLA fue el más abundante, seguido del ácido 3-4-dihidroxihidrocinámico y benzoico en todas las matrices. El medio de cultivo W2 tras la fermentación contenía unas concentraciones de PLA similares a las obtenidas en el MRS fermentado. Se evidenciaban valores entre los 19.86 a 30.00 mg/L en el MRS y de 19.86 a 30.00 mg/L en el medio W2 (excluyendo los resultados de la cepa *L. plantarum* SC13 por su baja producción en todos los medios de cultivo). Estos resultados se correspondían con los obtenidos en el estudio de la abundancia relativa. Se puede apreciar una menor presencia de PLA en el medio de cultivo W3 y aún menor en el W1. En el análisis de ácido 3-4-dihidroxihidrocinámico y benzoico se observó una tendencia similar a la descrita con el PLA.

En el análisis de la producción de estos metabolitos bacterianos se ve una relación distinta a la encontrada con el ácido láctico. Para este, la producción no está tan ligada a la presencia de monosacáridos como la lactosa o glucosa. En este caso parece que es necesaria la presencia de más nutrientes para la metabolización. Se pudo observar de forma general como los medios de cultivo con extracto de levadura triptona y diferentes sales (el MRS, y los medios de suero enriquecidos W2 y W3) había una mayor producción de PLA, 3-4-dihidroxihidrocinámico y benzoico. Por otro lado, estos resultados indican que las proteínas del suero de leche son un interesante sustituto a las de extracto de carne que emplea el MRS, ya que han evidenciado resultados similares en la producción de compuestos fenólicos. Entender los mecanismos detrás de la metabolización de estos compuestos es de interés para la ciencia ya que en la bibliografía se señala a estos compuestos como los principales agentes en la actividad antifúngica de las BAL. Hay diversos reportes de la presencia de PLA con acción fungistática contra hongos contaminantes de alimentos de distintas especies de *Aspergillus*, *Fusarium* y *Penicillium* (Ruggirello et al., 2019; Xu et al., 2021). Y con los ácidos 3-4-dihidroxihidrocinámico y benzoico se observa una tendencia similar (Broberg et al., 2007b; Peyer et al., 2016).

4.2.1.5. Caracterización cualitativa de la relación entre los metabolitos y la actividad antifúngica del suero lácteo fermentado mediante el análisis de componentes principales (PCA)

Con el objetivo de alcanzar una mayor comprensión de como los metabolitos antifúngicos producidos por las BAL ejercían su actividad antifúngica se cuantificaron y se relacionaron con el estudio cuantitativo de la acción antifúngica hecho con los mismos medios de cultivo fermentado. Para ello se relacionaron los resultados de la MFC contra *A. flavus* y *Penicillium* spp. con los datos obtenidos de la determinación de ácido láctico, PLA, ácido 3-4-dihidroxihidrocinámico y ácido benzoico en el MRS y suero lácteo fermentado. Este análisis se

realizó con un ensayo de medida del análisis de componentes principales (PCA). Los resultados se pueden observar en el **Punto 3.3. Fig 3** y **Punto 3.3. Fig 4**.

Tras el análisis de los datos se apreció que con el componente principal (PC) 1 y el PC 2 se alcanzaba una variabilidad acumulativa superior al 90%, por lo tanto, se prescindió del uso del PC 3 para este análisis. Tanto en el PCA obtenido con *A. flavus*, como el obtenido con *Penicillium* spp., se evidenció que no existían indicios suficientes como para separar los resultados obtenidos con los 3 sueros fermentados y el MRS ente sí. Los resultados del análisis de la MFC indicaron que, de media, los sueros fermentados evidenciaban una alta actividad antifúngica en comparación al MRS, no obstante, el análisis de PCA no fue capaz de separar los grupos solo con los metabolitos estudiados. Esto lleva a deducir que todavía hay una gran cantidad de metabolitos antifúngicos producidos por las BAL que normalmente no son analizados, pero intervienen de forma significativa. Ya desde los años 90, se considera que la producción por parte de las BAL de metabolitos antifúngicos está muy por debajo de las concentraciones con capacidad fungistática y antifúngica, no obstante, es la sinergia entre todos ellos lo que resulta en la actividad contra el crecimiento de hongos (Dieuleveux et al., 1998). El análisis biplot de ambos hongos reveló más datos de interés. Existía una tendencia inversa muy acentuada entre la mayor concentración de ácidos fenólicos y concentraciones antifúngicas menores, dato que cabía esperar ya la literatura recoge la alta eficacia de estos compuestos contra el crecimiento de hongos (Broberg et al., 2007b; Peyer et al., 2016b; Ruggirello et al., 2019). No obstante, la relación entre la bajada de la MFC y la concentración de láctico no es tan notable, pese a que en la literatura se ha descrito siempre que una mayor cantidad de este metabolito suele estar relacionada con un incremento actividades antifúngicas. Aquí se observa que efectivamente su presencia sí que está relacionada con menores MFC, pero una mayor concentración del metabolito no disminuye de forma exagerada la MFC. Probablemente esto se deba al mecanismo de acción del ácido, que facilita la entrada de estos metabolitos a través de la pared celular del hongo, pero per se, no tiene unas altas propiedades antifúngicas y se requerirían de cambios más drásticos en su concentración para observar un cambio en la MFC (Schnürer & Magnusson, 2005). Para una corroboración de los resultados de forma numérica, se realizó el test de Pearson r, en este test valores superiores a 0.8 o inferiores a -0.8 se relacionan con correlación positiva o negativa entre factores (**Punto 3.2. Table 5**). Los resultados evidenciaban correlaciones de la concentración de PLA, ácido 3-4-dihidroxihidrocinámico y ácido benzoico - 0.93, - 0.83 y - 0.88 en los ensayos con *Aspergillus* spp. y de - 0.85, - 0.84 y - 0.90 con *Penicillium* spp., respectivamente. Por lo tanto, se demostró la relación inversa entre concentraciones más altas de estos compuestos y valores de MFC más altos. Mientras que, para el ácido láctico, los valores obtenidos con este test fueron en torno a 0.5. Por lo general la bibliografía tiende a estudiar únicamente la presencia ácido láctico y los compuestos fenólicos, pero estos datos evidencian la necesidad incluir más compuestos a analizar

en nuevos trabajos para así poder entender de forma más correcta qué hay detrás de la actividad antifúngica que presentan las BAL (Dagnas et al., 2015; Ström et al., 2002).

4.2.2. Caracterización de los compuestos antifúngicos presentes en el bagazo de cerveza fermentado

En el estudio del bagazo de cerveza fermentado se determinó el pH a 0 y 72 h de fermentación y el contenido de ácido láctico y PLA presente en las muestras (**Punto 3.4. Table 2**). Las muestras analizadas fueron: medio de cultivo control, solo nutrientes sin el BB, y después el mismo medio de cultivo con concentraciones crecientes de BB (5, 10 y 20%). A tiempo 0 h, el pH de todas las muestras oscilaba entre valores de 6.30 a 5.96. Tras 72 h de fermentación, el valor del pH en los todos los medios de cultivo fue por debajo de 3.90, encontrándose los valores más pequeños de pH en los medios de cultivo con una mayor cantidad de bagazo. El incrementó en la concentración de bagazo reducía significativamente el pH. No se observaron diferencias entre las dos bacterias estudiadas. La relación entre la mayor bajada de pH y mayor concentración de BB se debe probablemente a la serie de proteínas y fibra digerible por las BAL que aporta este ingrediente al caldo de cultivo (Castro-Criado et al., 2023). Estos valores de pH más bajo suelen atribuirse a una mayor producción de metabolitos por parte de las BAL (Carr et al., 2002).

El estudio de la presencia de ácido láctico en las muestras evidenció concentraciones entre los 10.04 y 21.40 g/L de este metabolito. Se preciaba un aumento significativo de la concentración de ácido láctico al aumentar la proporción de BB en el medio de cultivo. Los medios con un 5%, 10% y 20% mostraron concentraciones medias de 13.60, 16.99 y 20.83 g/L, respectivamente. De igual manera, la producción del PLA también se vio afectada por la concentración de BB. Los medios de cultivo con una concentración superior al 10% de BB presentaban unas concentraciones significativamente mayores de este metabolito respecto al control. Mientras que en las muestras con un 20% de BB las concentraciones de PLA alcanzaba los 30.17 mg/L de media en el control se encontraba a una concentración de 22.40 mg/L. Entre las dos bacterias que se estudiaban la cepa *L. plantarum* L1 fue la que mayor producción de ácido láctico y PLA produjo.

La mayor presencia de BB en los medios de cultivo ayuda a la producción de nuevos metabolitos y un menor pH. El BB es una matriz rica en nutrientes como fibras con estructuras hechas con hidratos de carbono. Durante la fermentación por las BAL estos hidratos son aprovechados como fuente de carbono que les permite incrementar su producción de metabolitos (Moran-Aguilar et al., 2021). Por otro lado, como ya se ha comentado anteriormente una mayor concentración de ácido láctico y PLA no es determinante, pero sí que es un buen indicador de que

la matriz fermentada puede ser capaz tener actividades fungistáticas y antifúngicas contra contaminantes de alimentos (Debonne et al., 2020; Rajanikar et al., 2021).

