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Producción de ácidos lactobiónico/láctico y sistemas experimentales considerando la interacción entre microorganismos productores

Programa de Doctorado en Ingeniería Química,
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TESIS DOCTORAL

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RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

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RESUMEN (en español)

La búsqueda de alimentos novedosos que aporten un beneficio para la salud, más allá de sus propiedades nutricionales, constituye actualmente uno de los principales motores en el desarrollo de productos dentro de la industria alimentaria. En este contexto, la biotecnología ofrece enormes posibilidades para la obtención de productos fermentados con propiedades mejoradas, debido a la gran variedad de metabolitos producidos por los microorganismos fermentadores, muchos de ellos considerados compuestos bioactivos. Éste es el caso del ácido lactobiónico, un ácido orgánico con gran potencial en la industria alimentaria por sus propiedades tecnológicas y beneficiosas para la salud como prebiótico. En este contexto, esta tesis doctoral explora por primera vez el acoplamiento de la bioproducción de ácido lactobiónico por la bacteria *Pseudomonas taetrolens* a los procesos fermentativos tradicionales llevados a cabo por bacterias ácido lácticas, en concreto la bacteria probiótica Lactobacillus casei, para la obtención de productos lácteos enriquecidos en ácido lactobiónico.

El empleo de cultivos iniciadores mixtos es muy frecuente en fermentaciones alimentarias, pudiendo establecerse interacciones positivas o negativas entre la microbiota que determinan las características del producto final. Por ello, como primer paso imprescindible se estudió la interacción establecida entre *P. taetrolens* y *L. casei* en fermentaciones competitivas sobre un sustrato de suero de queso, monitorizando el proceso en términos de productividad, crecimiento y estado fisiológico de los microorganismos. Este estudio reveló una interacción de tipo amensalista entre *P. taetrolens* y *L. casei,* caracterizada por la exclusión competitiva de la Pseudomona debido a la producción de metabolitos antimicrobianos por *L. casei*.

El descubrimiento de esta interacción antagónica dio lugar a la exploración de diferentes estrategias fermentativas. Se diseñó así un sistema secuencial de dos etapas para permitir a P. taetrolens desarrollar su metabolismo sin la influencia negativa de la bacteria ácido láctica. Se establecieron los parámetros clave para el diseño del proceso, y se realizó su seguimiento en cada etapa. El sistema secuencial se reveló como una alternativa factible para el correcto desarrollo de las dos bacterias y la obtención de un producto final conteniendo ácido láctico y ácido lactobiónico. Se determinó también en este estudio el carácter simbiótico de la combinación de *L. casei* y ácido lactobiónico. A continuación, se realizó una aproximación a la aplicación industrial del proceso secuencial. El sustrato de suero de queso fue sustituido por leche de vaca y el proceso se escaló a nivel de biorreactor con control de pH. Se realizó el seguimiento del proceso y se llevó a cabo la caracterización del producto, en términos de composición química, física y microbiológica, haciendo hincapié en sus propiedades funcionales: su contenido elevado en el probiótico *L. casei* y el prebiótico lactobiónico, y bajo en lactosa. Asimismo, se evaluó el efecto del ácido lactobiónico sobre las propiedades texturales del producto. En esta tesis doctoral se exploró también otra alternativa para el control de cultivos mixtos: la encapsulación bacteriana. Para ello se llevaron a cabo fermentaciones mixtas encapsulando *P. taetrolens* y *L. casei* de forma alternativa en diferentes matrices de

aplicación alimentaria. La caracterización del proceso permitió determinar el potencial de la encapsulación para actuar como una barrera física que permite minimizar el antagonismo en cultivos mixtos.

La presente tesis doctoral, por tanto, aborda el diseño de nuevos bioprocesos para la obtención de productos fermentados funcionales, de carácter novedoso por su introducción de un compuesto emergente de gran interés como es el ácido lactobiónico, al mismo tiempo que aporta información útil para el desarrollo de estrategias de cultivo en fermentaciones mixtas competitivas.

RESUMEN (en Inglés)

The search for novel foods that provide a health benefit, beyond their nutritional properties, is currently one of the main drivers of product development in the food industry. In this context, biotechnology offers great possibilities for obtaining fermented products with enhanced properties, due to the plethora of metabolites produced by the fermenting microorganisms, many of them considered bioactive compounds. This is the case of lactobionic acid, an organic acid with great potential in the food industry due to its technological and healthy properties as a prebiotic compound. In this context, this thesis explores for the first time the coupling of the lactobionic acid bioproduction by the bacterium *Pseudomonas taetrolens* to the traditional fermentation processes carried out by lactic acid bacteria, specifically the probiotic strain *Lactobacillus casei*, for obtaining dairy products enriched with lactobionic acid.

The use of mixed starter cultures is very common in food fermentations. Positive or negative interactions can be established between the microbiota, which determine the characteristics of the final product. Therefore, as an essential first step, the interaction established between *P. taetrolens* and *L. casei* in competitive fermentations on a cheese whey substrate was studied, monitoring the process in terms of productivity, growth and physiological state of the microorganisms. This study revealed an amensalistic interaction between *P. taetrolens* and *L. casei*, characterized by the competitive exclusion of *Pseudomonas* due to the production of antimicrobial metabolites by *L. casei.*

The antagonistic interaction found between *P. taetrolens* and *L. casei* led to the exploration of different fermentative strategies. A two-stage sequential system was designed for allowing *P. taetrolens* to develop its metabolism without the negative influence of the lactic acid bacteria. The key parameters for the design of the process were established and monitored at each stage. The sequential system was revealed as a feasible alternative for the proper development of the two bacteria and for obtaining a final product containing lactic and lactobionic acids. The synbiotic nature of the combination of *L. casei* and lactobionic acid was also determined in this study. Subsequently, an approximation to the industrial application of the sequential process was carried out. The cheese whey was replaced by cow's milk as substrate, and the process was scaled at the bioreactor level with pH control. The process was monitored, and the product characterization was carried out, in terms of chemical, physical and microbiological composition, emphasizing its functional properties: its high content in the probiotic *L. casei* and the prebiotic lactobionic acid, and its low lactose concentration. Likewise, the effect of lactobionic acid on the textural properties of the product was evaluated. In this thesis, another alternative for the control of mixed cultures was also explored: bacterial encapsulation. Mixed fermentations were implemented by alternately entrapping *P. taetrolens* and *L. casei* in different matrixes of food application. The characterization of the process allowed to determine the potential of the encapsulation to act as a physical barrier that allows to minimize the antagonism in mixed cultures.

The present thesis, therefore, addresses the design of new bioprocesses to produce novel functional fermented products containing an emerging compound of great interest such as lactobionic acid, while it provides useful information for the development of culture strategies in competitive mixed fermentations.

SR. PRESIDENTE DE LA COMISIÓN ACADÉMICA DEL PROGRAMA DE DOCTORADO EN INGENIERÍA QUÍMICA, AMBIENTAL Y BIOALIMENTARIA

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ABSTRACT

The search for novel foods that provide a health benefit, beyond their nutritional properties, is currently one of the main drivers of product development in the food industry. In this context, biotechnology offers great possibilities for obtaining fermented products with enhanced properties, due to the plethora of metabolites produced by the fermenting microorganisms, many of them considered bioactive compounds. This is the case of lactobionic acid, an organic acid with great potential in the food industry due to its technological and health properties as a prebiotic compound. In this context, this thesis explores for the first time the coupling of the lactobionic acid bioproduction by the bacterium *Pseudomonas taetrolens* to the traditional fermentation processes carried out by lactic acid bacteria, specifically the probiotic strain *Lactobacillus casei*, for obtaining dairy products enriched with lactobionic acid.

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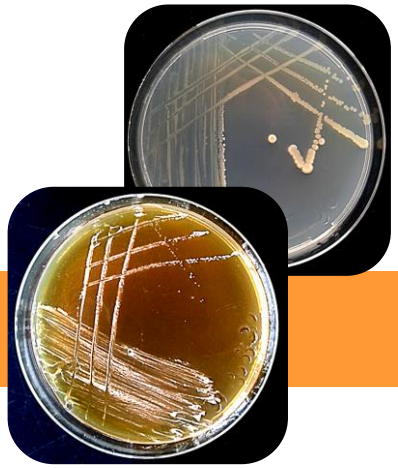

1. INTRODUCCIÓN

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1.1. Introducción

En los últimos años, la industria alimentaria ha tenido que adaptarse a cambios en la demanda de los consumidores, cada vez más conscientes y preocupados por la protección y mejora de su salud. El deseo de una mejor calidad de vida, directamente relacionado con una mayor longevidad, ha impulsado el crecimiento del mercado de los alimentos funcionales, definidos como aquellos alimentos destinados a ser consumidos dentro de una dieta normal, y que contienen componentes activos con el potencial de mejorar la salud o reducir el riesgo de enfermedad, más allá de su valor nutricional intrínseco (Cencic y Chingwaru, 2010; Moons et al., 2018). A nivel mundial, el mercado de estos productos se encuentra dominado por Estados Unidos y Japón, seguidos por Asia-Pacífico y Europa. Dentro de Europa, el mayor porcentaje de ventas lo integran Reino Unido, Francia y Alemania (Figura 1.1), aunque en España y Países Bajos el mercado está creciendo también de forma significativa (Annunziata y Vecchio, 2011).

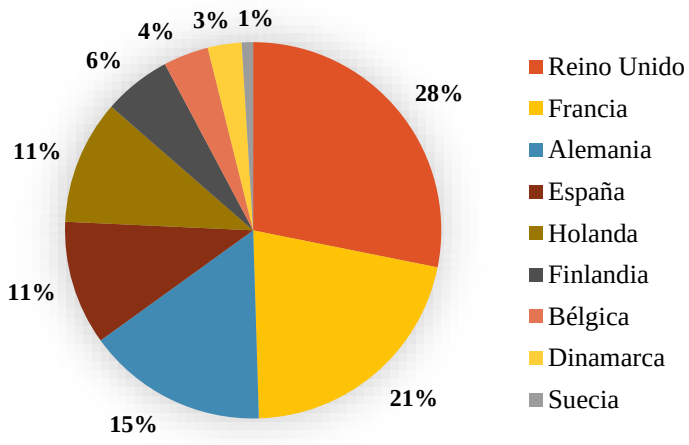


Figura 1.1. Distribución europea del mercado de alimentos funcionales.

En el año 2014, el 56% de los ciudadanos europeos afirmaba consumir alimentos y bebidas funcionales; el 19% al menos una vez a la semana. Junto con las bebidas funcionales, los productos lácteos suponen el mayor segmento de crecimiento, esperándose que para 2025 cubran el 40% de la demanda total de productos funcionales (Bogue et al., 2017). La versatilidad de los productos lácteos fermentados los convierte en una excelente plataforma para el desarrollo de nuevos alimentos funcionales (Caleja et al., 2015; Morais et al., 2014; Southee et al., 2016). La leche constituye por sí sola una fuente de componentes con potencial

funcional, como lactoferrina, ácido linoleico conjugado, fosfolípidos y calcio. Pero su fermentación permite añadir una gran variedad de constituyentes, desde bacterias beneficiosas (bacterias ácido lácticas, propiónicas o bifidobacterias), productos resultantes de su actividad metabólica (ácidos orgánicos, péptidos bioactivos o ácido fólico) o compuestos antimicrobianos como bacteriocinas, ácido láctico o peróxido de hidrógeno (Marsh et al., 2014; Sánchez et al., 2009).

Algunos de estos metabolitos microbianos han sido ampliamente estudiados por su presencia en alimentos fermentados consumidos desde la antigüedad. Este es el caso de los compuestos sintetizados por las bacterias ácido lácticas (BAL), por su presencia mayoritaria como cultivos iniciadores en las industrias de productos lácteos, panadería, carne, vegetales y bebidas alcohólicas. Dentro del amplio abanico de metabolitos producidos por las BAL, el ácido láctico ha sido empleado como agente coagulante, aromatizante y conservante durante siglos en los alimentos fermentados tradicionales. Recientemente se le han atribuido además propiedades funcionales, al descubrir su papel modulador de las respuestas proinflamatorias en células epiteliales y mieloides, y reductor de las especies reactivas de oxígeno en los enterocitos intestinales (Marco et al., 2017). Debido a su papel crucial en la industria láctea, su producción fermentativa se ha investigado de forma exhaustiva buscando mejorar la productividad de las BAL mediante el desarrollo de técnicas de co-cultivo, ingeniería metabólica y genética o inmovilización bacteriana (Abdel-Rahman et al., 2013; Eş et al., 2018).

Además del ácido láctico, existen otros ácidos orgánicos emergentes con un alto valor añadido y nuevas aplicaciones, que pueden obtenerse a partir de otras plataformas microbianas. Este es el caso del ácido lactobiónico, un polihidroxiácido derivado de la oxidación de la lactosa, cuya estructura (Figura 1.2) y propiedades fisicoquímicas le confieren aplicaciones alimentarias como agente potenciador del sabor, acidulante, antioxidante, estabilizante, espesante o inductor de la coagulación. Además de este papel tecnológico se le atribuyen propiedades saludables como estimulante de la absorción mineral y potencialmente prebiótico, al ser resistente a los enzimas digestivos y absorbido pobremente en el intestino delgado, pudiendo ser fermentado en el colon por bacterias probióticas beneficiosas para la salud (Adebola et al., 2014; Alonso et al., 2013; Gutiérrez et al., 2012b). Su uso como lactobionato de calcio (E-399) fue autorizado en Estados Unidos por la FDA en el año 2011 (Alonso et al., 2013).

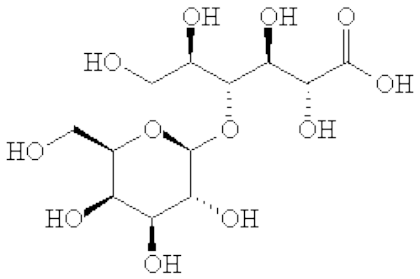


Figura 1.2. Estructura del ácido lactobiónico.

El interés en el ácido lactobiónico como ingrediente bioactivo ha estimulado la búsqueda de nuevas estrategias para su producción. En este contexto, su síntesis biotecnológica se ha revelado como una alternativa sostenible a la catálisis química a partir de lactosa refinada, demostrándose la idoneidad de matrices lácteas como sustrato para la bioconversión. Así, se han desarrollado metodologías eficientes y competitivas, con elevados rendimientos y productividades, empleando la bacteria *Pseudomonas taetrolens* sobre un sustrato residual de suero dulce de queso (Alonso et al., 2011, 2012, 2013). Otras estrategias de bioproducción de ácido lactobiónico desarrolladas hasta la fecha se basan en el empleo de sustratos sintéticos o utilizan células en condiciones de reposo, cepas patogénicas, mutantes o poco selectivas que generan subproductos como resultado de la oxidación de la lactosa (Kiryu et al., 2012; Malvessi et al., 2013; Murakami et al., 2006).