4.2.3. Caracterización de los compuestos antifúngicos presentes en el salvado de arroz fermentado

Para la caracterización del medio de cultivo a base de salvado de arroz (RB) se preparó medio de cultivo con el residuo de arroz a concentraciones de (5, 10 y 20%). Tras la fermentación, se determinaron los cambios de pH y las concentraciones de ácido láctico y PLA a las 72 h de estudio y se compararon con un control no inoculado (**Punto 3.5. Table S2**). A tiempo 0 h el pH de los medios de cultivo se encontraba en valores entre 6.04 y 6.22. Se podía apreciar que una mayor cantidad de RB incrementaba el pH ligeramente. Tras las 72 h de fermentación, el control sin inocular mantuvo valores similares a los originales, mientras que en las muestras fermentadas se produjo una bajada del pH hasta valores entre 3.54 a 3.68. No se observaron grandes diferencias entre las dos bacterias, ni entre los distintos medios fermentados, por lo tanto, se concluyó que la concentración de RB no influía en la disminución del pH.

La concentración de ácido láctico osciló entre los 18.10 y 32.60 g/L. En este caso la concentración de RB sí que modificaba la producción de ácido láctico de forma significativa. Esto se observaba en las concentraciones medias del compuesto se encontraban a 17.85, 21.45, y 27.70 g/L, en los medios de cultivo con un 5%, 10% y 20%, respectivamente. En contraposición, la presencia de PLA no se vio afectada por la concentración de RB. Se alcanzó de media una concentración de 30.52 mg/L, pero sin observarse grandes diferencias entre las muestras. Cabe indicar que ambos metabolitos estudiados se encontraban por debajo de los límites de detección en los controles. Lo cual indica que el RB es una matriz estable a contaminaciones durante su almacenamiento (De Koe & Juodeikiene, 2012).

La mayor producción de láctico en concentraciones crecientes de RB se puede deber a que la composición del RB suele tener un alto contenido de hidratos de carbono de cadena corta obtenidos durante la separación del germen y del grano (Sohail et al., 2017). Estos hidratos de carbono son fácilmente metabolizados por las BAL y produciendo de forma constante ácido láctico (Muñoz et al., 2011). En cuanto a las concentraciones de PLA son similares a las obtenidas en otros estudios desarrollados con fermentados de BAL donde la presencia de este metabolito se relaciona con una mayor preservación de pan contra la contaminación por hongos (Illueca et al., 2023; Omedi et al., 2021).

4.3. Reducción del crecimiento de los hongos en alimentos

4.3.1. Empleo del suero de leche fermentado en separadores de queso

4.3.1.1. Empleo del suero de leche fermentado en separadores de queso en pruebas "in vitro"

Tras una primera selección mediante pruebas *in vitro* de la actividad antifúngica y de los metabolitos encontrados en el suero lácteo fermentado por la BAL, se seleccionó el suero fermentado por la cepa *L. plantarum* BN17 para la producción de los separadores antifúngicos. A modo de prueba inicial, se realizó el estudio con uno de los separadores para observar si era capaz de tener actividad fungistática o antifúngica en pruebas *in vitro* contra tres especies distintas de *Penicillium* spp., que después, iban a ser empleadas en las pruebas sobre alimentos (**Punto 3.1. Table 6**). Los resultados evidenciaron que tanto el separador con tratamiento como el separador control (el propio plástico con el recubrimiento sin el tratamiento) retrasaban el crecimiento de los hongos un día respecto a un control sin separador. En las pruebas contra *P. verrucosum* el separador con el SLF lograba retrasar el crecimiento del moho un día más respecto al control.

Comparando estos resultados con la literatura, existen ejemplos de extractos empleados de una forma semejante mediante su incorporación al recubrimiento de alimentos. Como el uso de natamicina en envases de bebidas lácteas que presentan actividad contra hongos y levaduras contaminantes de lácteos (Naderi et al., 2022). Otro el ejemplo es la incorporación de aceites esenciales de orégano, el carvacrol, que lograba reducir la aparición de *Penicillium* y *Rhizopus* contaminantes de productos de bollería cuando era incorporado al envoltorio del alimento (Klinmalai et al., 2021). No obstante, en la bibliografía no se encuentran precedentes de una matriz fermentada sin una previa purificación como agente antifúngico incorporado a un plástico.

4.3.1.2. Empleo del suero de leche fermentado en separadores de queso en pruebas con alimentos

Los resultados de las pruebas del separador sobre alimentos se pueden observar en el **Punto 3.1. Table 7**. En las pruebas sobre alimentos, se observó un gran incremento de la actividad de los separadores respecto a las pruebas *in vitro*. La matriz es un factor muy importante de cara al crecimiento de los hongos. Su desarrollo en los quesos no es igual que en un medio ideado para el crecimiento de hongos como es el PDA. Centrándose en las pruebas con el alimento, se probaron dos separadores diferentes con su correspondiente control (con el mismo tamaño de recubrimiento y mismo pH), un separador con un recubrimiento de 12 µm (V1) y un segundo de 24 µm (V2). Paralelamente, se realizó un control sin separador para determinar si era el tratamiento o la reducción de la presión parcial de oxígeno lo que estaba afectando al crecimiento de los hongos. El separador V2 evidenció los mejores resultados consiguiendo retrasar el crecimiento de los hongos una media de 20 días. Cuando se compararon los resultados con el

control sin separador, el V2 logró extender la vida útil de los quesos un total de 15 días en los quesos contaminados por *P. commune*, de 13 días en los contaminados por *P. verrucosum* y 14 días en los quesos contaminados por *P. solitum*. Al equiparar los datos del control del separador V2 para descartar la reducción de oxígeno como agente determinante en la actividad fungistática se podía apreciar que el tratamiento V2 incrementaba la vida útil 4, 5 y 10 días en los quesos contaminados por *P. commune*, *P. verrucosum* y *P. solitum*. Tanto el control como el tratamiento alargaron la vida útil de los quesos contra los hongos una media de 11 días.

En la acción fungistática de los separadores estudiados, tanto de los tratamientos como del control, hay dos factores importantes interviniendo. El primero de ellos, ya anteriormente comentado, es la disminución en la presión de oxígeno. Los hongos del género *Penicillium* son microorganismos aerobios y, por ende, una reducción en la presencia de oxígeno conseguida por el uso de plástico en el separador es un factor a tener en cuenta (Pitt & Hocking, 2022). En segundo lugar, es conocido que los pH bajos, como los presentes en el recubrimiento añadido al separador, son capaces de retrasar el crecimiento de diversos hongos (Pitt & Hocking, 2022). No obstante, el incremento de la vida útil entre los separadores control y los tratamientos sí que se puede adjudicar a los metabolitos presentes en el suero fermentado por las BAL. La literatura asocia al género *Penicillium* como la principal causa de pérdidas de quesos por contaminación microbiana, tanto en Europa como en el resto del mundo. *Penicillium* spp. es el microrganismo más común en este tipo de alimentos (Lund et al., 1995; Ramos-Pereira et al., 2021).

Se ha probado otro tipo de ensayos en la literatura con objetivos similares. Entre los compuestos el que más se ha empleado es la natamicina. Estudios como Fajardo et al. (2010) evidencian un retraso en el crecimiento de *Penicillium* spp. de 14 días. Otros componentes han sido analizados con el mismo fin como el empleo de quitosano, aceites esenciales extraídos de nuez y cinamaldehído (compuesto orgánico que proporción el olor reconocido como canela). En estos ensayos se han obtenido incrementos de la vida útil de queso iguales o inferiores a los encontrados en esta tesis (Balaguer et al., 2013; Kõrge et al., 2020; Ortiz de Elguea-Culebras et al., 2019). Sin embargo, cabe destacar que la extracción de estos componentes y purificados implica una serie de pasos que complican el proceso en comparación a la preparación del tratamiento empleado en este estudio, lo que supone una ventaja en su uso.

4.3.2. Empleo del suero de leche fermentado en pan

4.3.2.1. Efecto del suero de leche fermentado en las cualidades tecnológicas de la masa del pan

En este estudio se buscaba probar suero lácteo fermentado por BAL como agente para la conservación de pan. Se pretendía hacer un producto con una mayor resistencia a la contaminación por hongos, pero que mantuviera las cualidades esperadas en un pan. Para ello, se evaluaron estas cualidades en comparación a un pan común. En el presente trabajo se prepararon tres panes con el ingrediente de suero lácteo fermentado (SLF) al 1%, 5% y 10%. Estos se compararon con un control de pan, un pan control con sal y un control de pan con propionato a las concentraciones más altas permitidas por la legislación (**Punto 3.3. Table 1** y **Punto 3.3. Table 2**).

En el estudio de la medida del pH y de la acidez titulable, se observó que los panes con un 1%, 5% y 10% del SLF, que, de forma previa a la fermentación de 1 h, el pH era significativamente inferior al de las muestras, pero esa diferencia desaparecía tras la fermentación. El valor de la acidez titulable también se veía afectado por la fermentación. Durante todo el proceso, los valores de las muestras fueron superiores a los presentes en el control. Se encontraron valores equivalentes de ácido láctico inferiores a los 5 g/L en los controles y superior a 7 g/L en los tratamientos. No se observaron diferencias significativas entre la adición de un 1% y 5% de ingrediente para estas dos medidas. Mientras que en el pan con un 10% de SLF este valor alcanzaba los 20 g/L. Estos valores de acidez y pH presentados en las muestras no son un factor negativo, de hecho, la mayor acidez está relacionada con una mayor resistencia a la contaminación del alimento y, además, estos ligeros cambios en la acidez están relacionados con una mayor aceptación por parte del consumidor

El recuento de las unidades formadoras totales y de la población de levaduras totales evidenció unas concentraciones similares entre los tratamientos a 1%, 5% y los controles, que oscilaban en torno a los 8.5 a 8.7 Log_{10} unidades formadoras de colonias por gramo de alimento (UFC/g). En el pan al 10% este valor bajó hasta las 6.5 Log_{10} UFC/g tras la fermentación. A esas concentraciones el ingrediente afectaba de forma negativa a la población de bacterias, lo que seguramente fue uno de los factores que impidió el crecimiento de la masa. La similitud entre recuento de los microorganismos presentes durante la fermentación de los tratamientos y el control es un resultado positivo ya que el ingrediente a base de SLF se desea que inhiba el crecimiento de hongos, pero no ha de afectar a la microbiota involucrada en la fermentación (Black et al., 2013).

El último valor que se estudió fue la expansión del pan. El tratamiento con 1% de SLF logró incrementar su tamaño de forma significativa en comparación a los controles, mientras que en el tratamiento al 5% se mantuvo. Este incremento se puede asociar al aumento en la presencia

de azucares fermentables por las levaduras que aporta el ingrediente SLF que, a su vez, aumenta la producción de CO_2 (Steinkraus, 2009). Por su parte, el pan con un 10% de SLF no logró formar una masa esponjosa. Se considera que fue debido al factor anteriormente mencionado y también a la falta de gluten al sustituir la harina por el ingrediente. El gluten es uno de los agentes más importantes en la formación alveolar en el pan (Belderok, 2000).

4.3.2.2. Efecto del suero de leche fermentado en las cualidades tecnológicas del pan

Los resultados del análisis de estos parámetros en el pan se pueden apreciar en el **Punto 3.3. Table 3**. En el pan se continuó observando una tendencia similar a la ya vista en la masa respecto a la acidez y el pH. Los valores de pH fueron ligeramente menores en los panes con un 5% y 10% de SLF. Esto se veía reflejado en una mayor acidez titulable. Como cabía esperar por las altas temperaturas alcanzadas durante el horneado, los recuentos de las UFC/g de pan dieron valores por debajo de las 100 UFC/g (Al-Nasser et al., 2021).

De igual forma, el volumen especifico siguió una tendencia similar a la obtenida en el aumento de volumen. El pan control evidenció unos valores ligeramente superiores al resto de panes. Entre los otros dos controles y el pan tratamiento al 1% y 5% no se observaron diferencias y, por último, el pan con un 10% de SLF presentaba unos valores bajos en comparación al resto. La bibliografía describe como una mayor sustitución de la harina de trigo con otros ingredientes sin gluten supone una mayor bajada del volumen especifico del pan (Fatemeh et al., 2022; Honda et al., 2021). La actividad de agua de los panes no se vio afectada por los tratamientos oscilando en valores entre 0.72 a 0.78, valores a los que se ha observado que los hongos contaminantes comienzan a tener dificultades al crecer (Pitt & Hocking, 2022).

El análisis colorimétrico de la corteza del pan no reveló diferencias significativas en la luminosidad (L*), rojo (a*), amarillo (b*) e índice de pardeamiento (BI) entre los controles y las muestras. En la bibliografía se describe cómo la adición de ingredientes que no colaboran en reacciones de pardeamiento suele influir en una reducción de a*, b* y BI (Cirlincione et al., 2022). Mientras que la adición de azucares y proteínas que colaboran en la reacción de Maillard aumentan estos valores (Fatemeh et al., 2022). No obstante, parece que este tratamiento no produjo ningún cambio. Finalmente, el análisis de la superficie alveolar reveló un aumento significativo de la proporción de alveolos en los panes tratamiento al 5% en comparación al pan control con sal. No se observó diferencias respecto al resto de panes (exceptuando el 10% sobre el cual no se realizó esta medida). Atributos como una mayor o igual expansión, porosidad, volumen especifico y color similar como las apreciadas en los panes tratamiento al 1% y 5% son las esperadas por un consumidor cuando selecciona un producto (Morais et al., 2014).

4.3.2.3. Metabolitos antifúngicos presentes en el pan con suero de leche fermentado

La identificación y cuantificación de los principales metabolitos producidos por las LAB y presentes en los panes con el SLF y los controles se realizó con el objetivo de ver si estos compuestos se encontraban en los tratamientos tras el proceso de panificación (**Punto 3.3. Table 4**). De entre todos los estudiados, los detectados en mayor abundancia fueron: ácido láctico, PLA, ácido benzoico y ácido ferúlico. El ácido láctico solo fue encontrado en concentraciones detectables en los panes tratamiento. La concentración de este metabolito se redujo ligeramente a lo largo de las etapas de procesado de este alimento. El ácido láctico es el metabolito más producido por la fermentación por LAB, y como se esperaba, la adición de una mayor cantidad de SLF incrementaba la presencia de este metabolito (Holzapfel & Wood, 2014). La concentración de este metabolito fue de 1.04 a 1.40 g/Kg de alimento y de 5.52 a 6.56 g/Kg en los panes con un 1% y 5% de SLF, respectivamente. Respecto al PLA fue el único que se encontró en mayores concentraciones en los panes con mayor cantidad de SLF. Se puede observar en las tablas que durante el proceso de fermentación se aprecia una disminución en su contenido, no obstante, el proceso de horneado no afecta a su concentración respecto al peso del pan. En las masas con un 5% de SLF la presencia de este metabolito al inicio es de 7.86 mg/Kg. Mientras que en las dos siguientes etapas decrece hasta los 3.49 y 3.65 mg/Kg. En los controles se contempló concentraciones de PLA cercanas a cero. El estudio del ácido benzoico y ácido ferúlico determinó que estos metabolitos se encontraban a bajas concentraciones en comparación a los anteriormente mencionados y de forma más irregular.

Diversos autores evidencian el alto espectro de actividad antifúngica del ácido láctico y PLA (Dagnas et al., 2015; Rajanikar et al., 2021). El ácido láctico está aprobado por la Unión Europea como aditivo antifúngico y su empleo es reconocido como seguro (EFSA, 2023). Por su parte, el PLA es otro componente que se está evaluado sus posibilidades de uso como agente antifúngico debido a su alta actividad contra estos microorganismos ya que en estudios de toxicidad sobre animales y celulares no les afecta negativamente (J. P. Wang et al., 2009).

4.3.2.4. Empleo del suero de leche fermentado en pan como agente de bioconservación contra hongos

Para la evaluación de si el ingrediente de SLF conseguía alargar la vida útil contra hogos contaminantes de alimentos se contaminaron rebanadas de los panes con *A. flavus* y con *P. verrucosum* evaluando la eficacia de este agente durante un periodo de 7 días. Primero, se examinó visualmente el crecimiento del hongo de diariamente, y segundo, tras siete días de incubación, se realizó un recuento de las esporas por gramo de alimento presentes en los panes (**Punto 3.3. Table 5** y **Punto 3.3. Fig. 3**). Ambos tratamientos alargaron la vida útil de los panes

en comparación a los controles. En el estudio de los panes contaminados por *A. flavus*, la vida útil de los panes se logró alargar hasta el día 5 en el pan con un 1% de SLF y hasta el sexto día en los panes con un 5% de SLF. En los controles, se comenzó a observar el crecimiento de los hongos después de, tan solo, 3 días de incubación. En el caso de los panes inoculados con *P. verrucosum*, el crecimiento del hongo solo fue visible en los controles a partir del día 4, en los panes tratamiento con un 1% y 5% de SLF se observó tras los 5 y 7 días de incubación, respectivamente. Por lo tanto, el empleo del ingrediente tenía efectos sobre la vida útil del alimento de forma efectiva.

Para corroborar estos resultados se realizó el recuento de las esporas presentes en cada pan después de los 7 días de incubación. Tras los 7 días no se apreciaban diferencias en los panes contaminados por *A. flavus*. En las pruebas realizadas sobre los panes contaminados por *P. verrucosum* sí que se contempló diferencias estadísticamente significativas entre el tratamiento con un 5% de SLF y los controles. Se podía observar una reducción de 2.4 Log_{10} de esporas por gramo de alimento al comparar el tratamiento al 5% con el pan control con propionato.

El empleo del SLF por BAL es una aplicación novedosa que no se ha explorado mucho en la bibliografía. Algunos artículos como Luz et al. (2021) sí que evidencian el potencial como agente antifúngico de SLF de queso de búfala por *L. plantarum* y *L. ghanensis* en panes de pita contaminados por *P. expansum*. También, otros estudios hay autores que han utilizado el suero lácteo para la preparación de iniciadores para panes de masa madre seleccionado BAL que ayudaban a la conservación del producto y del pan producido con esa masa contra la contaminación fúngica (Gerez et al., 2009). La eficacia del ingrediente de SLF es bastante interesante por dos razones. Primera, en la legislación española y de otros países de la Unión Europea se aprueba el empleo de productos lácteos como ingrediente al pan (BOE, 2019). Segundo, como ya se ha mencionado anteriormente, el empleo y adición de bacterias ácido lácticas en alimentos está aprobado como QPS y GRAS en Europa y los Estados Unidos y esto puede significar la menor adición de distintos conservantes de origen químico, de los cuales se busca reducir su empleo (EFSA, 2024; Ryan et al., 2015).

4.3.3. Empleo de bagazo de cerveza fermentado en pan

4.3.3.1. Efecto del bagazo de cerveza fermentado en las cualidades tecnológicas de la masa del pan y en el pan

El ingrediente seleccionado para el estudio tras las primeras pruebas de selección fue el medio de cultivo con BB al 20% fermentado por la cepa *L. plantarum* L1. Se estudió las diferentes cualidades tecnológicas de los panes con el ingrediente BB al 5% y 10% en comparación con panes control: control, control propionato, control BB sin fermentar al 5% y control BB sin fermentar al 10% (**Punto 3.4. Table 3**). Los resultados evidenciaron que la adición del BB (fermentado y no fermentado) reducía ligeramente la actividad de agua y la expansión de volumen de los panes en comparación a los controles. La humedad de los panes con un 10% de BB era ligeramente menor que en los panes control sin BB, no obstante, esta diferencia de valor estaba por debajo del 2%. Por lo tanto, se puede considerar un cambio irrelevante. Sin embargo, la retención de agua durante el proceso de horneado fue mayor en los panes con BB. Como cabía esperar por los resultados de expansión de volumen los panes con el ingrediente BB también presentaban un menor volumen específico.