Frente al empleo de sustratos sintéticos, la utilización biotecnológica del suero lácteo supone una oportunidad para revalorizar este subproducto industrial, de producción creciente y gran potencial contaminante. Durante la fabricación de 1 kg de queso se generan 9 kg de suero, conteniendo en el caso del suero dulce el 50% de los sólidos totales encontrados en la leche, la mayoría de la lactosa y el 20% de las proteínas. Su elevada demanda biológica y química de oxígeno (>30000mg O_2/L y >60000mg O_2/L, respectivamente), debido principalmente a su gran contenido en lactosa, hace que existan estrictas regulaciones ambientales para evitar el vertido de suero no tratado. Paralelamente, el valor intrínseco de sus componentes ha impulsado la búsqueda de estrategias para su aprovechamiento. En este contexto, se ha explorado su potencial no solo como fuente de productos proteicos bioactivos (Dullius et al., 2018; Yadav et al., 2015), sino también su utilización directa como sustrato para la obtención de metabolitos de interés, como ácidos orgánicos (Alonso et al., 2010, 2011), y para el desarrollo de bebidas funcionales. Dentro de esta categoría el producto más común son las mezclas de zumo de frutas o vegetales y suero no procesado, desproteinizado o permeado de ultrafiltración, pero también

productos estrictamente lácteos similares a batidos y leches aromatizadas o a bebidas lácteas fermentadas como la leche agria o el kéfir (Guimarães et al., 2018; Jelen, 2009). Además de la fortificación de estas bebidas con vitaminas y minerales se ha ensayado la introducción de compuestos bioactivos como sustancias prebióticas (Guimarães et al., 2018).

Los prebióticos que se utilizan actualmente a nivel industrial son principalmente mezclas de oligosacáridos no digeribles (fructooligosacáridos, xilooligosacáridos, polidextrosa y galactooligosacáridos), excepto en el caso de la inulina, que está conformada por una mezcla de fructooligo- y polisacáridos (Fazilah et al., 2018). Se incorporan a productos lácteos fermentados con el objetivo de mejorar sus características tecnológicas (textura y estabilidad) y su carácter funcional. Otras leches fermentadas y productos lácteos, conteniendo cultivos iniciadores tradicionales, son suplementados con prebióticos y bacterias probióticas (*Lactobacillus* y *Bifidobacterium*). Esta combinación de prebióticos y probióticos da lugar a productos conocidos como "simbióticos", en los que tiene lugar un efecto sinérgico que beneficia la salud del hospedador al mejorar la supervivencia e implantación de las cepas microbianas vivas en el tracto gastrointestinal (Pandey et al., 2015). Sin embargo, existen barreras reguladoras legislativas, así como científicas y económicas, que suponen un desafío al desarrollo y comercialización de estos productos simbióticos (Illanes y Guerrero, 2016). A nivel científico, el principal obstáculo es el conocimiento aún limitado acerca de la microbiota humana y las interacciones que se establecen entre los microorganismos, así como la necesidad de evaluar la existencia de un efecto realmente sinérgico entre probiótico y prebiótico, que es específico a nivel de cepa. Las barreras económicas se refieren al coste adicional que supone el lanzamiento de un producto que puede llevar a un análisis coste-beneficio negativo. La mayoría de los oligosacáridos con potencial prebiótico son obtenidos mediante síntesis enzimática y posterior purificación a partir de materias primas de bajo coste, como lactosa, sacarosa y derivados vegetales. Sin embargo, la cantidad de prebiótico alcanzada depende de factores como la fuente enzimática, la concentración y naturaleza del sustrato y las condiciones de reacción, siendo frecuente la obtención de rendimientos bajos y costes de producción elevados (Figueroa-González et al., 2011).

Actualmente se está investigando el desarrollo de nuevas técnicas y materias primas para la producción eficiente, sostenible y competitiva de prebióticos y alimentos simbióticos funcionales, contexto en el que cobra importancia la síntesis biotecnológica de metabolitos microbianos como el ácido lactobiónico. El empleo de sustratos lácteos (como suero o leche) para su producción permitiría obtener *"in situ"* productos fermentados con propiedades

bioactivas, sin la necesidad de utilizar materias primas o procesos adicionales. Por otra parte, resulta de interés el acoplamiento de la síntesis del ácido lactobiónico a la fermentación llevada a cabo de forma tradicional por las BAL, pudiendo aportar un valor añadido al producto gracias a las propiedades conferidas por su actividad metabólica, así como la posibilidad de dotarle de carácter simbiótico si se da una combinación adecuada de cepas.

El diseño de inóculos mixtos se configura como una fuente ilimitada de posibilidades para el desarrollo de nuevos alimentos fermentados, o alimentos con propiedades mejoradas. No obstante, resulta esencial el estudio de las interacciones que pueden surgir en los consorcios microbianos, así como el diseño de estrategias para optimizar el proceso fermentativo y por tanto las características del producto final.

1.2. Objetivos

Considerando el interés de la introducción del ácido lactobiónico en productos lácteos fermentados, el objetivo principal de esta tesis doctoral es el acoplamiento novedoso de su producción biotecnológica a los procesos fermentativos tradicionales llevados a cabo por las BAL. Como cepa productora de ácido lactobiónico se puede emplear la bacteria *P. taetrolens*, por su elevado rendimiento en la conversión de la lactosa presente en sustratos lácteos. Para el desarrollo de la fermentación ácido-láctica se selecciona la bacteria *Lactobacillus casei*, considerando su amplia utilización en procesos alimentarios y su carácter probiótico. En un bioproceso integrado, en el que ambos microorganismos son inoculados de forma simultánea, resultan de interés las interacciones y limitaciones que pueden ocurrir. En el presente trabajo se hace énfasis en la necesidad del estudio de dichas interacciones y la exploración de alternativas de cultivo que permitan optimizar el desarrollo de ambos microorganismos, pudiendo aportar información útil para el desarrollo de otras fermentaciones que impliquen la participación de consorcios microbianos.

Se han planteado los siguientes objetivos específicos:

- Desarrollar fermentaciones competitivas con células libres de *P. taetrolens* y *L. casei* sobre un sustrato lácteo, caracterizando crecimiento microbiano y productividad, y empleando la citometría de flujo para obtener información sobre el estado fisiológico de las células en cultivo mixto.

- Definir el tipo de interacción establecida entre *P. taetrolens* y *L. casei* cuando coexisten en el mismo nicho.

- Estudiar la capacidad de la cepa *L. casei* CECT 475 para emplear el ácido lactobiónico como fuente de carbono, elucidando el carácter simbiótico de una combinación de ambos.

- Desarrollar una estrategia de fermentación secuencial que permita el correcto desarrollo del metabolismo de *P. taetrolens* y *L. casei* y la co-producción de los ácidos láctico y lactobiónico.

- Aplicar el cultivo mixto sobre un sustrato de leche de vaca para la obtención de un alimento fermentado de interés industrial. Caracterizar el producto resultante en cuanto a su composición, propiedades microbiológicas y texturales.

- Estudiar la interacción de *P. taetrolens* y *L. casei* en fermentaciones competitivas con biomasa inmovilizada mediante encapsulación, evaluando la idoneidad de diferentes matrices encapsulantes.

1.3. Estructura de la memoria

Esta memoria de tesis doctoral se presenta como un compendio de publicaciones, enmarcadas en el ámbito de los estudios de interacción microbiana para su aplicación en la obtención de metabolitos de interés alimentario y nuevos alimentos. Todas las publicaciones siguen el esquema tradicional (resumen, introducción, materiales y métodos, resultados y discusión, y conclusiones) y han sido aceptadas o están siendo evaluadas por revistas incluidas en el *Science Citation Index*. La memoria consta de 9 capítulos, subdivididos en sus correspondientes apartados.

En el **capítulo 1**, correspondiente a la parte introductoria, se plantea la relevancia del estudio de procesos biotecnológicos para el diseño de cultivos iniciadores, la producción de metabolitos tradicionales como el ácido láctico, o emergentes como el ácido lactobiónico, y la obtención de alimentos novedosos con propiedades mejoradas y funcionales (subcapítulo **1.1**). En base a este marco de interés, en el subcapítulo **1.2** se exponen los objetivos de la presente tesis doctoral.

En el **capítulo 2**, correspondiente a las consideraciones teóricas, se recoge la importancia de los cultivos mixtos en la producción biotecnológica de bebidas y alimentos líquidos fermentados, tanto tradicionales como nuevos alimentos. A continuación, se resumen las principales características de las BAL, por su relevancia como *starters* en fermentaciones alimentarias, y se recogen las diferentes categorías de alimentos fermentados obtenidos mediante el cultivo mixto de BAL y otros microorganismos. En el siguiente apartado del capítulo se describen los tipos de interacción que pueden establecerse en los *starters* mixtos y se presentan algunos modelos cinéticos que se han desarrollado para describir dichas interacciones. Por último, se recogen las estrategias fermentativas que pueden implementarse para el control de los cultivos mixtos y las nuevas tecnologías desarrolladas para su estudio.

En el **capítulo 3** se describe de forma global la metodología experimental y las técnicas analíticas empleadas en la presente tesis doctoral. La metodología específica de cada estudio se puede consultar en la sección "Materiales y Métodos" dentro de la publicación correspondiente.

En el **capítulo 4** se presentan los resultados obtenidos, constituyendo la parte central del trabajo. Dentro de este capítulo, cada subcapítulo corresponde a un artículo publicado o enviado para su evaluación. Los tres primeros artículos corresponden a los estudios realizados con biomasa en suspensión. En el subcapítulo **4.1** se aborda el co-cultivo de las bacterias *L. casei* y *P. taetrolens*, en el contexto de la co-producción de los ácidos láctico y ácido lactobiónico para

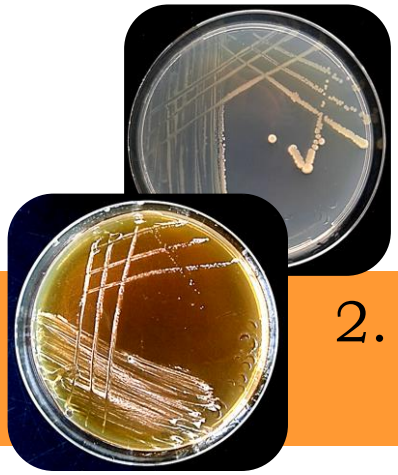
la posible obtención de un producto lácteo fermentado funcional. Los resultados obtenidos en este primer estudio se tomaron como base para el desarrollo de diferentes estrategias fermentativas, abordadas en los siguientes subcapítulos. Así, en el subcapítulo **4.2** se estudia el modelo fermentativo secuencial, atendiendo al diseño y a la viabilidad del proceso. En este apartado también se evalúa el carácter simbiótico del producto final conteniendo *L. casei* y ácido lactobiónico. En el subcapítulo **4.3** se realiza una aproximación a la aplicación industrial del proceso secuencial, haciendo hincapié en la caracterización química, física y microbiológica del producto. El subcapítulo **4.4** corresponde al estudio final realizado con biomasa inmovilizada. En él se aborda el efecto de la estrategia de encapsulación sobre la interacción de *L. casei* y *P. taetrolens* en cultivo mixto.

En el **capítulo 5** se recoge la discusión general de los resultados obtenidos y en el **capítulo 6** se exponen las principales conclusiones alcanzadas.

El **capítulo 7** recoge la bibliografía común a la presente memoria. Se omiten referencias específicas asociadas a cada subcapítulo de resultados, que pueden ser consultadas en la sección "Referencias" dentro de la publicación correspondiente.

El **capítulo 8** enumera las principales abreviaturas y símbolos empleados en la redacción de la memoria. El significado de los símbolos correspondientes a los modelos cinéticos recogidos en el capítulo 2 se detalla en la propia publicación.

Por último, en el **capítulo 9**, "Anexos", se recoge la difusión de la presente tesis doctoral en forma de artículos científicos y comunicaciones a congresos (**9.1**) y se presenta el informe con el factor de impacto de las revistas científicas de los artículos ya publicados (**9.2**).



2. CONSIDERACIONES TEÓRICAS

2. CONSIDERACIONES TEÓRICAS

2.1. Consorcios microbianos en fermentaciones mixtas

En este apartado se realiza una revisión bibliográfica sobre la participación de consorcios microbianos en alimentos fermentados, en base a su interés y enormes posibilidades para la mejora o diseño de alimentos novedosos. La revisión se centra en las fermentaciones mixtas en fase líquida, seleccionando los procesos y productos en los que intervienen BAL, por su papel mayoritario como cultivos iniciadores y su importancia en el desarrollo de alimentos funcionales. Se resumen las principales características de las BAL y sus productos metabólicos, haciendo énfasis en el efecto negativo que puede tener su capacidad para producir metabolitos con función antimicrobiana sobre la estabilidad de los consorcios microbianos. A continuación, se describen los principales productos alimentarios producidos por un consorcio de BAL y otros microorganismos, clasificándolos entre bebidas lácteas y productos obtenidos a partir de sustratos vegetales. Se recogen las materias primas y la microbiota empleada clásicamente en la elaboración de productos tradicionales, pero también las nuevas investigaciones para el diseño de consorcios microbianos y el desarrollo de alimentos novedosos. La revisión describe asimismo las distintas relaciones microbianas que se pueden encontrar en los productos fermentados objeto de estudio, centrándose en las interacciones indirectas positivas y negativas que se establecen entre las BAL, otras especies bacterianas y hongos. Se recogen también algunos de los modelos matemáticos que se han propuesto para el estudio de la cinética de estos cultivos mixtos. Por último, se explican diferentes estrategias que se han desarrollado y continúan explorándose para optimizar la bioconversión en las fermentaciones mixtas, minimizando el efecto de las interacciones negativas y potenciando las características deseadas en el producto final, finalizando con una breve introducción a las nuevas técnicas empleadas en el estudio de comunidades microbianas.