Las bajadas ligeras en el contenido de agua y la actividad de agua no son los suficientemente grandes como para implicar un desagrado hacia el producto por parte del consumidor (Genevois & de Escalada Pla, 2021). Por otro lado, la substitución de harina de trigo por otros ingredientes siempre afecta de forma negativa a valores como la expansión de volumen o el volumen especifico. Parte del objetivo en este tipo de estudios donde se prueban nuevos ingredientes siempre es que esta reducción sea lo más leve posible, igual que en los resultados obtenidos con el BB (Genevois & de Escalada Pla, 2021).

El color de la corteza, la porosidad y el recuento de la microbiota total a lo largo de la producción del pan no presentó diferencias significativas entre todos los panes. El consumidor espera que entre los panes comunes y otros tipos de panes como los integrales, no haya una gran diferencia de color en la corteza (Ramírez-Jiménez et al., 2000). Por otro lado, que la porosidad y el recuento de la microbiota sean semejantes es un buen indicador de que el tratamiento que se está estudiando no afecta de forma negativa al crecimiento de las levaduras y, por ende, al proceso de pacificación (Bayrock & Ingledew, 2004).

4.3.3.2. Metabolitos antifúngicos presentes en el pan con suero de leche fermentado

La preparación del ingrediente de BB a partir del medio de cultivo fermentado implicó un secado mediante liofilización que pudo afectar al contenido de compuestos antifúngicos presentes en el ingrediente. Por lo tanto, el primero paso fue realizar una evaluación de los metabolitos presentes en el ingrediente a base de BB fermentado y la comparación con los controles (**Punto 3.4. Table 4**). Los resultados evidenciaron que el ingrediente control tenía un pH que rondaba valores de 6.1 y se apreciaba una completa ausencia de ácido láctico y PLA. La ausencia de estos metabolitos era esperable ya que no se había sometido el ingrediente control a una fermentación con BAL. Por su parte, en el ingrediente fermentado los valores de pH bajaban de forma significativa hasta 3.8 y la cuantificación del ácido láctico y PLA evidenció concentraciones respectivas de 91.0 g/Kg y 135.5 mg/Kg.

Este estudio se repitió sobre las masas y los panes, los datos resultantes se pueden apreciar en el **Punto 3.4. Table 5**. En el estudio de las masas y de los panes se pudo determinar que el pH de los panes con ingrediente BB era significativamente menor a lo largo de todo el procesado. Durante la fermentación estos valores se mantenían estables y, solo tras el horneado, este valor incrementaba. Lo más probable es que este incremento se debiera a la perdida de agua durante el horneado (Onacik-Gür et al., 2022). El ácido láctico solo se encontró en las muestras con el BB fermentado. Su concentración no sufrió grandes variación a lo largo del procesado, lo cual indica que este metabolito resiste el proceso de fermentación y no se degrada ni evapora durante el horneado. Su concentración se mantuvo en medias de 4.2 g/Kg de alimento en los panes con un 5% de ingrediente y de 8.1 en los que contenían un 10% del ingrediente. Se observó una tendencia similar a la encontrada por el primer ácido al estudiar el PLA. De nuevo solo se pudo detectar este metabolito en las muestras fermentadas, su concentración se mantenía en las mismas unidades a lo largo del procesado del pan y se encontraba de forma más abundante cuando se empleaban concentraciones mayores del ingrediente.

La bajada del pH en los panes tratamiento es positiva no solo por el hecho evidente de que es eficaz para incrementar la vida útil en la contaminación contra diversos microorganismos (Gagiu et al., 2013). Además, estos sabores ácidos asociados normalmente a los metabolitos causantes de la bajada en el pH son altamente apreciados por el consumidor, ya que recuerdan a aromas típicos de los panes de masa madre (Canesin & Cazarin, 2021). La presencia de ácido láctico y PLA en estas concentraciones también es un buen indicador de la posible actividad contra el crecimiento de los hongos en pan. La presencia de estos dos ácidos normalmente está relacionada con una acción supresora de crecimiento de hongos tanto de forma individual como en sinergia con otros compuestos producidos por las BAL (Guimarães et al., 2018; Magnusson & Schnürer, 2001; Rajanikar et al., 2021).

4.3.3.3. Empleo de bagazo de cerveza fermentado en pan como agente de bioconservación contra hongos

Para la evaluación de la actividad antifúngica de los ingredientes a base de BB fermentado en panes, se estudió el crecimiento de *A. flavus* y *P. commune* a lo largo de 7 días de incubación (**Punto 3.4. Table 6**). Los panes fueron inoculados en diferentes puntos y se evaluó diariamente el número de puntos donde se apreciaba el crecimiento de cada hongo. En las pruebas realizadas con panes contaminados por *A. flavus* se observó como el hongo no comenzó a crecer hasta el día 4 en el control, control BB 5%, control BB 10%, y tratamiento BB 5%. En el pan con un 10% de BB fermentado no creció hasta el día 5. En el control propionato no se contempló crecimiento hasta el día 6. Cabe mencionar que a día 4, pese a apreciarse crecimiento fúngico en el pan con un 5%, solo se detectaba en un 60% de los puntos de inoculación, mientras que en el control eran en el 100%. Respecto a las pruebas contra *P. commune*, exceptuando el control con propionato que alargo la vida útil hasta el día 5, en el resto de los panes se apreció el crecimiento del hongo tras tres días de incubación. Sin embargo, se observaba que en el tratamiento con BB al 10% solo crecían hongos en un 25% de los puntos de inoculación mientras que en el control superaba el 75%.

Al revisar estos datos se ha de recordar que en la naturaleza las contaminaciones por hongo suelen ocurrir a concentraciones de esporas en torno a 3 o 4 unidades logarítmicas más bajas a las presentes en este estudio. Como se pretendía evaluar la eficacia del tratamiento se aceleró aumentando la concentración a la que se contaminaron las muestras (Pitt & Hocking, 2022). El uso de conservantes de origen químico se está relacionado con problemas con la salud y se buscan nuevas alternativas a su empleo en alimentos (Mohammadzadeh-Aghdash et al., 2019). Como el empleo de las BAL, que en esta tesis han sido utilizadas de forma novedosa para la obtención de un ingrediente a base de bagazo de cerveza fermentado, por lo tanto, por sus características entraría dentro de los ingredientes que se podría añadir a un pan aprobados por la Unión Europea (EFSA, 2024). Entre los pocos reportes encontrados en la literatura donde se emplea de forma similar el BB se puede citar la fermentación con *Metarhizium anisopliae* con el objetivo de purificar enzimas con actividad contra la pared de hongos (Aita et al., 2019).

4.3.4. Empleo de salvado de arroz fermentado en pan

4.3.4.1. Efecto del salvado de arroz fermentado en las cualidades tecnológicas de la masa del pan y el pan

De igual forma que en los anteriores panes, se observó como la adición de un nuevo ingrediente afectaba a las cualidades del pan (**Punto 3.5. Table S3**). En este caso tras las pruebas *in vitro* anteriores se seleccionó como ingrediente el medio de cultivo con un 20% de RB fermentado por la cepa *L. plantarum* H1. El estudio del pH revelo como en los panes control se apreciaban valores de pH superiores a 5.00 durante toda la preparación, mientras que en los panes con un 10%, el ingrediente RB fermentado mostraba valores que rondaban los 4.40 de media y en los panes con un 20% bajaba a un 4.13 de media. No hubo grandes diferencias a lo largo del proceso de panificado. Los valores de la acidez titulable correspondían con los resultados observados con el pH. Cuanto mayor cantidad de ingrediente RB fermentado, mayor acidez presentaban los panes. Mientras que en el pan tratamiento se encontraban valores de acidez equivalentes a los 27.5, 26.5 y 20.3 g equivalentes de ácido láctico por Kg de alimento respectivamente en la masa, masa fermentada y pan, este valor disminuyó a 8.7, 6.1 y 3.2 en el pan control. Este valor no se vio afectado por el proceso de fermentación, pero sí que el horneado resultó en una bajada de esta unidad con respecto al anterior punto en el procesado. Otra vez se encontró un resultado semejante al observado en los anteriores estudios realizados en esta tesis. De forma esperada y deseada, los panes con el ingrediente fermentado presentaban una mayor acidez que es de interés por la aparición de nuevos aromas y sabores además de ayudar en la actividad contra el crecimiento de hongos.

En el estudio de la expansión de volumen se apreciaba como la sustitución de harina por el ingrediente reducía este parámetro ligeramente ya que la sustitución de la harina por el ingrediente afectaba a la formación de alveolos (Barros et al., 2022). Y consecuentemente esto se vio reflejado también en el volumen específico, donde, de nuevo podía verse como los panes con tratamiento presentaban unos valores inferiores.

Finalmente, los dos panes tratamiento presentaron Aw significativamente menores al resto de controles, presentado Aw por debajo de 0.83, mientras que en el resto de los controles este paramento superaba los 0.95. La Aw está normalmente relacionada con el pH de la matriz, los pH bajos suelen estar relacionados con una menor Aw (Bell & Labuza, 1994). En el estudio de la humedad de los panes se observó una mayor humedad en el control con un 10% de ingrediente y en el tratamiento con un 10% en comparación al pan control y al pan control propionato. No se apreciaron diferencias entre el resto de las muestras.

4.3.4.2. Metabolitos antifúngicos presentes en el pan con suero de leche fermentado

Tras la preparación del ingrediente fermentado mediante liofilización del medio de cultivo se estudió y cuantificó los metabolitos antifúngicos presentes en los mismos (**Punto 3.5. Table 1**). El pH en el ingrediente control rondaba valores de 6.2 mientras que en el ingrediente fermentado el valor se reducía hasta 3.7. En el control no se detectaron cantidades cuantificables ni de ácido láctico y PLA, mientras que en el tratamiento la concentración se elevaba a 103.4 g/Kg y 182.3 mg/Kg de ingrediente, respectivamente.

Los resultados de estos análisis en el pan se pueden apreciar en el **Punto 3.5. Table 2**. Como resultado de esto, el ácido láctico solo fue detectado en los panes que incorporaban el ingrediente a base de RB fermentado. No se detectó este metabolito en ninguno de los controles. La concentración a la que se detectó el ácido láctico en el pan con 10% de ingrediente fermentado fue de media 9.93 g/Kg de alimento y de PLA sobre los 0.29 mg/Kg. Los valores de estos mismos metabolitos incrementaban en el pan con un 20% de ingrediente fermentado hasta los 16.0 g/Kg de ácido láctico y 1.00 mg/Kg de PLA. Ambos metabolitos evidenciaron una gran estabilidad al proceso de preparación de los panes. La posibilidad de utilizar este tipo de ingredientes para el control de hongos reduciendo de esta forma el uso de aditivos por petición del consumidor es de alto interés en la industria. El ácido láctico es un aditivo aprobado y su consumo es seguro en humanos, no obstante, su aparición en el etiquetado del alimento puede generar rechazo. La sustitución de estos aditivos por ingredientes, donde el conservante se encuentra de forma natural, con los que el consumidor pueda sentirse tranquilo ayuda a la aceptación de los alimentos. Ejemplo de esto es cuando se etiqueta la acción de zumo de limón por su alto contenido en ácido cítrico en vez de usar directamente el aditivo y tener que añadir a la etiqueta el uso de E-330, que es el propio ácido cítrico (Rangel-Vargas et al., 2021).

4.3.4.3. Empleo de bagazo de cerveza fermentado en pan como agente de bioconservación contra hongos

Los resultados de como el ingrediente fermentado afectaba a la vida útil de panes se estudiaron a lo largo de 7 días (**Punto 3.5. Table 3**). En el último día de evaluación, se realizó un recuento de las esporas por gramo de alimento. En los panes contaminados por *A. flavus* los panes tratamiento lograban retrasar la aparición del hongo un día, se apreció el crecimiento del hongo tras 5 días, respecto al control y a los controles con el ingrediente RB sin fermentar, después de 4 días de incubación. Y solo un día menos respecto al control con propionato donde se comenzó a observar el crecimiento a partir del día 6. También se debe mencionar que al quinto día de incubación en el pan con RB fermentado al 20%, el número de puntos de inoculación donde creció el hongo era inferior al 50%.

En el estudio de los panes inoculados con *P. commune* el crecimiento del hongo se observó tras 3 días de incubación en los panes control, en los panes con un 10% de RB control y el tratamiento con un 10% de RB fermentado. Después de 4 días se detectó crecimiento del hongo en el control con un 20% de RB. Y después de 5 días de incubación en el control propionato y el pan tratamiento con un 20% de RB fermentado. Se puede observar que en este caso el tratamiento lograba unos resultados similares a los observados en el control con aditivo antifúngico.

Con el objetivo de realizar un análisis más exhaustivo, se hizo el recuento de las esporas de hongo por gramo pan (**Punto 3.5. Table 4**). El tratamiento con un 20% de RB fermentado y el control propionato fueron los panes que demostraron de forma significativa una mayor reducción del crecimiento de ambos hongos. El tratamiento al 20% de RB fermentado presentaba unas concentraciones de hongo de 4.7 y 4.4 Log_{10} de esporas por gramos de aliento en los panes contaminados por *A. flavus* y *P. commune*, respectivamente. Mientras que en el control estos mismos hongos alcanzaron poblaciones de 5.4 y 5.2 Log_{10} de esporas por gramo. Estos resultados coincidían con los obtenidos en el estudio de la vida útil.

La investigación sobre nuevas técnicas de conservación de alimentos es altamente respaldada por las autoridades y órganos reguladores del área de la alimentación (Nasrollahzadeh et al., 2022). Entre todas estas nuevas estrategias, el empleo de las BAL en bioconservación es una de las que mayor interés presenta gracias su consideración como GRAS y QPS (Schmidt et al., 2019). Además, el empleo de estos microorganismos para la revalorización de residuos de la industria no solo lleva nuevas vías para la conservación de alimentos, sino que abre las puertas a la incorporación de procesos dentro del marco de la economía circular al que se pretende llegar mundialmente para el horizonte 2030 (United Nations, 2015b). No existe en la literatura actualmente más ejemplos de empleos semejantes de las BAL con el RB. Sí que se han realizado pruebas con otros microorganismos como la producción de un extracto fenólico de RB fermentado por *Rhizopus oryzae* para la bioconservación de panes contra *Penicillium* spp. o también la fermentación de este residuo con *Rhizosphere streptomyces* para el control de *Fusarium* spp. a nivel de campo (Denardi-Souza et al., 2018; Sari et al., 2021).

4.4. Reducción de micotoxinas en pan

4.4.1. Reducción de micotoxinas en pan con el ingrediente de bagazo de cerveza fermentado

El perfil de micotoxinas producidas por los hongos en los panes con bagazo de cerveza fermentado fue analizado (**Punto 3.4. Fig 2**). Este ensayo no se realizó con *P. commune* debido a que no producía ningún metabolito toxico regulado. Las micotoxinas estudiadas fueron la aflatoxina B1, B2 G1 y G2, las que frecuentemente se monitorizan en los alimentos contaminados

por *A. flavus* (Romero-Sánchez et al., 2022). En los resultados del análisis se aprecia como la aflatoxina B1 y G2 fueron detectadas en todas las muestras. El pan control fue la única muestra donde se encontrar las 4 micotoxinas a concentraciones detectables. El pan tratamiento 10% de BB fermentado evidenció unos resultados muy positivos al mostrar reducciones de la aparición de micotoxinas semejantes a las obtenidas con el control propionato. En estos dos panes se apreciaba una total ausencia de aflatoxinas B2 y G1 además de presentar una alta reducción de la presencia de aflatoxina B1 respecto al pan control. Los panes control 5%, control 10% y tratamiento 10% también lograron reducir la aparición total de aflatoxinas más de un 60% respecto al control. El control con propionato fue el tratamiento más efectivo al reducir la aparición total de micotoxinas en un 98%, seguido por el pan tratamiento al 10% de BB donde la reducción llegó a ser del 86%. En la comparación del tratamiento 10% con su correspondiente control se observa una reducción significativa de la concentración de aflatoxina B1 de más de 100 µg/Kg, por lo tanto, se puede considerar que la fermentación por BAL incrementa la bioactividad del ingrediente contra la producción de toxinas fúngicas.

Los resultados demuestran que la adición del BB incluso sin fermentar también presentaba actividad contra la producción de micotoxinas. Esto se puede deber a los cambios asociados en la composición del propio alimento, al sustituir la harina por otro ingrediente (Pitt & Hocking, 2022). O a que el propio bagazo de cerveza aporta metabolitos que intervienen es esta actividad. Las plantas, como el arroz, de forma similar, se ven afectadas por la contaminación con hongos y han desarrollado una serie de mecanismos de defensa contra estos microrganismos (S. Wang et al., 2009).

Tras una revisión de la literatura en no se han encontrado aplicaciones iguales a las estudiadas en este trabajo. Pero sí que se observan en la literatura el uso de otras técnicas de bioconservación para controlar la aparición y presencia de micotoxinas en el pan. Entre los ejemplos se puede apreciar la selección de cepas específicas de *Pediococcus pentosaceus* y *Saccharomyces cerevisiae* que reducían ligeramente la concentración de aflatoxina en panes producidos con harinas contaminadas (Jin et al., 2021). O la detección y aislamiento de un péptido producido por una cepa de *P. pentosaceus* que lograba disminuir la presencia de aflatoxina hasta un 85% (Ebrahimi et al., 2020).

4.4.2. Reducción de micotoxinas en pan con el ingrediente de salvado de arroz fermentado

De forma similar se estudió la aparición de micotoxinas en los panes con el ingrediente de RB fermentado y contaminados por *A. flavus* (**Punto 3.5. Table 5**). En este caso la aflatoxina B1 fue la única detectada en todas las muestras, alcanzado concentraciones de 615 µg/Kg en el pan control, de 8 µg/Kg en el control propionato y de 12 µg/Kg en el tratamiento. La aflatoxina B2 fue el segundo metabolito encontrado en mayor cantidad, solo fue observado en el pan control a concentraciones de 92 µg/Kg y en el control con un 10% de ingrediente a concentraciones de 14 µg/Kg.

Al hacer la comparación del pan control con el pan control con un 10% de ingrediente se observó una reducción del 74% de aflatoxina B1. Cuando se comparaba el control con el resto de los panes se observó que esta reducción superaba el 93%. No se observó diferencias estadísticamente significativas en la concentración de micotoxinas entre el pan control propionato, el pan control con RB al 20% y ambos panes tratamiento.