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Estado: Enviada para su evaluación al *Food Research International.*

Liquid-phase food fermentations with microbial consortia involving lactic acid bacteria

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Abstract

Microbial associations are frequent in traditional fermented foods and beverages, conferring upon them their characteristic organoleptic, physical and nutritional properties. Moreover, the search for novel products that satisfy the needs of consumers, especially foods with health-giving properties, means that new combinations of microorganisms are tested on a wide variety of substrates. In these microbial consortia, lactic acid bacteria (LAB) are frequently the major bacterial starters, usually combined with other species such as propionic and acetic acid bacteria and yeasts. The ability of LAB to produce a wide variety of metabolites is a determining factor in the establishment of associations with the interacting microbiota, which can be positive, negative or neutral. In liquid-phase fermentations, the control of these interactions represents a great challenge, due to the rapid rate of bioconversion and the direct liberation of the metabolites into the medium. Therefore, the understanding of the co-culture dynamics is of vital importance. The present review compiles data referring to the microbial consortia involving LAB, traditionally used to obtain artisanal products, as well as the new associations proposed and tested for the development of novel fermented liquid foods and beverages. The different types of interactions that have been found in these microbial consortia are discussed, including some mathematical models that have been proposed to simulate and control the development of fermentations. Some of the strategies, techniques and devices that are being developed and implemented to improve the efficiency of co-cultures are finally presented.

Keywords: fermented beverages; mixed cultures; bacterial interactions; operational strategies.

Contents

1. Introduction

In the modern world, biotechnological processes represent a growing segment in a wide range of industrial sectors, involving both processes aimed at the cleaning of waste streams as well as others for obtaining energy and new products. Regardless of the type of bioprocess, the intervention of consortia of microorganisms is more frequent than that of pure cultures, and frequently the consortia make an important contribution to providing the product with the desired characteristics (Kleerebezem and Loosdrecht, 2007; Smid and Lacroix, 2013). In general, it has been seen that complex consortia are more versatile and robust than pure cultures,

even providing greater resistance to bacteriophage attack (Smid and Lacroix, 2013). But the coexistence of microorganisms in the same niche can lead to positive or negative interactions, having effects on their patterns of growth, adaptation and development, their morphology and their ability to synthesize proteins and secondary metabolites (Bertrand et al., 2014; Nai and Meyer, 2018). Some of these secondary metabolites are used as chemical signals for communication or competition. However, in axenic cultures and under laboratory cultivation conditions in which microorganisms have an excess of nutrients, high water availability and buffered temperature and pH, the genes responsible for secondary metabolism may remain silent (Yunita et al., 2018). It is evident that understanding the influence of interactions in the generation of secondary metabolites is essential if it is wished to modify the chemical composition of food products and maximize the biotechnological synthesis of new bioactive substances (Akone et al., 2016; Bertrand et al., 2014; Englezos et al., 2018). For these reasons, the number of investigations dealing with mixed fermentations in the food field has been constantly increasing over the last two decades (Figure 1).

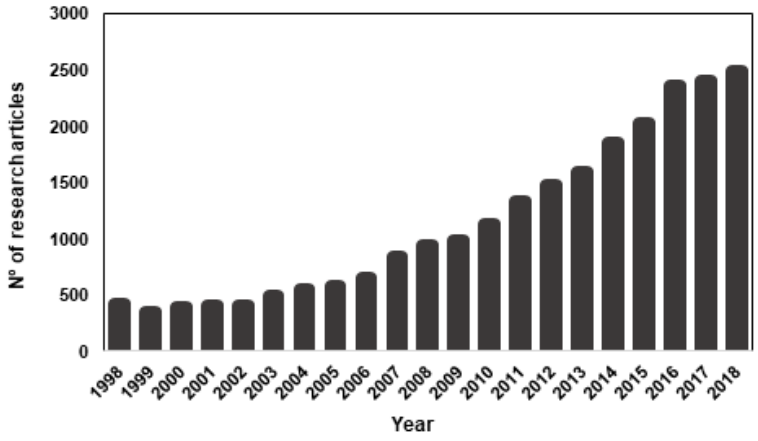

Figure 1 - Evolution of the number of research articles hosted by ScienceDirect website dealing with mixed fermentations in the food field.

Natural fermentation is the oldest technique for obtaining food products with improved properties and preservation capacity during storage. Based on the raw material employed, nine groups of fermented foods can be distinguished: fermented cereals, vegetables, legumes, roots/tubers, milk, meat and fish products, miscellaneous fermented products and alcoholic beverages (Tamang et al., 2016). Most traditional food fermentation processes involve mixed cultures established naturally, in which not only the starter microorganisms participate, but also nonstarter microbiota that contribute to the final product characteristics, particularly in

non-sterile bioprocesses (Gardner et al., 2001; Lu et al., 2018b; Papalexandratou et al., 2011; Yunita et al., 2018). Although spontaneous fermentation is used in the manufacture of most recognized traditional artisanal products, it may lead to greater variation in the sensory characteristics of these products. For this reason, in modern production systems the control of the microbiota by the use of commercial starter cultures is applied to regulate processes, even in the case of traditional products (Franciosa et al., 2018; Gardner et al., 2001; Terzić-Vidojević et al., 2015). The selection and characterization of starter cultures is essential, not only to standardize the organoleptic properties of the product, but also to guarantee its safety by preventing the uncontrolled proliferation of nonstarter microbiota.

The use of biotechnology in the design and production of foods and beverages has led to the development of artificial communities capable of producing an end-product with the desired characteristics. Different combinations of microorganisms allow researchers and manufacturers to make processes more efficient and create novel fermented products with better physical, organoleptic, nutritional or health characteristics (Ahmed et al., 2013; Cheirsilp et al., 2003; Chen et al., 2017). In this regard, a large proportion of recent studies dealing with new combinations of strains, on a great variety of food matrixes, share the goal of designing foods and beverages with functional properties beyond their nutritional value (Coman et al., 2012; Freire et al., 2018; Holko et al., 2013; Lu et al., 2018a).

In food microbial consortia, bacteria, yeasts and fungi may be present. In most associations, both those involved in traditional artisanal fermentation processes and novel industrial starters, LAB are present as major bacterial components, while other groups, such as *Brachybacterium, Brevibacterium,* and *Propionibacterium* constitute secondary bacterial participants (Tamang et al., 2015). LAB also have an important role in the design of new functional foods, because of the probiotic properties of some strains (Coman et al., 2012; Freire et al., 2018; Kedia et al., 2007; Khan et al., 2011; Lu et al., 2018a). Due to its versatility, cheese is one of the fermented foods in which the design of new starters involving microbial consortia has been extensively carried out (Albenzio et al., 2010; Burns et al., 2012; Cruz et al., 2009; Speranza et al., 2018). In solid matrixes such as cheese it is possible to conduct confrontation studies between microorganisms, distinguishing their morphology and being able to delimit their area of chemical interaction (Bertrand et al., 2014).

However, a very important segment in fermented foods that involve LAB coexisting with other microorganisms is that of beverages and products obtained from liquid matrixes, including traditional and artisanal products (e.g. acidified milks and fermented alcoholic beverages) along

with novel products. While in solid-state fermentations the substrates are transformed slowly and steadily, in liquid phase fermentations the process takes place quite quickly (Subramaniyam and Vimala, 2012). This rapid bioconversion, together with the direct release of the metabolites into the culture medium tends to increase the difficulty involved in carrying out studies into interactive behaviour. Nonetheless, reaching an understanding of the relations established in mixed fermentations is extremely important, because these interactions are decisive for quality and economy in processes as delicate as, for example, winemaking (Alexandre et al., 2004).

Within this context, the present review aims to assemble the available updated information on food fermentations in the liquid phase generated by consortia of microorganisms that include LAB (Figure 2).

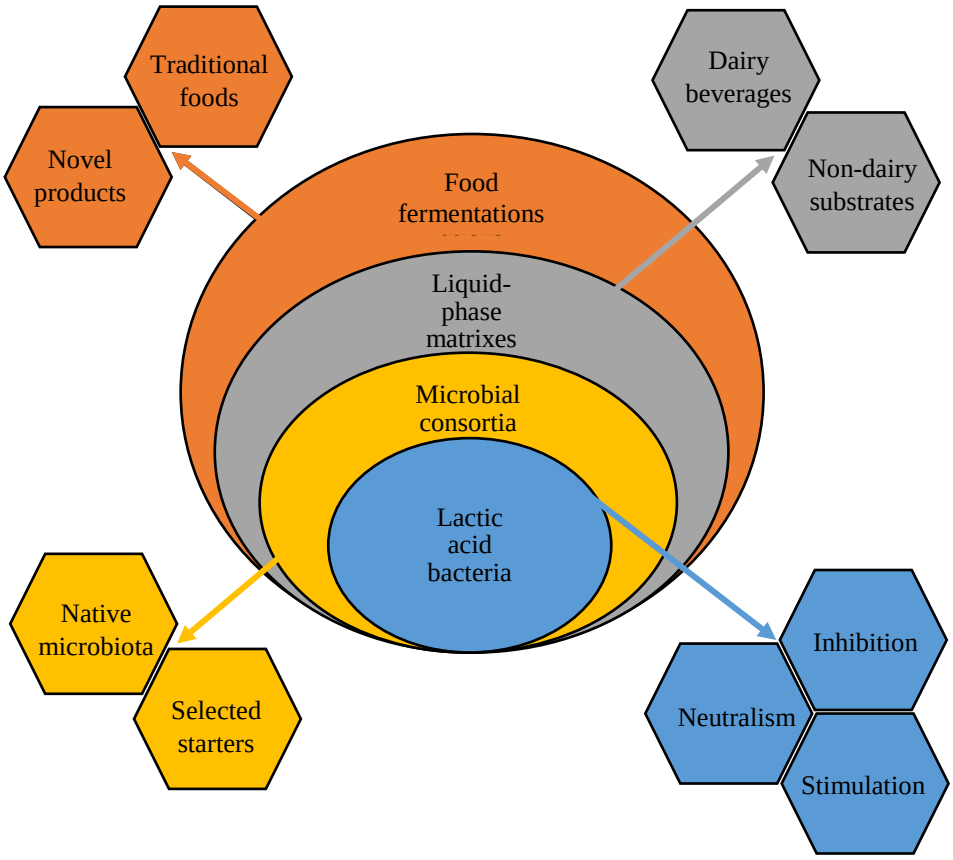

Figure 2 – Aspects to consider around food fermentations in liquid phase involving microbial consortia with LAB.

2. Lactic acid bacteria in mixed food fermentations

As stated above, LAB are the predominant microorganisms present in many traditional fermented foods and beverages, often in combination with other species and cooperating to generate the final product. Thus, the association of bacteria and fungi is very common in fermented dairy products, including alcoholic milks and moldy milks, as well as in cocoa, fruit or cereal fermentations (Tamang et al., 2016). But LAB can also act in consortia with other bacterial species, such as the combination with propionic acid bacteria (PAB) that occurs in semi-hard cheeses with "eyes" (Huc et al., 2014).

LAB are a large group of about 380 bacterial species belonging to 40 genera. The most common genera are *Bifidobacterium, Enterococcus, Lactobacillus, Leuconostoc, Lactococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella* (Kavitake et al., 2017; Tamang et al., 2015), isolated from grains, green plants, dairy and meat products, fermenting vegetables and the mucosal surfaces of animals (Rattanachaikunsopon and Phumkhachorn, 2010). They are Gram-positive, catalase-negative bacteria with the ability to produce large amounts of lactic acid. In this regard, they can be mainly divided in two groups, based on the end-products formed during glucose fermentation. In the case of homofermentative LAB (*Streptococcus*, *Pediococcus*, *Lactococcus* and some lactobacilli) lactic acid is the major end-product of glucose fermentation, two moles of lactate being synthesized per mole of glucose by the Embden-Meyerhof-Parnas pathway. Heterofermentative LAB (*Weissella, Leuconostoc* and some species of *Lactobacillus*) generate equimolar amounts of lactate, CO_2 and ethanol from glucose via the hexose monophosphate or pentose pathway (Rattanachaikunsopon and Phumkhachorn, 2010).

Therefore, in addition to the lactic acid resulting from glucose fermentation, the different LAB strains can produce a variety of compounds such as other organic acids, ethanol, diacetyl, exopolysaccharides, hydrogen peroxide, several enzymes and bacteriocins or bactericidal proteins during lactic fermentations. These metabolites provide the fermented foods with their characteristic texture, colour, flavour and aroma and provide the LAB with functional properties such as antioxidant, fibrinolytic activity and probiotic effects (Kavitake et al., 2017).

The ability of LAB to synthesize such a plethora of metabolites is of enormous significance in the context of their interaction with other microorganisms. Bacteriocins are ribosomally synthesized and extracellularly released peptides or protein molecules with bactericidal or bacteriostatic activities on species closely related to the producers, that is, other LAB strains,

but also on pathogenic and food spoilage microorganisms (Jamuna and Jeevaratnam, 2004). The production of bacteriocins and other antimicrobial factors, such as the accumulation of lactic acid, can be an obstacle to obtaining stable consortia in mixed fermentations (García et al., 2017a), but they allow spoilage or pathogenic microbiota to be excluded from fermented products.

In traditional fermented foods raw materials were mainly spontaneously fermented by the native LAB, but nowadays the protective role of LAB is being consciously exploited in the design of new foods with improved health safety and extended shelf-life, thus avoiding the addition of synthetic preservatives (Casaburi et al., 2016; Siroli et al., 2015). Furthermore, selective addition of LAB starter cultures is being carried out in the dairy, baking, meat, vegetable and alcoholic beverage industries, not only to exclude pathogens, but also to improve the bioprocessing parameters, by accelerating and controlling the process and enhancing the quality of final products (Kavitake et al., 2017; Sánchez et al., 2012; Wouters et al., 2013). The selection of autochthonous starters, compared to allochthonous starters, can be preferable for rapid adaptation and acidification (Mamhoud et al., 2016). For this purpose, LAB strains with the proper physiological and metabolic features are isolated from natural habitats and fermented foods (Abushelaibi et al., 2017; Salvucci et al., 2016). However, turning to the context of mixed fermentations, it must be remembered that the use of LAB in isolation can lead to loss of diversity and the generation of different metabolic activities. As a result, changes in the growth rates, the adaptation to particular substrates and competitive behaviour can occur, giving different results from those observed in natural consortia (Rattanachaikunsopon and Phumkhachorn, 2010).

It has been seen that the ability of LAB to control the development of a mixed fermentation, becoming the dominant partner, tends to be greater in the case of the homofermentative bacteria due to the rapid acidification of the medium. Therefore, although heterofermentative LAB are preferable for some food applications due to their greater influence on the sensory quality of products, they may be displaced in the process of microbial succession. Moon et al. (2018) reported that the control capacity of heterofermentative LAB, such as *Leuconostoc*, may be based on their bacteriocin-enhancing abilities.