Basándonos en estos resultados se puede considerar que la fermentación en este caso tampoco era el único factor interviniendo en la metabolización de las toxinas. Ya sea debido al cambio en la composición de harinas, la presencia de metabolitos que alteran la producción de metabolitos por parte de los hongos, o incluso la presencia de antinutrienes como quelantes que dificultarían la metabolización de los nutrientes del pan por parte del hongo (Pitt & Hocking, 2022). Este tipo de compuestos son muy abundantes en cereales como el arroz (López-Moreno et al., 2022).

Aun así, la fermentación con las BAL sigue siendo un factor clave en la actividad. Al comparar la aparición de aflatoxina B1 en el pan control 10% con el pan tratamiento al 10% se apreciaba que en el primero había una concentración de 157 µg/Kg, mientras que en tratamiento era de 12 µg/Kg. Es decir, una reducción del 92%. Muchos autores han señalado la relación entre la fermentación con BAL y la reducción de la aparición de aflatoxinas en pan mediante la adición de diversos ingredientes como masa madre o diferentes extractos (De Koe & Juodeikiene, 2012; Illueca et al., 2023). Diversos estudios *in vitro* evidencian esta actividad por parte de las BAL como en Rämö et al. (2022) donde varias cepas de BAL lograron reducir la aparición de aflatoxina hasta un 90% en extractos que simulan zumos de fruta.

5. CONCLUSIONS

CONCLUSIONES

5. CONCLUSIONES

1. El suero lácteo fermentado por las BAL presentó actividad antifúngica contra hongos de los géneros *Alternaria*, *Aspergillus*, *Botrytis*, *Fusarium* y *Penicillium* en las pruebas *in vitro*. Siendo las cepas *L. plantarum* BN17 y *L. plantarum* SC3 las que mejores resultados evidenciaron, alcanzando concentraciones con actividad fungicida de hasta 13 g/L contra los hongos más sensibles al tratamiento.

2. En la comparación con ensayos de la actividad antifúngica entre distintos medios de cultivo a base de suero de leche y el MRS los resultados indicaban que el suero lácteo fermentado tenía una mayor actividad antifúngica que el MRS fermentado. El enriquecimiento de los medios de cultivo a base de suero lácteo incrementaba la actividad antifúngica tras la fermentación con BAL.

3. El empleo de bagazo de cerveza fermentado por BAL fue efectivo inhibiendo el crecimiento de *Aspergillus flavus*, *Penicillium commune*, y *Alternaria alternata*. La cepa *L. plantarum* L1 evidenció los mejores resultados en estos ensayos.

4. El medio de cultivo a base de salvado de arroz presentó actividad en la fermentación con BAL frente a *Aspergillus flavus*, *Penicillium commune* y *Alternaria alternata*. Entre las cepas estudiadas *L. plantarum* H1 presentó la mayor actividad antifúngica contra estos hongos.

5. El estudio del perfil de metabolitos antifúngicos presentes en los medios de cultivo fermentados por BAL a base de suero lácteo, bagazo de cerveza y salvado de arroz evidenció un decrecimiento en el pH con respecto a los controles. Esta reducción del pH se debía a la aparición de metabolitos antifúngicos como ácido láctico y el PLA. Los medios de cultivo a base de salvado de arroz fueron donde se observaron las mayores concentraciones de estos metabolitos.

6. El desarrollo de un separador de lonchas de queso con un recubrimiento a base de suero lácteo fermentado logró alargar la vida útil de lonchas contaminadas por *P. commune, P. verrucosum y P. solitum* una media de 20 días, en comparación a un queso sin separador. Y una media de 6 días en comparación a un separador control.

7. Se determinó cómo la adición de un ingrediente bioactivo a base de suero lácteo, bagazo de cerveza y salvado de arroz fermentado afectaba a las diferentes cualidades tecnológicas del pan. Los resultados evidenciaban que los panes con el ingrediente activo presentaban una menor acidez, pero la adición del ingrediente no afectaba de forma drástica al color y ni al proceso de panificación con respecto a panes control.

8. El análisis y cuantificación de metabolitos antifúngicos detectados en los ingredientes y en el pan evidenció como la fermentación y horneado no tenían efectos drásticos sobre a la presencia de ácido láctico y el PLA.

9. La adición del ingrediente a base de suero fermentado al 5% lograba alargar la vida útil de panes contaminados por *A. flavus y P. verrucosum* hasta un total de 6 días. Tres días más que los panes control con propionato de calcio. Tras 7 días de incubación, el recuento de esporas de *P. verrucosum* todavía era 2.4 Log_{10} de esporas por gramos de alimento en comparación a los controles.

10. El ingrediente a base de bagazo de cerveza fermentado a un 10% lograba alargar la vida útil del pan hasta 5 días en los panes contaminados por *A. flavus* y 3 días en los panes contaminados por *P. commune*. En la comparación de estos resultados con los panes control propionato se apreciaba que el control propionato solo alargaba la vida útil un día más con respecto al tratamiento.

11. El uso de salvado de arroz fermentado como ingrediente activo en pan una concentración del 20% evitó el crecimiento de *A. flavus* hasta 5 días, uno menos que el control propionato. Y en el estudio de la conservación contra la contaminación por *P. commune* la aparición del hongo se retrasó hasta el quinto día de incubación en el tratamiento con salvado al 20% y el control propionato. En el recuento de las esporas tras 7 días de incubación se apreció que tanto el tratamiento con salvado al 20% y el control propionato presentaban una reducción significativa de las esporas por gramos de pan en comparación al pan control.

12. Los ingredientes fermentados a base de bagazo de cerveza y salvado de arroz reducían de forma significativa la aparición de micotoxinas en panes contaminados por *A. flavus*. La reducción evidenciada en comparación al pan control por el bagazo de cerveza fue de un 86% y del 74% con salvado de arroz, valores que se acercaban a la obtenida con el control propionato, de un 95%.

Conclusión final

El empleo de residuos de la industria alimentaria como medio de cultivo para la fermentación de BAL puede utilizarse para la preparación de ingredientes o agentes con actividad ante el control de hongos y toxinas en los alimentos de forma innovadora. Esta vía de revalorización de subproductos es un desarrollo dentro de la economía circular, ya que emplea residuos para darles un nuevo valor dentro de la industria alimentaria. En segundo lugar, el desarrollo de estos ingredientes ofrece alternativas al empleo de los conservantes alimentarios que se utilizan de forma común y que las autoridades instan a reducir su consumo.

Todos los procedimientos de preparación de los ingredientes y agentes antifúngicos elaborados en esta tesis son simples y fácilmente escalables a procesos industriales. Esto abre la puerta a su futura aplicación en productos que lleguen al mercado.

5. CONCLUSIONS

1. The milk whey fermented by LAB evidenced antifungal activity against fungi of the genera *Alternaria*, *Aspergillus*, *Botrytis*, *Fusarium* and *Penicillium* in *in vitro* tests. The *L. plantarum* BN17 and *L. plantarum* SC3 strains showed the best results, reaching concentrations with fungicidal activity of up to 13 g/L against the fungi most sensitive to the treatment.

2. When comparing the antifungal test from different milk whey-based culture media and the MRS the results showed that fermented whey had greater antifungal activity than fermented MRS. The complementation of the culture medium whey-based increased the antifungal activity after fermentation with LAB.

3. The use of beer bagasse fermented by LAB was effective in inhibiting the growth of *Aspergillus flavus*, *Penicillium commune*, and *Alternaria alternata*. The *L. plantarum* L1 strain showed the best results in these tests.

4. The culture medium based on rice bran fermented by LAB showed activity against *Aspergillus flavus*, *Penicillium commune* and *Alternaria alternata*. Among the strains studied, *L. plantarum* H1 presented the highest antifungal activity against these fungi.

5. The study of the profile of antifungal metabolites present in the culture media fermented by LAB based on whey, beer bagasse and rice bran showed a decrease in pH with respect to the controls. This drop in pH was due to the appearance of antifungal metabolites such as lactic acid and phenyllactic acid. The highest occurrence of these metabolites was detected in the rice-bran based medium.

6. The development of film with a coating incorporating fermented whey managed to extend the shelf life of cheese slices contaminated by *P. commune*, *P. verrucosum* and *P. solitum* by an average of 20 days, compared to a no film control. And an average of 6 days compared to a control film.

7. The addition of a bioactive ingredient based on whey, beer bagasse and fermented rice bran affected the different technological qualities of the bread showed that the breads with the active ingredient had lower acidity, but the addition of the ingredient did not drastically affect the color or the baking process when compared to control bread.

8. The analysis and quantification of antifungal metabolites detected in the ingredients and in the bread showed how fermentation and baking did not drastically changed the concentrations of lactic acid and phenyllactic acid.

9. The addition of the ingredient based on 5% fermented whey managed to extend the shelf life of breads contaminated by *A. flavus* and *P. verrucosu*m up to a total of 6. Three days longer than control breads with calcium propionate. After 7 days of incubation the spore count of *P. verrucosum* was still 2.4 Log_{10} spores per gram of food compared to the controls.

10. The beer bagasse ingredient used at a 10% concentration was able to extend the shelf life of bread by up to 5 days in bread contaminated by A. flavus and 3 days in bread contaminated by *P. commune*. In the comparison of these results with the propionate control breads, it was seen that the propionate control only extended the shelf life one more day compared to this treatment.

11. The use of fermented rice bran as an active ingredient in bread at a concentration of 20% prevented the growth of *A. flavus* for up to 5 days, one less than the propionate control. And in the study of conservation against contamination by *P. commune*, the appearance of the fungus was delayed until 5 days of incubation in the treatment with 20% bran and the propionate control. The spore count after 7 days of incubation showed that both the 20% bran treatment and the propionate control presented a significant reduction in spores per gram of bread in comparison to the control bread.