3. Liquid food matrixes and interacting microbiota

3.1. Dairy beverages

Traditional fermented dairy foods can be classified in two categories, according to the microorganisms involved. In the first place there are those in which the fermentative process involves only bacteria (including mesophilic and thermophilic microorganisms). Within this category would be the product known as "yogurt", whose name can only be applied commercially to those acidified milks produced by the exclusive action of the LAB strains *Lactobacillus delbrueckii subsp. bulgaricus* and *Streptococcus thermophilus.* But within this first group there are other acidified milks in which, along with the LAB, other bacterial species participate, the LAB usually being dominant. The second category corresponds to fungal-lactic fermentations, involving LAB and yeasts cooperating to generate an acidified milk with characteristic properties (Tamang et al., 2016).

One of the most popular acidified milks obtained through a mixed fermentation system involving LAB and other microorganisms is kefir (Table 1). Kefir is a traditional fermented milk beverage from the Caucasus and Anatolia regions, which is obtained by inoculating milk with microbiologically complex kefir grains. Kefir grains are composed of a microbial consortium embedded in a matrix of kefiran, a water-soluble polysaccharide, which is considered to have antibacterial and antitumor properties and to be a modulator of the gut immune system (Hamet et al., 2013). In the microbial consortium, genera of LAB such as *Lactobacillus, Leuconostoc, Lactococcus* and *Streptococcus* are predominant, *Lactobacillus kefiranofaciens* and *Lactobacillus kefiri* being the dominant species. These two species are thought to be the key LAB in the formation of kefir grains, due to their strong auto-aggregation capacity (Wang et al., 2012). In addition, *L. kefiranofaciens subsp. kefiranofaciens* has been reported to be the organism mainly responsible for the production of kefiran (Hamet et al., 2013).

LAB coexist in kefir with *Pseudomonas, Acinetobacter* and *Acetobacter* (Garofalo et al., 2015), along with yeast species belonging to *Saccharomyces, Kazachstania, Kluyveromyces, Pichia, Issatchenkia* and *Dekkera. Kluyveromyces marxianus,* a lactose-positive species, has been reported as the most dominant yeast found in kefir grains. Its presence, therefore, ensures the metabolism of lactose through alcoholic fermentation, while the presence of lactose-negative yeasts plays an important role in the development of the typical flavour and aroma of kefir

(Dertli and Çon, 2017; Wang et al., 2008). Want et al. (2012) also reported that yeasts play a role in the formation of the kefir grain, with *K. marxianus* and *Pichia fermentans* showing high auto-aggregation abilities.

The consortium of microorganisms in kefir generates a variety of volatile compounds, including alcohols, aldehydes, carboxylic acids, esters, ketones and sulphur compounds that are responsible for the kefir's aromatic profile, carboxylic acids being the most abundant. Nevertheless, the specific microflora of kefir grains and the chemical properties of the final product differs depending on their origin (Wang et al., 2008) and production method (temperature and time of incubation, agitation, ratio of grain to milk, etc.). Differences in the yeast count, affecting ethanol levels, have been detected when different types of milk are employed as substrate. These changes in yeast populations can be attributed to the different production of acids by LAB from pyruvic acid (Gul et al., 2015).

There are other traditional fermented milks whose production process involves a consortium of LAB and yeasts, such as the koumiss or the moldy milks (Tamang et al., 2016). Koumiss is a slightly alcoholic drink popular in Central Asia, which is obtained by inoculating mare's milk with LAB and yeasts belonging mainly to the genera *Kluyveromyces, Saccharomyces* and *Candida.* The proteolytic and lipolytic activities of yeasts gives the koumiss its typical flavour and taste. An influence of the geographical location on the yeast populations has been reported (Mu et al., 2012), just as in kefir. Viili is a ropy, moldy milk from Scandinavia. It is produced by the action of LAB, mainly exopolysaccharide-producing strains of *Lactococcus lactis ssp. cremoris* and a surface-growing, yeast-like fungus, of the *Geotrichum candidum* species (Wang et al., 2008). Most traditional viili cultures also contain yeasts that are believed to stimulate the LAB production of exopolysaccharides.

Besides the fungal-lactic fermentations, there are traditional acidified milks produced by bacterial consortia, in which yeasts do not intervene. This is the case of the combination of LAB and acetic acid bacteria (AAB), one of the most common starters in Japan (Nakasaki et al., 2008). For instance, *L. lactis ssp. cremoris* and *Acetobacter orientalis,* along with *Leuconostoc* and *Gluconobacter* species, are present in the "Caspian Sea yogurt", a fermented milk product from the Caucasus region (Kiryu et al., 2009). *A. orientalis* oxidizes the lactose present in cow milk generating lactobionic acid, an organic acid that is potentially prebiotic.

The appreciated health-giving properties of these traditional fermented milks, together with the growing demand from consumers for products with functional and enhanced properties, has

stimulated the search for novel microbial associations involving LAB and the use of new substrates for the design of new fermented dairy products.

Table 1 – Food matrixes and interacting microbiota in traditional and novel fermented beverages.

Food matrix	Microbiota	Product	References
Dairy substrate	LAB, AAB, yeasts	Kefir	Dertli and Çon (2017), Garofalo et al. (2015), Gul et al. (2015), Hamet et al. (2013), Wang et al. (2008, 2012)
	LAB, yeasts	Koumiss	Mu et al. (2012)
	LAB, yeasts	Viili	Wang et al. (2008)
	LAB, yeasts	Dairy Kombucha	Hrnjez et al. (2014), Malbaša et al. (2009)
	LAB, AAB	Acidified milk	Nakasaki et al. (2008), Kiryu et al. (2009)
	LAB, *Pseudomonas taetrolens*	Acidified milk	García et al. (2017b, 2018)
	LAB, PAB	Acidified milk	Ruijschop et al. (2008), Schwenninger and Meile (2004), Suomalainen and Mäyrä-Mäkinen (1999)
Fruit	LAB, yeasts	Wine	Alexandre et al. (2004)
	LAB, yeasts	Cider	Herrero et al. (1999), Sánchez et al. (2010)
	LAB, yeasts	Kombucha fruit beverage	Zubaidah et al. (2018)
	LAB, yeasts	Fruit-based beverage	Lu et al. (2018a)
Cereal	LAB, aerobic bacteria, yeasts	Sake	Blandino et al. (2003)
	LAB, aerobic bacteria, yeasts	Chinese yellow rice wine	Lv et al. (2012)
	LAB, aerobic bacteria, yeasts	Chicha	Blandino et al. (2003)
	LAB, AAB, yeasts	Kefir beer	Fiorda et al. (2016)
	LAB, AAB, yeasts	Lambic beer	Roos et al. (2018), Spitaels et al. (2015)
	LAB, yeasts	Cereal-based beverages	Freire et al. (2017), Kedia et al. (2007)
Vegetable	LAB, yeasts	Kefir juice	Corona et al. (2016)
Legume	LAB, yeasts	Kefir soybean beverage	Norberto et al. (2018)
	LAB, yeasts	Legume-based beverage	Santos et al. (2014)

The combination of LAB and PAB with the aim of improving the protective effect of LAB antimicrobial compounds has been explored, based on the lower effectiveness of LAB bacteriocins against Gram-negative bacteria, yeasts and moulds. Suomalainen and Mäyrä-Mäkinen (1999) developed a protective culture composed of *Lactobacillus rhamnosus* DSM 7061 and *Propionibacterium freudenreichii* ssp. *shermanii* JS (DSM 7067), which was added to the traditional yogurt starter. The protective co-culture did not interfere with the basic starter, inhibiting yeasts and moulds and generating a fermented milk with a prolonged shelf life. Other authors have tested the addition of other propionic acid strains, such as *Propionibacterium*

jensenii, to the yogurt starters and probiotic LAB like *Lactobacillus paracasei* subsp. *paracasei* (Ekinci et al., 2008; Ranadheera et al. 2012; Schwenninger and Meile, 2004). The antiyeast activity found in fermented milks was attributed to the synergistic action of microorganisms. Combinations of LAB and PAB have also been tested with the aim of producing dairy beverages with satiating potential (Ruijschop et al., 2008), based on the role of the propionic acid as a satiety-inducing trigger.

In relation to lactobionic acid, García et al. (2017a, 2017b, 2018) tested dual mixed fermentations of the probiotic *Lactobacillus casei* CECT 475 and the lactobionic acid producer *Pseudomonas taetrolens* LMG 2336. This strain was chosen due to its 100% selectivity in the bioconversion of lactose, giving high yields and productivities (Alonso et al., 2011). The ability of *L. casei* CECT 475 to use lactobionic acid for its growth, in the absence of another carbon source, was determined (García et al., 2017b) as an essential condition for a truly synergistic effect to occur between a probiotic microorganism and a prebiotic compound. The co-inoculation of *L. casei* and *P. taetrolens* on a dairy substrate, therefore, allowed a fermented product with synbiotic properties and lower lactose content than traditional yogurts to be obtained (García et al., 2018).

The application of the kombucha inoculum for milk fermentation has also been proposed by several authors (Hrnjez et al., 2014; Malbaša et al., 2009). Kombucha inoculum contains LAB (*Lactobacillus kefiranofaciens* subsp. *kefirgranu*), yeasts (*Pichia, Zygosaccharomyces, Saccharomyces, Brettanomyces, Torulaspora, Schizosaccharomyces, Saccharomycodes,* and *Candida*) and non-lactic acid bacteria of the *Acetobacter* and *Gluconobacter* genera. This microbial consortium has been traditionally used for fermentation of sweetened black or green tea. Yeasts convert sucrose to organic acids, carbon dioxide and ethanol. Ethanol is used by AAB to synthesize acetaldehyde and acetic acid. AAB also employ glucose to produce cellulose and gluconic acid (Marsh et al., 2014). The final fermented product contains ethanol, carbon dioxide, high concentrations of acids and other health-promoting metabolites. The application of kombucha as inoculum in milk substrates would generate dairy fermented products similar to yogurt or kefir (Hrnjez et al., 2014).

The search for new mixed starters in dairy fermentations does not only have the objective of obtaining products with improved organoleptic and health properties, but also that of permitting technological improvements. Thus, the combination of yeasts with the traditional yogurt starter (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*) has been tested as a way of obtaining products with extended stability during storage (Liu and Tsao, 2009).

3.2. Fruit, vegetable and cereal substrates

Microbial consortia involving LAB are present in the manufacture of traditional fermented alcoholic beverages such as wine and cider. During the vinification of most red and certain white and sparkling wines, there is an alcoholic fermentation, performed mainly by *Saccharomyces cerevisiae*, with a secondary malolactic fermentation implemented by LAB, which improves the flavour and aroma of the final product (Alexandre et al., 2004). *Lactobacillus, Oenococcus, Pediococcus* and *Leuconostoc* have been reported as the predominant genera during malolactic fermentation in winemaking, *Oenococcus oeni* being considered the best alternative due to its high resistance to the acidic environment and high ethanol levels (Sánchez et al., 2010). The LAB inoculation is usually carried out after the alcoholic fermentation. The growth of the LAB population occurs then until 10^6 CFU mL^{-1} or more, and the malolactic fermentation begins, with the decarboxylation of L-malic acid into L-lactic acid. The same interaction between yeasts and malolactic fermentation is found in cider making (Herrero et al., 1999). Nevertheless, while *O. oeni* has been revealed as the dominant species in winemaking, in cider production there is no clear predominance, but a microbial succession (Sánchez et al., 2010).

Among the products derived from cereal substrates, there are traditional varieties of beer produced not only by the action of *S. cerevisiae*, but by a consortium of microorganisms involving LAB. Belgian lambic beer is traditionally produced by spontaneous fermentation by *Enterobacteriaceae,* LAB species such as *Lactobacillus brevis* and *Pediococcus damnosus,* and yeasts like *S. cerevisiae, S. pastorianus* and *Dekkera (Brettanomyces) bruxellensis* (Roos et al., 2018; Spitaels et al., 2015). Current reviews of the microbial profile, employing techniques such as the MALDI-TOF MS for species identification, have revealed that in modern industrial processes an initial acidification is carried out that prevents the development of *Enterobacteria.* However, the presence of *Acetobacter* species has been discovered (Roos et al., 2018) and the resulting production of acetic acid, together with the LAB metabolism, is responsible for the acidic flavours of lambic beers.

Besides beers, there are other traditional fermented beverages that are based on a cereal substrate. This is the case of sake (wine rice) and Chinese yellow rice wine, both alcoholic drinks which are prepared from rice in a fermentation process involving LAB (*Weissella, Pediococcus*), aerobic bacteria, wild yeasts and sake yeasts (Blandino et al., 2003; Lv et al., 2012). Another example of a traditional beverage based on cereal fermentation, but less well-

known, is chicha, produced from corn fermented by a mixed culture of *S. cerevisiae, Aspergillus* sp., *Acetobacter* sp. and LAB of the genus *Lactobacillus* and *Leuconostoc.* The nutrient profile of cereals makes them an appropriate substrate for LAB growth. The fermentation carried out by LAB can, in addition, eliminate the "green" and unpleasant flavour which is associated with raw cereals, thus improving their organoleptic properties (Peyer et al., 2016).

Within the range of new functional foods, an important segment is also composed of cereal, legume, tuber and fruit-based beverages. These products have become significant in the context of a greater prevalence of lactose intolerances, and the preference for low cholesterol products, leading consumers to avoid dairy beverages. The survivability and growth of LAB, especially probiotic microorganisms, on vegetable substrates can represent a challenge owing to the acidic conditions of the medium (Granato et al., 2010).

Corona et al. (2016) studied the development of kefir inoculum on carrot, fennel, melon, onion, tomato and strawberry juices, finding the same co-dominance of LAB and yeasts as in milky kefir. The possibility of replacing milk by a wide variety of non-dairy matrixes, including honey, soybean and malt, has been tested for obtaining kefir beverages (Fiorda et al., 2016; Norberto et al., 2018; Rodrigues et al., 2016). In the case of substrates such as honey and soybean, which are attributed antioxidant properties due to their high flavonoid content, the fermentation of kefir grains allowed their functional qualities to be increased.

Other authors have studied the development of kombucha inoculums on a fruit substrate, finding improved bioactive properties due to fermentation (Zubaidah et al., 2018). Lu et al. (2018a) designed a new non-dairy beverage involving probiotic *Lactobacillus casei* and the yeast *Williopsis saturnus* var. *saturnus* NCYC22 on a substrate of durian pulp. They implemented a sequential inoculation and reported an improvement in LAB viability and enhanced organoleptic properties with the yeast-*L. casei* pair.

With regard to the design of new starters, Santos et al. (2014) developed a fermented peanut-soy milk involving a combination of different LAB species, including the probiotics *Lactobacillus rhamnosus* LR 32, *Lactobacillus acidophilus* LACA 4, *Pediococcus acidilactici* UFLA BFFCX 27.1 and *Lactococcus lactis* CCT 0360, and the yeast *Saccharomyces cerevisiae*. They found that the combination with yeasts and other LAB species allows shorter fermentation times to be used, counteracting the long time required by probiotic bacteria to reach low pH values. Co-culture fermentations of *Torulaspora delbrueckii* CCMA 0235 with *Lactobacillus plantarum* CCMA 0743 and *L. acidophilus* LAC-04 in order to obtain a non-

dairy beverage on a mixed rice and cassava substrate were tested by Freire et al. (2017). They observed an improvement in the growth of the yeast due to the acid environment generated by the LAB metabolism. In relation to cereal-based beverages, Kedia et al. (2007) tested the combination of *Lactobacillus reuteri* 11951 and yeasts from a malt substrate. An improvement in LAB growth was observed but this was accompanied by a decrease in yeast viability.

4. Types of interactions

The joint action of the mixed starters involved in the production of fermented beverages is responsible for the characteristic properties of each final product. But there are different kinds of relations that may be established between microorganisms (Table 2). First, interactions can be direct or indirect. Second, the effects of interaction on the microorganisms involved can be positive, neutral or negative (Sieuwerts et al., 2008). Based on these effects, five types of indirect interactions are generally distinguished (Fig. 3): mutualism and competition (affecting both strains), commensalism and amensalism (affecting only one of the two strains), and neutralism (Sieuwerts et al., 2008; Smid and Lacroix, 2013). Direct interactions refer to predation and parasitism (Viljoen, 2001).

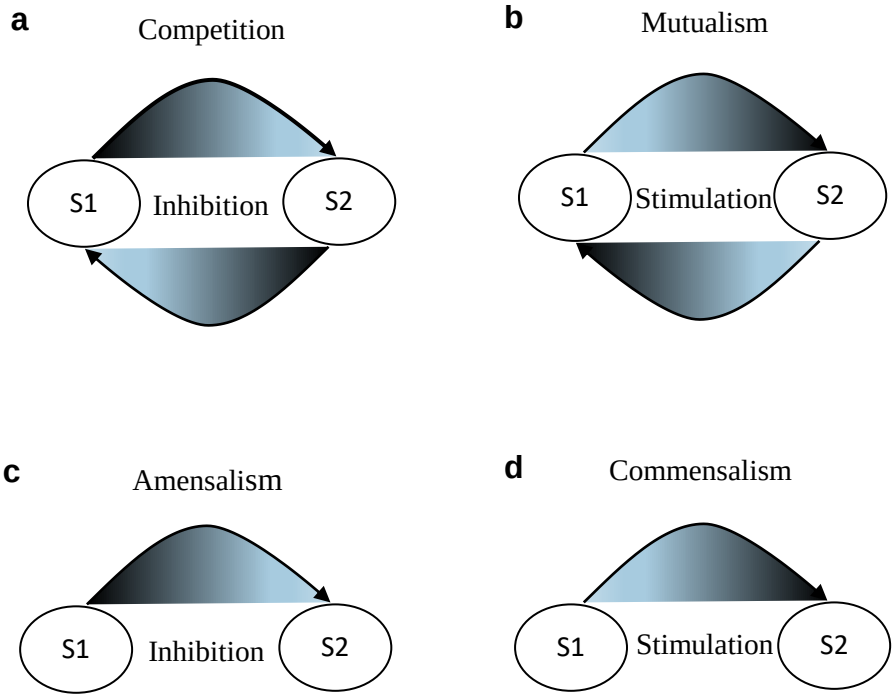

Figure 3 - Scheme of the four most relevant microbial interactions between two strains (S1 and S2) in mixed food fermentations.

Understanding the interaction mechanisms in mixed fermentations is essential for improving the productivity of starters and the quality of the final product. However, sometimes it can be difficult to separate interactions into the discrete groups mentioned above, since more than one interaction can occur simultaneously (Viljoen, 2001). Moreover, the radically different relationships that can be found, even between the same groups of microorganisms, highlights the fact the type of interaction depends very much on the specific strains involved.

Table 2 – Interactions studied in beverages fermented by a consortium of microorganisms.

Interaction	Microbiota	Result	Product	References
Competition	*Lactobacillus paracasei, Candida colliculosa, Saccharomyces dairenensis, Dekkera bruxellensis*	Global inhibition	Acidified milk	Gadaga et al. (2001)
Amensalism	*Oenococcus oeni, Saccharomyces cerevisiae*	LAB inhibition	Wine	Alexandre et al. (2004), Nehme et al. (2010)
	Oenococcus oeni, Saccharomyces cerevisiae	*Saccharomyces cerevisiae* inhibition	Wine	Alexandre et al. (2004)
	Leuconostoc oenos, Saccharomyces cerevisiae	*Saccharomyces cerevisiae* inhibition	Cider	Herrero et al. (1999)
	Bifidobacterium animalis, Williopsis saturnus	*Williopsis saturnus* inhibition	Fruit-based beverage	Lu et al. (2018a)
	L. casei, P. taetrolens	*P. taetrolens* inhibition	Acidified dairy product	García et al. (2017a)
Mutualism	*Lactobacillus kefiranofaciens, Saccharomyces cerevisiae*	Global stimulation	Milky kefir	Cheirsilp et al. (2003)
	LAB, yeasts		Water kefir	Stadie et al. (2013)
	Lactobacillus sp., Gluconacetobacter sp.		Kefir, Kombucha	Yang et al. (2010)
Commensalism	LAB, yeasts	LAB stimulation	Acidified milk	Liu and Tsao (2009), Viljoen (2001)
	Oenococcus oeni, Saccharomyces cerevisiae	LAB stimulation	Wine	Alexandre et al. (2004)
	LAB, yeasts	LAB stimulation	Cereal-based beverage	Kedia et al. (2007)
	L. casei, *Williopsis saturnus*	LAB stimulation	Fruit-based beverage	Lu et al. (2018a)
	LAB, PAB	PAB stimulation	Acidified milk	Ranadheera et al. (2012), Smid and Lacroix (2013)

4.1. Competition

In a competition, two or more microorganisms compete for energy sources and nutrients during fermentation. This kind of interaction affects all interacting parts negatively (Fig. 3a), although the predominance of the strongest microorganism may be temporarily established (Smid and Lacroix, 2013). In general, there are three decisive factors for the establishment of a competitive interaction: high cell density, high niche overlap between species and high spatial mixing. The coexistence of competitors, therefore, is favoured by spatial separation, something that can occur more easily in solid or semi-solid media than in liquid-phase fermentations (Ghoul and Mitri, 2016).

Microorganisms have two mechanisms of competition: indirectly, through the exploitation of resources, and directly, through interference competition in which individual cells damage one another (Ghoul and Mitri, 2016). In exploitative competition, one strain consumes a limiting resource, thus restricting its availability to the other competitor. Carbon sources are usually found in high concentration in substrates but in milk fermentations, for example, nitrogen is limiting. Therefore, in mixed dairy fermentations, the ability to use amino acids determines the capacity to survive and grow. In the first stage of fermentation, microorganisms compete for the amino acids free in milk and later they do the same for the peptides resulting from the activity of proteolytic enzymes (Sieuwerts et al., 2008). Competitive exploitation also occurs when one strain exploits the products secreted by another strain for its own use. Digestive enzymes or siderophores, for instance, are costly to synthesize and are released outside the producing cell, where other microorganisms of the consortium can benefit from them (Ghoul and Mitri, 2016). In interference competition, the synthesis of antimicrobial compounds may be involved. Gadaga et al. (2001) described antagonistic interactions between some LAB and yeast strains in a traditional African fermented milk due to the production of inhibitory substances. They found suppression of *Lactobacillus* growth that could be attributed to the production by the yeast of free fatty acids that exert a negative effect on the LAB. In another case, lower viable counts of yeasts were reported when co-cultured with *Lactobacillus. paracasei subsp. paracasei* Lb11, implying an antagonistic effect between LAB and yeasts.

4.2. Amensalism

As represented in Fig. 3c, amensalism is characterized by an interaction in which one of the microorganisms negatively affects the other, without being affected itself (Pommier et al., 2005;

Sieuwerts et al., 2008). This type of relation is typically found in food fermentations involving LAB, due their production of large amounts of acid and antimicrobial compounds, such as bacteriocins, which restrict the growth of sensitive microorganisms, while the producing strains remain unaffected.

Cases of amensalism have also been detected in food consortia involving LAB and yeasts, in which yeasts exert a negative effect on LAB and vice versa. In winemaking, the ability of certain yeast strains to inhibit the growth of malolactic bacteria, thus exerting a negative impact on the acidification of the wine, has been commonly reported. The ethanol produced by yeasts during alcoholic fermentation is not the only cause of this negative impact, and in fact affects the growth of LAB more than their malolactic activity. Other inhibitory metabolites have been identified, such as the sulphur dioxide (SO_2) produced by certain strains of *S. cerevisiae*, and medium chain fatty acids such as decanoic acid (Alexandre et al., 2004). Nehme et al. (2010) also discovered an inhibitory peptidic fraction synthesized by *S. cerevisiae* which is strain dependant.

In winemaking, the sequential process is the most widely used practice, but studies have been carried out testing simultaneous fermentation (Massera et al., 2009). When the simultaneous process is implemented, reverse amensalism may occur: the inhibition of yeast by LAB activity. It was determined that acetic acid, propionate and acetate limit the growth and alcohol production of *S. cerevisiae*. Another factor responsible for the inhibition could be the yeast cell wall degradation induced by LAB (Alexandre et al., 2004). This negative effect of the LAB on yeasts has also been observed in the process of cider production. Herrero et al. (1999) studied the coexistence of *S. cerevisiae* and *Leuconostoc oenos* on a substrate of apple juice. They reported a delay in the initial sugar consumption during the alcoholic fermentation due to the presence of *L. oenos*.

Yeast inhibition by LAB has also been reported in the design of new non-dairy fermented beverages including probiotic *Bifidobacteria*, due to their production of high acetic acid concentrations. Acetic acid penetrates the yeast cells, causing the acidification of the cytosol and membrane permeabilization, and consequently blocking their metabolic activity and growth (Lu et al., 2018a).

In addition to the LAB-yeast pairing, LAB dominance has also been observed during its coexistence with other bacterial species. García et al. (2017a) found an amensalistic interaction between *L. casei* and *P. taetrolens* coexisting in culture to obtain acidified milks enriched in

lactobionic acid. The possible release of inhibitory lactic metabolites such as bacteriocins, and the accumulation of lactic acid in the fermentation medium, caused inhibition of the growth of *P. taetrolens*. The physiological status of the microorganisms was monitored using flow cytometry, which detected the appearance of a viable but non-culturable (VBNC) population of *P. taetrolens* after only 8 hours from inoculation because of the stress. In terms of production, coexistence resulted in a reduction in the lactobionic acid synthesized by 87% compared to pure cultures of *P. taetrolens*. In contrast, *L. casei* was able to preserve its viability and productivity in the presence of the *Pseudomona,* not being damaged or improved.

4.3. Mutualism

In mutualism, all microorganisms involved benefit from the interaction (Fig. 3b). This is the typical relation that occurs between *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* in yogurt, improving the growth rate of both bacteria (Sieuwerts et al., 2008). But this kind of synergy can also be triggered when LAB combine with other microorganisms.

In general, mutualism occurs between LAB and yeasts in milky kefir. In this product, yeasts provide growth factors to the bacteria (amino acids, vitamins and other compounds), and the bacterial end-products are employed by yeasts as energy sources, resulting in a stable product (Viljoen, 2001). In this context, Cheirsilp et al. (2003) described a mutualistic interaction between the dominant LAB species in kefir, *Lactobacillus kefiranofaciens*, and a strain of *Saccharomyces cerevisiae* (IFO0216) capable of employing lactic acid as a carbon source. In addition to the decrease in lactic acid concentration, the yeast synthesizes metabolites that also stimulate the development of *L. kefiranofaciens* and kefiran production. The yeast employs the fermentation products of *L. kefiranofaciens* (lactic acid, glucose and galactose) as energy sources, establishing the mutualistic relationship.

Stadie et al. (2013) reported the same mutualistic interaction between the predominant LAB and yeasts used as inoculum in water kefir: *Lactobacillus hordei, L. nagelii, Zygotorulaspora florentina* and *Saccharomyces cerevisiae*. Higher cell yields in co-cultivations were found, compared with single water kefir isolates, for both LAB and yeasts. *L. hordei* and *L. nagelii* are considered auxotrophic microorganisms, needing some amino acids and vitamin B_6 for their growth. Stadie et al. (2013) found that these essential nutrients are released by yeasts when

stimulated by the presence of the LAB. The improvement in yeast growth was attributed to the drop in the pH of the medium caused by the synthesis of lactic and acetic acids by LAB.

Mutualism has also been found between LAB and other bacteria. In fermented beverages such as kefir and kombucha, a symbiotic interaction has been described between LAB and AAB (Marsh et al., 2014; Yang et al., 2010). Yang et al. determined the capacity of *Lactobacillus* sp. to support the growth of *Gluconacetobacter* sp. A4. The xylitol and acetic acid produced by LAB were used by G. sp. A4 for growth and to synthesize d-saccharic acid 1,4 lactone, one of the components of kombucha with functional properties.

4.4. Commensalism

Commensalism occurs when one of the microorganisms that coexist is favoured by the interaction, while the other does not suffer any effect (Fig. 3d), either good or bad (Sieuwerts et al., 2008). A commensalistic relation was described by Viljoen et al. (2001) between *Lactobacillus acidophilus* and *Kluyveromyces fragilis* in acidophilus-yeast milk. The presence of lactose-fermenting yeasts exerts a stimulating action, increasing the number of viable LAB. Liu and Tsao (2009) also described an enhancement in viability of LAB when co-cultured with yeasts, improving the stability of cultures of fermented milks and yogurts during storage. Nevertheless, they found that yeast extracts had only a limited effect, which implies that there must be other mechanisms that enhance LAB viability in addition to the synthesis of nutrients and growth factors.