12. In the study of the capacity of fermented ingredients based on beer bagasse and rice bran to reduce the appearance of mycotoxins in bread contaminated by *A. flavus*, the studied treatments showed a significant reduction in aflatoxin occurrence. When comparing the results to the control, the aflatoxin reduction obtained by the bread with beer bagasse was 86% and 74% with rice bran, values that were close to that obtained with the propionate control, 95%.

Final conclusion

Byproducts from the food industry as a culture medium for the fermentation of LAB can be used for the preparation of ingredients or agents with activity against fungi and toxins in food in novel ways. This revalorization of food residue is a development within the circular economy, since it uses waste to give them a new value within the food industry. Secondly, the development of these ingredients also offers alternatives to the use of food preservatives that are commonly used and that authorities urge to reduce.

All the preparation procedures for the ingredients and antifungal agents developed in this Thesis are simple and easily scalable to industrial processes. This opens the door to its future application in products that reach the market.

6. REFERENCES

REFERENCIAS

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ANNEX 1: ASEAN MAXIMUM USE LEVELS OF FOOD ADDITIVES, (2021). (testimony of Aesan)

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ANNEX

Anexo

International Journal of Dairy Technology

SDT Society of Dairy Technology

doi: 10.1111/1471-0307.12847

RESEARCH ARTICLE

Antifungal properties of whey fermented by lactic acid bacteria in films for the preservation of cheese slices

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Fungal deterioration by Penicillium *species in refrigerated storage conditions is one of the main risk factors for health issues and food waste in the dairy industry. The purpose of this study was to evaluate the antifungal properties of a plastic film carrying whey fermented by lactic acid bacteria (LAB). The results evidenced that a film containing whey fermented using lactic acid bacteria inhibited the growth of* Penicillium commune, Penicillium verrucosum *and* Penicillium solitum *for 23, 20 and 17 days, respectively.*

Keywords Lactic acid bacteria, Antifungal activity, Cheese, Edible films.

INTRODUCTION

Currently, food contamination by toxigenic fungi is one of the main risks in the food industry. The spoilage of food by these microorganisms not only deteriorates the food but may also release a series of secondary metabolites, called mycotoxins, that are abundant in raw foods such as cereals and nuts (Fazekas *et al.* 2002). Within the group of toxigenic fungi, those belonging to the genus *Penicillium* are one of the main producers of these metabolites. Mycotoxins can cause neurotoxic and nephrotoxic effects, lesions in the circulatory system, intestinal disorders, and neurological and genotoxic effects (Marin *et al.* 2013). Therefore, the preservation of food against these fungi is a major concern for the industry.

The use of synthetic antifungal compounds is the most common response from the industry to avoid fungal contamination. However, the use of these compounds has been related to pathologies. Additionally, there is an increase in the consumer doubts towards this type of food preservatives and a rising demand for 'healthier' foods (Hanafy *et al.* 2021). Therefore, new and safer methods are being studied to avoid fungal contamination of food.

The use of lactic acid bacteria (LAB) in the preservation against toxigenic fungi contamination is postulated as an alternative to these synthetic origin compounds. Furthermore, the addition of LAB to food is allowed by the European Union, since they are classified as 'Qualified Presumption of Safety' (QPS) by this entity (Leuschner *et al.* 2010). There are several examples in the bibliography of their application as preservatives against the toxigenic fungi and their toxins (Li *et al.* 2012; Mcnair *et al.* 2018; Ngolong Ngea *et al.* 2021): the application of *Lactiplantibacillus plantarum* strain to biopreserve grapes from *Aspergillus flavus* contamination (Lappa *et al.* 2018), or the screening of several LAB for the preservation of bread against *Penicillium* genera contamination (Valerio *et al.* 2009).

Whey is one of the main wastes in the dairy industry. Processing of this leftover is a concerning problem for this industry. Whey is mainly processed as residual water or used as fertiliser and livestock feed particularly in less developed countries (Verma and Subudhi 2021). According to the objectives established by the World Health Organization (WHO) in the goals of 2030 for sustainable development, new methods must be found for the reuse of industrial waste

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Films with lactic acid bacteria-fermented whey for cheese biopreservation.

Food Bioscience 53 (2023) 102586

Contents lists available at ScienceDirect

Food Bioscience

journal homepage: www.elsevier.com/locate/fbio

Revalorization by lactic acid bacterial fermentation of goat whey from cheese industry as a potential antifungal agent

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ARTICLE INFO

Keywords:
Industrial waste
Lactic acid bacteria
Revalorization
Antifungal
Food preservation

ABSTRACT

The industry is continuously searching for new methods to reduce the use of chemical preservatives while still produce safer foods for the consumer. Goat whey is a waste from the cheese industry which is primarily treated as a residue. It is rich in different nutrients and has the potential to be used as a matrix for lactic acid bacteria fermentation. This work aims to find a revalorization of whey for the production of a natural origin antifungal agent by a fermentation that can be used as food ingredient. First different whey culture mediums were developed and fermented. Quantification of different antifungal molecules in whey culture mediums in comparation to a fermentation of De Man, Rogosa and Sharpe (MRS) culture medium evidenced that the production of this antifungal metabolites was similar. The *in vitro* qualitative and quantitative assays evidenced that the whey-based mediums increased the antifungal activity of the CFS in comparation to the MRS. The fermented whey produced bigger inhibition halos (above 1 cm) in the agar diffusion test in comparation to the fermented MRS (bellow 0.6 cm). Alike, in the minimal fungicidal concentration test the lowest antifungal concentrations were detected on the fermented whey mediums, averaging antifungal concentrations lower than 130 g/L. Finally, a multivariable data analysis determined that more antifungal metabolites yet to be detected were affecting in the antifungal activity from the whey samples. The fermented whey has the potential to be a tool in reducing the use of chemical preservatives in foods.

1. Introduction

Contamination by toxigenic fungi is one of the main concerns for the food industry. These fungi not only deteriorate the food, also releases a serie of toxic secondary metabolites, called mycotoxins, that are abundant in raw foods such as cereals and nuts (Romero-Sánchez et al., 2022). Within the group of toxigenic fungi, those belonging to the genus *Aspergillus* and *Penicillium* are the main producers of the most common mycotoxins in foods, aflatoxins (AF), especially aflatoxin B1 (AFB1) and aflatoxin B2 (AFB2), and ochratoxin A (OTA). The consumption of these mycotoxins have been related to a wide spectrum of toxigenic effects. Carcinogenesis, genotoxicity and problems in the reproductive and immune system by the contamination of AF. In the case of OTA thse effect are neurotoxic, teratogenic and nephrotoxic effects among many others. (Marin et al., 2013).

The use of antifungals of synthetic origin is the common industry response to avoid fungal contamination (Císarová et al., 2020). However, use of these compounds has been related to pathologies. New methods are being studied to avoid fungal contamination of food. Additionally, all this leads to an increase in consumer doubts towards this type of food preservative and an increase in the demand for "healthier" foods (Hanafy et al., 2021). Innovative techniques like fermentation or addition of lactic acid bacteria (LAB) for the preservation against toxigenic fungi contamination and the reduction of the toxins is postulated as a great alternative to these synthetic origin compounds (Freire et al., 2020; Murugesan et al., 2015; Zheng et al., 2020). Furthermore, the addition of LAB to food is allowed by the European Union, since they are classified as "Qualified Presumption of Safety" (QPS) by this entity (Leuschner et al., 2010). There is several examples in the bibliography of their application as preservatives against the toxigenic fungi and their toxins, especially from the *Lactiplantibacillus plantarum* (*L. plantarum*) species (Li et al., 2012; Mcnair

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https://doi.org/10.1016/j.fbio.2023.102586
Received 12 January 2023; Received in revised form 15 March 2023; Accepted 23 March 2023
Available online 26 March 2023

LWT - Food Science and Technology 174 (2023) 114427

Contents lists available at ScienceDirect

LWT

journal homepage: www.elsevier.com/locate/lwt

Evaluation of shelf life and technological properties of bread elaborated with lactic acid bacteria fermented whey as a bio-preservation ingredient

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ARTICLE INFO

Keywords:
Antifungal activity
Lactic acid bacteria
Bread
Aspergillus
Penicillium

ABSTRACT

Nowadays, food lost by fungal contamination is one of the main safety problems for the bakery industry. As the consumer is rejecting the use of regular food additives new methods are being investigated. One of the more promising alternatives is the use of microorganism in the preservation of food, bio-preservation. In this article the ability of a lactic acid bacteria fermented whey was assessed as bio-preservative ingredient to inhibit the growth of *Aspergillus* sp. and *Penicillium* sp. in bread. The rheological, mechanical and antifungal properties of 3 different concentrations of this ingredient were studied. Results evidence that the addition lactic acid bacteria fermented whey in a of a 5% concentration did not modify drastically the quality of the bread, increased the occurrence of antifungal metabolites such as lactic acid and phenyllactic acid and managed to extend the shelf life from bread contaminated with *Aspergillus flavus* and *Penicillium verrucosum* and significantly reduced the fungal population of *Penicillium verrucosum* in the bread after 7 days in comparation to a regular bread.