Although less frequently, commensalistic relations have also been reported between yeasts and LAB during winemaking, in which yeast stimulates malolactic bacteria. This stimulation seems to be related to the rate of yeast autolysis and its release of substances such as nitrogenous components (Alexandre et al., 2004). As stated before, amensalistic interactions have been determined more typically between LAB and yeasts in winemaking, so strain compatibility is a determining factor (Alexandre et al., 2004). But the must composition and the timing of inoculation also influence the success of the process (Massera et al., 2009). Thus, simultaneous inoculation can be beneficial for the development of malolactic fermentation, for the simple reason that the LAB find a substrate in which all the nutrients are available, and also because they can adapt gradually to the increasing concentration of alcohol. In co-culture, bacteria are present from the beginning, having enough time to grow properly and consume the malic acid in a medium free of the yeast inhibitory compounds (Nehme et al., 2010). Massera et al (2009)

studied the early inoculation of *O. oeni*, 12 h after yeast inoculation. They found a reduction of between 7 and 14 days in fermentation time with respect to the traditional inoculation practice, without any negative effect either on LAB or the yeast population.

Commensalistic interactions in which LAB are stimulated by yeasts have also been reported in novel non-dairy beverages. Kedia et al. (2007) found an increase in the concentration of *L. reuteri* in mixed cultures with a yeast strain due to the supply of essential nutrients by the yeast metabolism. An improvement in probiotic *L. casei* L26 survival was reported by Lu et al. (2018) when adding a culture of *W. saturnus* var. *saturnus* NCYC22.

But commensalistic interactions have also been detected between LAB and other bacterial species. Thus, a typical case of commensalism occurs between LAB and PAB, although on this occasion the PAB is favoured by the action of LAB. It was seen that the lactic acid synthesized by LAB stimulates the growth of the PAB and is used as a preferential carbon source to produce acetic and propionic acids (Smid and Lacroix, 2013). Ranadheera et al. (2012) also reported an improvement in growth and viability of *Propionibacterium jensenii* 702 due to the proteolytic activity of *L. delbrueckii* subsp. *bulgaricus* liberating essential amino acids in a goat milk substrate.

5. Modelling the growth dynamics in mixed cultures

Although mathematical models are powerful devices for simulating and understanding the development of fermentations, there are only a limited number of studies on the kinetics of mixed cultures.

5.1. The Classical Model

An early theoretical model for the interaction between two species competing in the same ecological niche was developed as an extension of the Logistic Model of single population growth. This Classical Model of competition is given by the following equations (Vadasz et al., 2003):

$$\frac{dx_1}{dt} = [\mu_1 - \gamma_1(h_1X_1 + h_2X_2)]X_1$$

$$\frac{dx_2}{dt} = [\mu_2 - \gamma_2(h_1X_1 + h_2X_2)]X_2$$

Where X_1 and X_2 correspond to the biomass concentration of species 1 and 2 (gL^{-1}) and μ_1 and μ_2 their corresponding maximum specific growth rates in isolation (h^{-1}). γ_1 and γ_2 represent food utilization factors for each species, which are dependent, for instance, on their metabolic capacity to use the available nutrients.

But in this classical model, only nutrient depletion impacts on the death rates of the respective species. According to this system, one or both species involved is doomed to die, coexistence being impossible unless a limiting condition is applied, according to "Volterra's Competitive Exclusion Principle" (Vadasz et al., 2003).

5.2. Models considering the production of inhibitory metabolites

The Classical Model has been modified to reflect the more complex situations that take place in microbial interactions. Coefficients have been introduced that consider not only the effect of substrate availability on the death rate, but also the presence of metabolic toxins and other environmental factors.

In this regard, a microbial culture containing two species in liquid-phase can be represented by the following equations (Kedia et al., 2009; Vadasz et al., 2003):

$$\frac{dx_1}{dt} = [\mu_1 - \gamma_1(h_{11}X_1 + h_{12}X_2)]X_1$$

$$\frac{dx_2}{dt} = [\mu_2 - \gamma_2(h_{21}X_1 + h_{22}X_2)]X_2$$

Coefficients can be combined in the following way:

$$\frac{dx_1}{dt} = (\mu_1 - \beta_{11}X_1 - \beta_{12}X_2)$$

$$\frac{dx_2}{dt} = (\mu_2 - \beta_{22}X_2 - \beta_{21}X_1)$$

In this Modified Classical Model, the coefficient β_{ii} represents a factor which regulates the population density by not only considering nutrient depletion but also the possible presence of toxic or harmful compounds of metabolic or other origin.

This model constitutes a "macroscopic" approach, introducing the possible global effect of an antimicrobial substance as a lumped parameter (Vadasz et al., 2003). An example of a

"microscopic" approach is that proposed by Ramon-Portugal et al. (1997), since it tries to describe in a specific way all possible known effects.

Ramon-Portugal et al. (1997) proposed a mathematical model for a typical case of amensalism in mixed liquid-phase fermentations, involving a killer strain which produces an antimicrobial compound, and a sensitive microorganism. They segregated the microbial biomass into four populations: the viable killer cells ($X_{v,k}$), the viable sensitive cells ($X_{v,s}$), the dead killer cells ($X_{d,k}$) and the dead sensitive cells ($X_{d,s}$).

In the case of the sensitive microorganism, in addition to the natural death rate, a term was introduced to consider the killer effect due to the toxicity of the medium, resulting in the following equations:

$$r_{d,k} = \mu_{d,k} X_{v,k}$$

$$r_{d,s} = \mu_{d,s} X_{v,s} + K X_{v,s} T$$

Where $r_{d,s}$ is the death rate of the sensitive strain and $r_{d,k}$ is the death rate of the killer strain (cells $mL^{-1}h^{-1}$); μ is the specific "natural" death rate (h^{-1}), K is the killing rate ($g^{-1}Lh^{-1}$) and T is the concentration of the antimicrobial substance in the medium (gL^{-1}).

This kinetic model was proposed after growing mixed cultures in bioreactors and determining biomass by microscopic counting and spread plating using the methylene blue-staining method (Ramon-Portugal et al., 1997). However, there are certain limitations to quantifying the biomass concentration of each strain in mixed liquid fermentations. Therefore, membrane bioreactors (MBR) are used as a specific tool for studying indirect interactions between microorganisms (Brandam et al., 2016; Nehme et al., 2010; Pommier et al., 2005). In MBR, microorganisms are kept in a homogenous liquid phase separated by a porous membrane that allows substrates and metabolites to pass through. The different strains grow separately, and are sampled distinctly, and no further differentiation is necessary (Albasi et al., 2001; Manjarrez et al., 2000).

Pommier et al. (2005) reviewed the kinetic model of Ramon-Portugal by using an MBR and they detected the existence of a lag phase before the killing effect begins. In the first stage (S1), therefore, both the dominant and the sensitive strain would only be affected by "natural" death. Pommier et al. (2005) developed a two-step kinetic model. The expression of the sensitive cell death rate in S1, therefore, was modified as follows:

$$r'_{d,s} = \mu_{d,s} X_{v,s}$$

This lag phase would correspond to the time necessary for the toxin to reach a sufficient concentration to cause the inhibition of the sensitive strain. It has been seen that the synthesis of LAB antimicrobial compounds such as bacteriocins begins at the end of the exponential growth phase, reaching its highest concentration in the middle of the stationary phase (Jamuna and Jeevaratnam, 2004). The delay in the onset of the inhibitory effect has also been reported by other authors in amensalistic interactions involving LAB (García et al., 2017a).

5.3. Models considering the effect of substrate consumption

Membrane bioreactors have also been used to develop mathematical models based on the effect of the concentration of substrates, comparing substrate consumption between pure and mixed fermentations. Brandam et al. (2016) designed an equation for predicting L-malic acid consumption by mixed LAB cultures in winemaking. They were based on the kinetic model previously developed by Fahimi et al. (2014) for evaluating and quantifying the link between the specific growth of *Oenococcus oeni* and the specific L-malic acid consumption. Fahimi et al. (2014) proposed the following equation:

$$v = k_i \times \mu \times \frac{[mal]}{[k_{mal}] + [mal]'}$$

Where v is the specific L-malic acid consumption rate ($gL^{-1}h^{-1}$), μ is the specific growth rate (h^{-1}) and [*mal*] is the L-malic acid concentration (gL^{-1}). k_i is a parameter representing the coefficient of proportionality between v and μ. k_{mal} (gL^{-1}) is a substrate limitation parameter so that a low value of k_{mal} means that the microorganism is able to grow with a low concentration of malic acid in the medium, and a high value implies that it requires a certain minimum concentration of substrate to allow successful growth.

In mixed fermentations, the consumption of L-malic acid can be calculated using the experimental data of biomass concentration for the interacting microorganisms (Brandam et al., 2016):

$$\frac{d[mal]}{dt} = X_{s1,mixed} \times k_1 \times \mu_1 \times \frac{[mal]}{[mal] + k_{mal1}} + X_{s2,mixed} \times k_2 \times \mu_2 \times \frac{[mal]}{[mal] + k_{mal2}}$$

In this equation, s1 and s2 are the interacting strains; X is the biomass concentration (optical density units, OD) and t the time in hours.

Comparing predicted substrate consumption with the experimentally determined consumption makes it possible to determine if the presence of one strain causes an effect on the intrinsic capacity of the other strain to consume L-malic acid. If there is no interaction, both consumption values will be identical. This system allowed Brandam et al. (2016) to detect competitive, commensalistic and predominance interactions (coexistence affected the growth of the faster strain negatively and that of the slower one positively).

5.4. Models to formulate the effect of stimulatory metabolites

In addition to the effect of environmental factors, substrate concentrations and antimicrobial compounds, the effect of stimulatory metabolites should also be considered in commensalistic and mutualistic interactions. In this regard, Aghababaie et al. (2015) designed a Monod type model to describe the symbiotic interaction between the LAB starters of yogurt (*L. bulgaricus* and *S. thermophilus*). Previous studies had considered the acidifying activity of the yogurt starter system without introducing an interaction factor (Sodini and Latrille, 2000). However, both microorganisms produce metabolites which are favourable for the growth of the other strain. Non-structured kinetic models, such as the Monod, Haldane and Gompertz models, are widely used for describing microbial growth and product formation (Zajše and Goršek, 2009).

In the model developed by Aghababaie et al. (2015), the effect of *L. bulgaricus* on *S. thermophilus* is described by the following equation:

$$f_L^S = Z\frac{C_{pep}}{C_{pep} + K_{pep}}$$

Where Z is a coefficient, C_{pep} is the concentration of metabolites produced by *L. bulgaricus* which stimulate the growth of *S. thermophilus* (μgL^{-1}) and K_{pep} is the Monod parameter for metabolites produced by *L. bulgaricus* (μgL^{-1}). The effect of *S. thermophilus* on *L. bulgaricus* would be given by a parallel equation.

6. Strategies to improve the bioconversion parameters in mixed food fermentations

The possible negative interactions between microbiota in liquid-phase co-cultures, together with the possibility of enhancing their benefits, has promoted the development and testing of different techniques for implementing mixed fermentations. Due to their food interest and the capacity of LAB to produce a wide range of interacting metabolites, many studies involve

microbial consortia of these bacteria, developing culture techniques that could be applied to other combinations of strains in which the metabolic relationship is similar (Table 3).

6.1. Sequential inoculation

As mentioned above, sequential inoculation has been used traditionally in wine and cider making. Despite the benefits found by simultaneously inoculating LAB and yeasts in the must (Massera et al., 2009; Nehme et al., 2010), the introduction of LAB once the alcoholic fermentation has been completed avoids the excessive synthesis of d-lactate and acetate from sugars due to the heterofermentative metabolism of LAB. In addition, it allows the products of yeast excretion and autolysis to be available for the LAB from the beginning of malolactic fermentation (Herrero et al., 1999).

In a sequential fermentation system, differentiated stages can be implemented in which different operational conditions are applied, thus optimizing all subprocesses. In wine and cider-making, the establishment of a temperature of 15 ºC during alcoholic fermentation prevents the formation of undesirable volatile end-products due to the yeast metabolism. However, a higher temperature of 22 ºC has been reported as optimal for malic acid degradation, decreasing the time necessary for its complete consumption from 15 to 9 days (Herrero et al., 1999).

This advantage of sequential design in allowing the use of the most favourable conditions for each microorganism in turn is currently being explored in the development of novel dairy and non-dairy fermented beverages involving LAB (García et al., 2017b, 2018; Lu et al. 2018a). Lu et al. (2018a) introduced a LAB probiotic culture into a durian pulp substrate, incubating it in the first place at 37 ºC, until the maximum cell population was reached. In the second stage, a culture of *W. saturnus var. saturnus* NCYC22 was inoculated and cultivated at 30 ºC. The introduction of the yeast resulted in an improvement in the survival of *L. casei* L26, while the yeast population remained stable. However, the sequential co-culture design was not effective for the pair *Bifidobacterium animalis* subsp. *lactis* B94-yeast due to inhibition of the yeast.

In sequential design it should be considered that microorganisms in consortia may interact and influence each other's metabolism through physical contact, via signalling molecules or by generating changes in the physicochemical properties of the environment which trigger a response in the other strains (Sieuwerts et al., 2008). Consequently, even if there is no coexistence, the development of the sensitive strain may be inhibited in a medium fermented by the dominant microorganism.

In this regard, García et al. (2017b, 2018) prevented the competitive exclusion exerted by LAB antimicrobial compounds on the sensitive *P. taetrolens* by inoculating LAB in second place in dairy substrates. *L. casei* was able to develop in the *P. taetrolens* fermented medium achieving a lactic acid yield/substrate of 0.95 gg^{-1}, revealing the global process effectiveness.

Therefore, in addition to optimizing the environmental conditions for each microorganism, sequential inoculation can avoid possible amensalistic interactions between the new microbial consortia explored, by inoculating first the sensitive strain to favour the correct development of its metabolism and introducing the inhibitory strain in a second stage. In this regard, a key parameter in sequential fermentations is the duration of the interval between first and second inoculation (Ciani et al., 2014). The period when only the first microorganism is active can affect the duration of its own metabolic activity but consequently also the concentration of substrates, products and other metabolites interacting with the second microorganism. The length of the first stage will also be the determining factor for the pH value of the medium. In the case of LAB, it has been reported that a pH value of 5 corresponds to the maximum acceptable level of acidity for the onset of this second stage (García et al., 2017b).

Table 3 – Summary of strategies to improve the bioconversion parameters in mixed food fermentations.

Strategy	References
Sequential inoculation	García et al. (2017b, 2018), Herrero et al. (1999), Lu et al. (2018a)
Immobilization through encapsulation	Devanthi et al. (2018a, 2018b), Doleyres et al. (2004a, 2004b), Grattepanche et al. (2007)
Long-term continuous culture	Doleyres et al. (2004a, 2004b), Grattepanche et al. (2007)
Suppression of antimicrobial production	Ariana and Hamedi (2017), Liu et al. (2006), Maldonado et al. (2004), Ruiz-Barba et al. (2010)
Enhancement of antimicrobial production	Domínguez-Manzano and Jiménez-Díaz (2013)

6.2. Microbial encapsulation and long-term continuous culture

Microbial encapsulation is considered to be the most promising technique for providing microorganisms with a protective environment against adverse conditions and is mainly used in dairy foods for keeping probiotics alive. In food processing the addition of large particles can affect the texture and sensory properties of the product, a risk which restricts the size of the

capsules that might be used for protective purposes and leads to microencapsulation being generally considered as the most appropriate technology. Most microcapsules produced for food applications are generated by extrusion and emulsion techniques (Heidebach et al., 2012), spray-coating, spray-drying and gel-particle technologies also being applied (Champagne and Fustier, 2007). Alginate, starch, gellan-gum, xanthan, carrageenan, locust-bean-gum, cellulosic materials and mixtures thereof are commonly selected as supporting materials.

It has been seen that these encapsulating biopolymers can act as a physical barrier limiting mass transfer between the bead core and the external environment (Heidebach et al., 2012). Other authors have reported that the encapsulation process itself would increase the expression of stress-related genes, thus helping cells to cope with the stress generated by the inhibitors. Accordingly, entrapment may increase the microorganism's ability to ferment inhibitor-containing media (Westman et al., 2012).

With its effectiveness based on the limited diffusion of inhibitory substances such as metabolic products, organic acids and bacteriocins through the bead, and the greater stress resistance of the entrapped cells, encapsulation is being tested as another strategy to produce competitive mixed cultures (Smid and Lacroix, 2013).

In this context, Doleyres et al. (2004a) reported that immobilized cell technology can be used to control the strain ratio of a mixed culture involving dominant LAB (*Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* MD) and a less competitive strain (*Bifidobacterium longum* ATCC 15707). They designed a two-stage fermentation system with two reactors in series, continuously fed with MRS medium. In the first stage (R1) both strains were grown encapsulated separately in k-carrageenan/locust bean gum gel beads. Then, the second bioreactor (R2) was inoculated with free cells released from the first reactor. Encapsulated cells of *B. longum* were more resistant in R1 to lactic and acetic acids synthesized by *L. diacetylactis*, thus avoiding its competitive exclusion. In subsequent studies Doleyres et al. (2004b) reported a global increase in the stress tolerance, for both *L. diacetylactis* and *B. longum*, after the process was completed. Bacteria were more resistant to freeze-drying, hydrogen peroxide, simulated gastrointestinal conditions, antibiotics and the bacteriocin nisin Z.

Grattepanche et al. (2007) tested the same continuous inoculation and prefermentation system with an antagonistic mixed culture of a nisin Z producer strain (*Lactococcus lactis* ssp. *lactis* biovar. *diacetylactis* UL719) and two nisin-sensitive strains: *Lactococcus lactis* ssp. *cremoris* ATCC19257 (highly acidifying) and *Lactobacillus rhamnosus* RW-9595M (exopolysaccharide

producer) on a milky substrate. They reported an increase in nisin tolerance for *L. rhamnosus*, leading to an enhanced synthesis of exopolysaccharide. They also reported higher milk acidification capacity for *L. diacetylactis* and *L. rhamnosus*.

Encapsulation strategy, but without prefermentation, has also been tested in antagonistic mixed cultures of LAB and yeasts for producing soy sauce. During the second stage of soy sauce fermentation (*moromi*), the predominant microbiota are the LAB *Tetragenococcus halophilus* and the yeast *Zygosaccharomyces rouxii*, which synthesize volatile compounds, amino acids, peptides and sugars that contribute to the final flavour. However, an antagonistic relation is established between the two microorganisms in which alcoholic fermentation due to *Z. rouxii* can be supressed by acetic and lactic acids synthesized by the LAB, and excessive alcohol production by *Z. rouxii*, together with the aerobic culture conditions, can cause growth inhibition of the LAB (Devanthi et al. 2018a, 2018b). Devanthi et al. (2018a) investigated the possibility of a sequential inoculation, introducing *Z. rouxii* in a second stage. Although they observed an enhanced flavour profile in this sequential process, the antagonistic interaction remained. However, the application of a water-oil-water double emulsion for segregating *T. halophilus* and Z. *rouxii* allowed the antagonistic interaction between them to be minimized (Devanthi et al., 2018b). The oil layer functioned as a physical barrier, preventing the passage of inhibitory metabolites, but the existence of possible physiological changes in *T. halophilus*, increasing its tolerance against *Z. rouxii* inhibition, was also reported.

6.3. Suppression and enhancement of LAB production of antimicrobials

In mixed cultures, LAB bacteriocins allow the producer strains to control the fermentation. It has been seen that the synthesis of bacteriocins by some strains is a constitutive process. However, in other cases bacteriocin production is controlled by a three-component regulatory system, involving specific autoinducer peptides, a histidine protein kinase and a response regulator, working *via* quorum sensing, and this system can be activated by co-culture with bacteriocin-inducing-strains (Ruiz-Barba et al., 2010). This phenomenon has been investigated by some authors to enhance LAB survival by stimulating their production of bacteriocins through co-cultivation with inducing strains (Maldonado et al., 2004; Ruiz-Barba et al., 2010). Other strategies are based on the introduction of auxiliary strains to consume lactic acid, whose accumulation in the fermentation medium inhibits the biosynthesis of bacteriocins such as nisin (Ariana and Hamedi, 2017; Liu et al., 2006).

The undesirable side-effects of competitive interactions have also motivated studies investigating ways to suppress bacteriocin production. Domínguez-Manzano and Jiménez-Díaz (2013) reported a reduction in bacteriocin synthesis by co-culturing the bacteriocin-producer strains *Lactobacillus plantarum* NC8C and *Enterococcus faecium* 6T1a. In *E. faecium* there was a decrease in the enterocin activity from 5420 BU/ml in single cultures to 720 BU/ml in co-cultures. In the case of *L. plantarum*, plantaricin activity was completely supressed. The same phenomenon was found for bacteriocin-producing co-cultures involving different strains, proving that this is not specific to the *L. plantarum – E. faecium* combination. Other research, employing a two-compartment system, has shown that the bacteriocin suppression is not cell-cell contact-dependent.

7. Novel microbiological tools for monitoring food ecosystems

The need to improve the efficiency and competitiveness of industrial processes for the manufacture of traditional fermented foods, as well as the growing demand for novel and functional products, requires the use of modern techniques for the investigation and development of microbial co-cultures. In addition to MBR and sequential and encapsulating designs, new microbiological tools such as miniaturized versions of classical cultivations, microfluidic devices and 3D-bioprinting, constitute new devices for the study of microbial communities (Nai and Meyer, 2018).

Moreover, the presence of VBNC populations in food systems highlights the relevance of experimental techniques such as flow cytometry as an alternative to the traditional microbiological techniques (plate counts, isolation, and biochemical identification) for monitoring the physiological state of cells in microbial co-cultures (García et al., 2017a). At industrial level, traditional techniques are being replaced by culture-independent molecular methods that are directly applied to a sample. D/TGGE is widely used in food fermentations for profiling microbial populations and following their dynamics over time (Cocolin et al., 2013). However, in recent years, food microbiota studies are more commonly based on sequencing, including high-throughput sequencing (HTS), metataxonomics and metagenome sequencing. Metagenomics allows us not only to determine the taxonomic composition of the food ecosystem, but also and more importantly, to determine the relationships between microorganisms, their activities and function, identifying the enzymes synthesized, their metabolic pathways and mechanisms of action (Franciosa et al., 2018).

8. Conclusions

The growing demand of consumers for new functional products, containing the lowest possible amount of chemically synthesized additives, will imply in the future market an increasing presence of foods and beverages that have been biotechnologically manufactured. The possibility of organoleptic improvement and the enrichment in bioactive compounds derived from the use of microbial co-cultures, instead of pure starters, makes the understanding of the interactions between strains indispensable. It is of particular interest to elucidate the role of LAB in these associations, as participating or even predominant microorganisms in the vast majority of food fermentative processes.

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3. MATERIALES Y MÉTODOS

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3.1. Microorganismos

Se emplearon dos bacterias capaces de producir ácidos orgánicos mediante el aprovechamiento de la lactosa contenida en sustratos lácteos.

Como BAL se empleó la cepa probiótica *Lactobacillus casei* CECT 475 (Colección Española de Cultivos Tipo, Valencia, España). Se trata de una bacteria anaerobia facultativa, Gram-positiva, ampliamente utilizada en la industria alimentaria dentro del mercado de los alimentos funcionales debido a sus propiedades potencialmente probióticas. *L. casei* se mantuvo congelada a -20 ºC en una solución de glicerol al 40% (v/v), siendo posteriormente subcultivada en placas de medio MRS comercial (de Man Rogosa and Sharpe, Biokar Diagnostics, Francia) con 2% (p/v) de agar. Las placas se incubaron durante 48 horas a 30 ºC y se almacenaron a 4 ºC hasta su uso.

Para la producción de ácido lactobiónico se empleó la bacteria *Pseudomonas taetrolens* LMG 2336 (Colección Coordinada Belga de Microorganismos, Gante, Bélgica). Es una bacteria estrictamente aerobia, Gram-negativa, no patogénica, de tipo silvestre y capaz de oxidar la lactosa con un 100% de selectividad para el ácido lactobiónico y elevados rendimientos de conversión. *P. taetrolens* se mantuvo conservada de igual forma en solución de glicerol, y a continuación fue subcultivada en placas de agar de medio NB (Nutrient Broth, conteniendo 1 g/L de extracto de carne, 2 g/L de extracto de levadura, 5 g/L de peptona y 5 g/L de NaCl). Las placas fueron incubadas durante 48 horas a 30 ºC y preservadas a 4 ºC.

3.2. Preparación de sustratos

3.2.1. Suero dulce de queso

Se empleó permeado de ultrafiltración de suero dulce de queso suministrado por Industrias Lácteas Asturianas S.A. (Navia, Asturias, España), con una composición inicial de 200 g/L de lactosa y una pequeña concentración de sales minerales (12-15% de los sólidos totales, que a su vez suponen menos del 5% en el permeado de ultrafiltración). Se empleó el método de Bradford (Bradford, 1976), determinándose un contenido insignificante de proteínas.

El permeado de suero se diluyó con agua destilada hasta alcanzar una concentración idónea de lactosa de 40 g/L y se ajustó su pH a 6.5 mediante la adición de una solución de NaOH 6 M. A continuación, el suero fue esterilizado empleando un sistema de microfiltración tangencial, equipado con un cassette de membranas de PVDF y 0.22 μm de tamaño de poro (Millipore, California, Estados Unidos).

3.2.2. Leche desnatada de vaca

Se utilizó leche desnatada de vaca UHT, con un contenido de 47 g/L de lactosa, 31 g/L de proteína y un pH de 6.7-6.8. Para asegurar su esterilidad, antes del desarrollo de las fermentaciones la leche se calentó en un baño de agua a 90 ºC durante 10 minutos (Yousseef et al., 2016).

3.3. Preparación de inóculos

3.3.1. Fermentaciones sobre suero dulce de queso

L. casei se reactivó en primer lugar bajo condiciones microaerófilas. A partir de una placa de medio MRS, incubada durante 48 horas a 30 ºC, se inoculó una botella de vidrio Pyrex de 250 mL de capacidad, conteniendo 250 mL de MRS líquido (relación de volumen de trabajo: aire de 1:0). Este preinóculo se cultivó en un incubador orbital (modelo G25, New Brunswick Scientific Co., Nueva Jersey, Estados Unidos) a 37 ºC, sin agitación, durante 16 horas. A continuación, 10 mL de este precultivo (en fase de crecimiento exponencial) se utilizaron para inocular 90 mL de suero de queso (nivel de inóculo del 10% v/v), que se incubaron a 37 ºC y 100 rpm durante 20 horas. Posteriormente se emplearon células en crecimiento activo de este cultivo en suero como inóculo para las fermentaciones. El crecimiento previo en suero permite a los microorganismos posteriormente adaptarse mejor a las condiciones del suero de queso, menos rico en nutrientes que su medio típico de crecimiento.

P. taetrolens se reactivó en condiciones aerobias, partiendo de una placa de NB incubada a 30 ºC durante 48 horas. Se inoculó en primer lugar un matraz Erlenmeyer de 500 mL de capacidad con 100 mL de medio líquido NB (relación de volumen de trabajo: aire de 1:4), siendo incubado a 30 ºC y 250 rpm durante 10 horas. Se empleó la biomasa de 10 mL de este preinóculo para inocular 100 mL de suero de queso, cultivando dicho inóculo a 30 ºC y 250 rpm durante 12 horas.

Ambos medios de crecimiento, así como todo el material utilizado durante los experimentos, se esterilizaron en autoclave (modelo AES-75, Raypa Steam Sterilizer, Barcelona, España) a 121 ºC durante 20 minutos.

3.3.2. Fermentaciones sobre leche desnatada de vaca

En el caso de las fermentaciones desarrolladas sobre leche de vaca se prepararon únicamente los precultivos de cada microorganismo en los medios de crecimiento MRS y NB, bajo las condiciones descritas anteriormente, y las fermentaciones se inocularon directamente a partir de ellos. No se realizó un segundo precultivo en leche, debido a que su acidificación durante la incubación provocaría la floculación de las caseínas, imposibilitando su uso posterior como inóculo.

3.4. Fermentaciones en cultivo puro

Para poder conocer la influencia del co-cultivo de *L. casei* y *P. taetrolens* sobre su viabilidad y comportamiento fermentativo, se llevaron a cabo a modo de control fermentaciones de ambos microorganismos en cultivo puro. Las fermentaciones en cultivo puro se desarrollaron en las condiciones más favorables para cada microorganismo, en base a las optimizadas en trabajos previos (Alonso et al., 2010; 2011) teniendo en cuenta el carácter anaerobio y termófilo de *L. casei* y el metabolismo aerobio y mesófilo de *P. taetrolens*, y que se detallarán en los siguientes subapartados. En todos los casos se empleó un porcentaje de inóculo del 10% (v/v).

A lo largo de los cultivos se tomaron muestras periódicas para la medición del pH del medio de cultivo y la realización de diferentes determinaciones, en función del ensayo. Las muestras recogidas se centrifugaron a 16000 x *g* durante 5 minutos (centrífuga Eppendorf, modelo 5415 D, Hamburgo, Alemania), separando la biomasa del medio de cultivo. La biomasa recogida se empleó para realizar el seguimiento del crecimiento bacteriano mediante medida de la densidad óptica, recuento de Unidades Formadoras de Colonias (UFC) sobre placas en medio sólido y monitorización del estado fisiológico de las células mediante citometría de flujo. El sobrenadante libre de células se utilizó para la cuantificación de la lactosa y los ácidos orgánicos mediante Cromatografía Líquida de Alta Eficacia (HPLC). En los casos pertinentes se analizaron las propiedades texturales del producto final.