1. Introduction

Approximately around a 25% of the food worldwide is contaminated with filamentous fungi and the toxins produced by this microorganism (H. Cao, Jiang, et al., 2021). As a result of this fact, fungal and mycotoxigenic contamination have become a severe problem which cost food industry million euros per year and threatens the life of the consumers with their toxigenic effects (Russo et al., 2017; Zain, 2011). Among all the food-contaminant species of moulds, bakery products are especially susceptible to contamination from the *Aspergillus*, *Penicillium*, *Fusarium* and *Rhisopus* (Debonne et al., 2021). *Aspergillus* and *Penicillium* genera are considered the most harmful to human health. This fungal species are the main producers of aflatoxins (AF) and ochratoxin (OTA) in cereal and different cereal-based foods (Bryła et al., 2021). AF ingestion is related to hepatotoxic, mutagenic and carcinogenic activities in animals, and is classified as Group 2B by the International Agency for Research on Cancer (IARC) (Bennett & Klich, 2003; Gupta, 1994). OTA is also related to nephrotoxic, teratogenic, embryotoxic, genotoxic, neurotoxic suppression of the immune system effects (Belkacem-Hanfi et al., 2014).

In the production of bread, the temperatures reached during the baking stage destroy the fungal vegetative forms, but some of the spores are resistant to this treatment and grow in the final product. In addition, post preparation practices lead to an increasing contamination of the bread (Cizeikiene et al., 2013; Jideani & Vogt, 2016). It is interesting to find effective fungicides resistant to high temperatures to prevent the growth of moulds and mycotoxin production after the bakery stage. Chemical preservatives, such as calcium propionateand calcium acetate are commonly added to bread in order to prevent fungal and further toxin contamination. (Perincherry et al., 2019). Nevertheless, there is an increasing demand from the consumer for "natural" and "healthier" food by the reduction of the concentration from these chemical preservatives present in food (Sadiq et al., 2019). As a result, novel technological approaches are being studied to reduce fungal contamination with safer techniques. Use of lactic acid bacteria (LAB) as bio-protective agents postulates as a great alternative to the synthetic origin preservatives.

Many reports in the bibliography show evidence of the antifungal effect of this microorganism (Chen et al., 2021; Illueca et al., 2021). The use of these bacteria as bio-preservative agents has already being proved. Álvarez et al. (2021) used edible coatings incorporating LAB to prolong the shelf life of cherry tomatoes against *Fusarium* sp. and *Rhisopus* sp. Also Omedi et al. (2021) evidence the bio-preservation potential of bread contaminated by *Cladosporium*, *Aspergillus* and

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https://doi.org/10.1016/j.lwt.2023.114427
Received 24 October 2022; Received in revised form 30 December 2022; Accepted 3 January 2023
Available online 4 January 2023

Food Bioscience 58 (2024) 103588

Contents lists available at ScienceDirect

Food Bioscience

journal homepage: www.elsevier.com/locate/fbio

Revalorization of beer brewing waste as an antifungal ingredient for bread biopreservation

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ARTICLE INFO

Keywords:
Beer bagasse
Lactic acid bacteria
Shelf life
Antifungal activity
Toxigenic fungi
Food waste

ABSTRACT

This study assesses the potential revalorization of beer bagasse (BB), an important food industry waste, as antifungal ingredient in bread, by lactic acid bacterial (LAB) fermentation of this food waste. Initially, *in vitro* studies of the metabolite and antifungal activity were performed for the preparation of a microbial culture medium for the LAB. The culture medium with a 20% BB was determined as the optimal for the bacterial growth. Therefore, bread was prepared with a 5% and 10% (w/w) of a dried powder of this fermented culture medium as ingredient. The fermentation and heat treatment during baking did not compromise the antifungal metabolites in the bread. In the trials of the augment of the shelf-life the bread with a 10% concentration of the ingredient extended the shelf life of the bread by a 1 day and 3 days against *Aspergillus flavus* and *Penicillium commune*, respectively, compared to a regular bred. When studying the mycotoxin production in the bread the addition of the ingredient at a 10% concentration resulted in an 86% reduction in aflatoxin concentration compared to the control bread. Moreover, this ingredient did not importantly deteriorate the technological parameters of the bread.

1. Introduction

Nowadays, there is an increasing interest in finding alternative uses for the by-products produced by the food industry. With a world growing population that has surpasses seven billion humans in the planet the United Nations is encouraging the investigation to identifying ways for more sustainable use of resources and the revalorization the food wastes (United Nations, 2015). Beer is the most consumed alcoholic drink in globally, among all beverages is the third in consumption after water and tea (Habschied et al., 2020). Beer is mainly produced with a malt in a process known as brewing (Albanese et al., 2017). During the production of beer the main waste is the brewer's spent grain also called beer bagasse (BB). Approximately 5–6 kg of bagasse are produced for every 100 litters of beer (Del Rio Osorio et al., 2021). This bagasse brewing is a solid residue primarily used for feed stock, as a fertilized in for crops and for biogas production (Kwoczynski & Čmelík, 2021; Loomis et al., 2020). This uses are not enough to significantly reduce the quantities of this waste, therefore, ways for the conversion of the bagasse are being studied (Martínez et al., 2012).

Bagasse is the residue produced from the filtration of the wort during the brewing process. This filtered residue is mainly composed of the malt seeds after the fermentation, therefore, is a matrix rich in nutrients. The composition of malt bagasse after the beer processing contains a 73% of carbohydrates (64% of them fibber), 24% protein content, 4% lipids and 2% ashes (Tassoni et al., 2020). Several studies repot the fermentation of this matrix for the production of compounds with value for the industry. In the investigation Moran-Aguilar et al. (2021) it is reported the production of cellulases and xylanases with an *Aspergillus niger* fermentation. In other examples, by the fermentation with other microorganism this matrix has been used for the production of biofuels, organic acids and biosurfactants among many other products (Khonngam & Salakkam, 2019; Pérez-Bibbins et al., 2015).

There is a growing interest from consumers in the ingredients used in the food industry, which has led to an increased demand for a reduction of the chemical preservatives (Cosentino et al., 2018). One of the alternatives more widely investigated in recent years is the use of lactic acid bacteria (LAB) for the biopreservation of foods (Li et al., 2020; Mcnair et al., 2018). LAB fermentation has been proven to extend the shelf life of foods against fungal contamination. Biopreservation with this microorganism has the potential to partially or totally reduce the

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https://doi.org/10.1016/j.fbio.2024.103588
Received 24 October 2023; Received in revised form 27 December 2023; Accepted 5 January 2024
Available online 19 January 2024

Food Bioscience 58 (2024) 103703

Contents lists available at ScienceDirect

Food Bioscience

journal homepage: www.elsevier.com/locate/fbio

Revalorization of rice bran as a potential ingredient for reducing fungal contamination in bread by lactic acid bacterial fermentation

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ARTICLE INFO

Keywords:
Rice bran
Lactic acid bacteria
Shelf life
Antifungal activity
Toxigenic fungi

ABSTRACT

Rice bran (RB) is a by-product of the rice industry that reaches a waste of 63 million tonnes per year. In order to promote the development of the circular economy, it is important to revalorize the by-products generated by the food industry. Lactic acid bacteria (LAB) can ferment multiple substrates generating antifungal compounds such as organic acids, phenolic acids, or bacteriocins, so the use of LAB can be a good strategy for the revalorization of food industry by-products. In addition, RB can be a good substrate for the lactic acid bacteria fermentation due to its content of carbohydrates and nitrogenous compounds. This study explored the potential of incorporating RB into a lactic acid bacterial medium for antifungal bread ingredient development. We evaluated the *in vitro* antifungal activity and metabolite production of LAB-fermented RB media. *L. plantarum* H1 fermentation with 20 % RB demonstrated the most effective inhibition of fungal growth and antifungal metabolite production. This formulation was chosen for bread preparation without compromising dough and bread quality. The bread with 20 % fermented RB extended shelf life like the propionate control, with one day less for *Penicillium commune* and *Aspergillus flavus*-contaminated bread. The fungal population after seven days showed no significant differences compared to the propionate control. Additionally, aflatoxin levels significantly decreased in bread with 20 % RB, achieving a 96 % reduction, like the propionate control. This research not only extends the shelf life of commonly consumed foods but also reduces waste from this food industry's important by-product.

1. Introduction

Society's challenge is to sustainably feed over seven billion people on a resource-limited planet. Revaluing food industry by-products offers a solution to reduce hunger and optimize resource utilization (United Nations, 2015). Rice, a vital global food staple (Zhuang et al., 2019), generates a by-product known as rice bran (RB) during milling, producing over 63 million tons annually. Typically, RB is used for fuel or livestock feed (Webber et al., 2014). RB is nutrient-rich, containing unsaturated lipids, polysaccharides, and proteins, as well as bioactive compounds like flavonoids, tocopherols, and phenolic acids, which can be harnessed through fermentation (Goufo & Trindade, 2014). Therefore, the potential for RB revalorization holds promise for addressing food security and resource sustainability challenges.

RB is a nutrient and bioactive-rich by-product that presents an ideal substrate for lactic acid bacteria (LAB) fermentation (Alauddin et al., 2019). LAB-mediated biopreservation has gained significant traction as a food preservation technique in recent years (Li et al., 2020; Mcnair et al., 2018). Increasing consumer awareness of food ingredients has driven demand for reduced chemical preservatives (Cosentino et al., 2018).

Lactic acid fermentation has proven effective in extending the shelf life of foods, offering the potential to significantly reduce or eliminate the use of chemical preservatives (Punia Bangar et al., 2022). This antifungal activity arises from a diverse spectrum of antifungal compounds produced during fermentation. Organic acids, bioactive peptides, and phenolic acids represent some of the bioactive compounds identified as effective against contaminating microorganisms during LAB fermentation (Peyer et al., 2016).

The bread serves as a versatile food matrix where numerous ingredients can be incorporated to modify rheology, extend shelf life, and enhance flavour. The literature showcases the utilization of various

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https://doi.org/10.1016/j.fbio.2024.103703
Received 9 November 2023; Received in revised form 30 January 2024; Accepted 31 January 2024
Available online 1 February 2024