Todas las fermentaciones (tanto en cultivo puro como co-cultivos) se realizaron por duplicado como experimentos independientes, presentándose para cada parámetro resultados que corresponden a la media de al menos tres medidas.

3.4.1. Fermentaciones en incubador orbital

Para aportar las condiciones adecuadas de temperatura y agitación se hizo uso del mismo incubador orbital empleado para el cultivo de los inóculos.

Las fermentaciones de *L. casei* se desarrollaron en botellas Pyrex de 500 mL de capacidad, conteniendo 400 o 500 mL de medio de cultivo (en función del experimento). Como condiciones de incubación se seleccionaron 37 ºC y 100 rpm de agitación.

Para las fermentaciones en cultivo puro de *P. taetrolens* se utilizaron botellas de 2 L, conteniendo 400 mL de medio en todos los casos, y se fijaron 30 ºC de temperatura y 250 rpm de agitación.

3.4.2. Fermentaciones en biorreactor con agitación mecánica

Las fermentaciones se escalaron a un biorreactor de 2 L con agitación mecánica y 1 L de volumen de trabajo (modelo BioFlo 110, New Brunswick Scientific Co. Inc.). Este biorreactor está equipado con un sistema digital de control, un pH metro (Mettler Toledo, Greifensee, Suiza) y un electrodo polarográfico para la medida del oxígeno disuelto (modelo InPro 6830, Mettler Toledo), pudiendo así monitorizar de forma continua los valores de pH y tensión de oxígeno disuelto.

Figura 3.1. Biorreactor BioFlo 110.

Para las fermentaciones de *L. casei* se fijó la temperatura en 37 ºC y la agitación en 50 rpm, sin aireación.

En el caso de *P. taetrolens* se seleccionaron 30 ºC de temperatura, 350 rpm de agitación y 1 Lpm de aireación suministrada mediante burbujeo. Para prevenir la formación de espuma debido a la elevada agitación se adicionó de forma automática la emulsión antiespumante Y-30 (Sigma-Aldrich, Steinheim, Alemania), diluida 1:10 en agua destilada. Para maximizar la producción de ácido lactobiónico se empleó una estrategia de control de pH basada en dejar variar libremente el pH durante la fase de crecimiento exponencial de *P. taetrolens*, manteniéndolo fijo a 6.5 durante la fase estacionaria mediante la adición automática de NaOH 2 M (Alonso et al., 2012).

3.5. Fermentación simultánea con biomasa libre

Las fermentaciones mixtas con biomasa libre se ensayaron sobre suero de queso en el incubador orbital y bajo dos conjuntos de condiciones operacionales diferentes: las más favorables a *L. casei* y las más favorables a *P. taetrolens*, correspondiendo a las detalladas para el cultivo puro de cada microorganismo (en cuanto a temperatura, agitación y relación volumen de trabajo: aire). Para cada bacteria se empleó un porcentaje de inóculo del 10% v/v, añadiendo al medio de fermentación de forma simultánea la biomasa correspondiente de ambos microorganismos (habiendo retirado el medio de precultivo mediante centrifugación). Se tomaron periódicamente muestras para la medida del pH del medio, la determinación global de la densidad óptica y el recuento de UFC. Cada muestra se sembró en placas de agar de medio MRS y NB, pudiendo realizar el recuento de colonias de cada bacteria de forma independiente. Además, a lo largo de la fermentación se cuantificaron lactosa, ácido láctico y ácido lactobiónico por HPLC, y se monitorizó el estado fisiológico de los microorganismos por citometría de flujo.

3.6. Fermentación secuencial con biomasa libre

El sistema de fermentación secuencial se desarrolló sobre suero de queso en el incubador orbital, siguiendo el esquema mostrado en la Figura 3.2.

Se inoculó una botella de 2 L de capacidad conteniendo 400 mL de suero de queso con biomasa de *P. taetrolens* procedente de los precultivos en suero (porcentaje de inóculo del 10% v/v). El cultivo se incubó a 30 ºC y 250 rpm hasta que la acumulación de ácido lactobiónico hizo

descender el pH del medio hasta un valor de 5. En ese momento, la biomasa de *P. taetrolens* se eliminó mediante centrifugación a 12000 *x g* durante 10 minutos (centrífuga Kubota, modelo 6700, Tokio, Japón). Una vez retirada la biomasa de *P. taetrolens*, el medio oxidado se transfirió en condiciones de esterilidad a una botella de 500 mL y se inoculó con biomasa procedente del precultivo en suero de *L. casei* (nivel de inóculo del 10% v/v). La fermentación de *L. casei* se desarrolló a 37 ºC y 100 rpm durante 48 horas. El valor de pH 5 había sido establecido en ensayos previos como el valor de acidez inicial aceptable para la inoculación de *L. casei*.

A lo largo de las dos etapas del proceso se tomaron muestras para la determinación del pH, densidad óptica, recuento de UFC y cuantificación de lactosa y ácidos orgánicos.

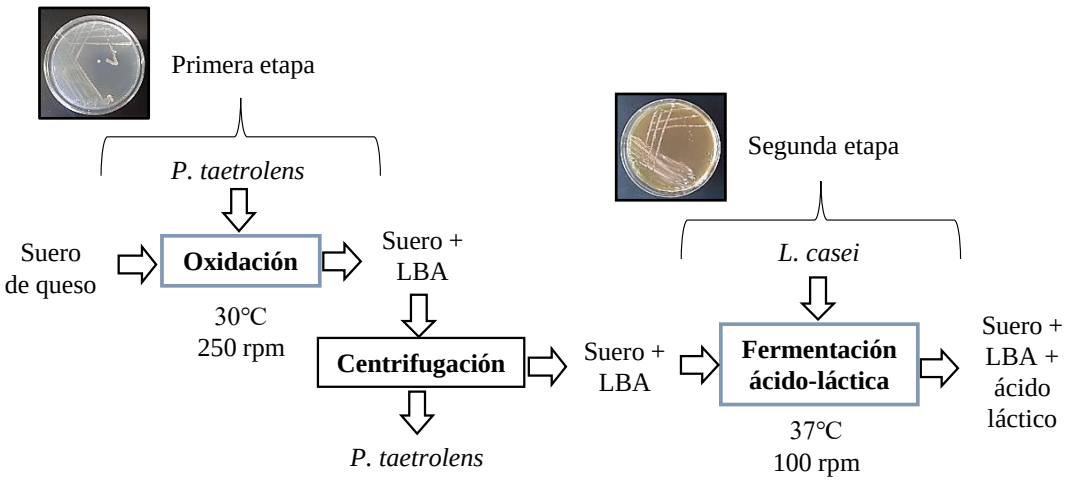

Figura 3.2. Diagrama del proceso de fermentación secuencial sobre suero de queso.

3.7. Estudio del consumo de ácido lactobiónico por *L. casei*

Se evaluó la capacidad de la cepa *L. casei* CECT 475 para utilizar el ácido lactobiónico en ausencia de otra fuente de carbono. Para ello se preparó un medio MRS líquido basal según la composición reflejada en la Tabla 3.1 (Adebola et al., 2014).

Una vez preparado el MRS basal, se ajustó su pH a un valor de 6.2 mediante la adición de NaOH 6M, y a continuación se esterilizó en autoclave a 121 ºC durante 15 minutos. Este medio basal se suplementó con ácido lactobiónico comercial (Sigma-Aldrich) en las siguientes concentraciones: 0.5, 1, 2.5 y 5% (p/v). Las mismas concentraciones de glucosa (Panreac, Barcelona, España) se emplearon para suplementar MRS basal constituyendo controles positivos. Como control negativo se empleó MRS basal sin ninguna fuente de carbono.

Se inocularon botellas Pyrex de 250 mL de capacidad, conteniendo 250 mL del MRS basal o suplementado, con biomasa de *L. casei* procedente de una placa de MRS incubada durante 48 horas a 30 ºC. Los cultivos se incubaron en el agitador orbital a 37 ºC, sin agitación, durante 52 horas. Se tomaron muestras de forma periódica para la medida de la densidad óptica y la cuantificación glucosa, ácido lactobiónico y ácido láctico.

Tabla 3.1. Composición del MRS basal.

MRS basal
10 g/L peptona
5 g/L extracto de levadura
5 g/L $C_2H_3NaO_2$ $2H_2O$
2 g/L K_2HPO_4 $3H_2O$
2 g/L $(NH_4)_3C_6H_5O_7$ $2H_2O$
0.2. g/L $MgSO_4$ $7H_2O$
0.05 g/L $MnSO_4$ $4H_2O$
1 mL Tween 80

3.8. Obtención de un alimento fermentado simbiótico

3.8.1. Diseño del bioproceso

La fermentación secuencial se ensayó en primer lugar a nivel de incubador orbital, escalando el proceso posteriormente al biorreactor con agitación mecánica y control de pH (Figura 3.3).

A nivel de incubador orbital se siguió exactamente el esquema detallado previamente para la fermentación secuencial sobre suero de queso, en cuanto a volúmenes, porcentaje de inóculo y condiciones operacionales, introduciendo pequeñas modificaciones para adaptar el protocolo al sustrato de leche. Así, en lugar de retirar la biomasa de *P. taetrolens* por centrifugación, el medio oxidado se sometió a calentamiento en baño de agua a 90 ºC durante 10 minutos (Yousseef et al., 2016). Por otra parte, la fermentación ácido-láctica se detuvo a las 24 horas (en vez de 48 horas), debido a la coagulación de las caseínas por la acidificación del medio.

A nivel de biorreactor con agitación mecánica y control de pH, se inoculó 1 L de leche esterilizada en primer lugar con biomasa de *P. taetrolens* (porcentaje de inóculo del 10% v/v),

aplicándose las mismas condiciones operacionales detalladas para *P. taetrolens* en cultivo puro (30 ºC, 350 rpm, 1 Lpm, adición de antiespumante y mantenimiento del pH en 6.5 durante la fase estacionaria). La posibilidad de introducir esta estrategia de control de pH eliminó el riesgo de inhibición ácida de *L. casei*, de manera que el factor determinante para detener la oxidación de *P. taetrolens* fue el contenido de lactosa del medio. En base a la información recogida en el cultivo puro de *P. taetrolens* bajo control de pH, su biomasa se inactivó cuando aún quedaba suficiente lactosa disponible para la fermentación de *L. casei*. El medio oxidado conteniendo *P. taetrolens* se bombeó fuera del biorreactor, se calentó en el baño de agua a 90 ºC durante 10 minutos, se enfrió, se inoculó con *L. casei* (10% v/v) y se reintrodujo nuevamente en el biorreactor preservando las condiciones de esterilidad. La bacteria láctica se incubó a 37 ºC, 50 rpm, sin aireación y dejando variar libremente el pH hasta la coagulación de las proteínas.

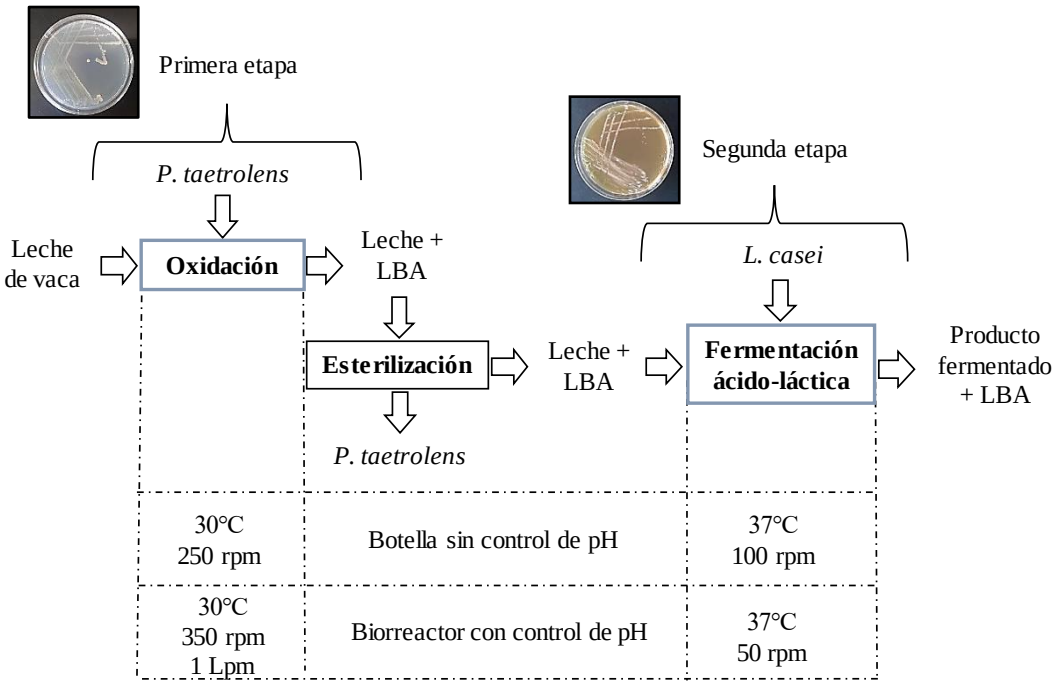

Figura 3.3. Diagrama del proceso de fermentación secuencial sobre leche de vaca.

A lo largo de todo del proceso se registraron el pH y el oxígeno disuelto y se tomaron muestras para el recuento de UFC y la cuantificación de lactosa y ácidos orgánicos. Además, se realizó la caracterización textural del producto fermentado final.

Sobre el fermentado final también se determinó el nivel de separación espontánea del suero, fenómeno conocido como sinéresis, empleando el método del sifón (Amatayakul et al., 2006). Se tomaron alícuotas de 50 mL del producto y se preservaron en un vaso a 4 ºC. A continuación,

se pesaron (balanza CB Complet, Cobos, Barcelona, España) y se mantuvieron en un ángulo de 45º para acumular el suero en un extremo del vaso. Se empleó una jeringa para sifonar el suero y las muestras fueron nuevamente pesadas. La sinéresis se calculó como porcentaje de suero en peso sobre el peso inicial de la muestra.

3.8.2. Adición de texturizante

Las mismas fermentaciones secuenciales sobre leche de vaca, con y sin control de pH, se llevaron a cabo añadiendo el texturizante k-carragenano (Sigma-Aldrich) al medio de fermentación. Se ensayaron concentraciones del 0.1% y 0.5% (p/v) en fermentaciones sin y con control de pH, respectivamente. La concentración fue mayor en las fermentaciones con control de pH para contrarrestar el efecto de la agitación interna del biorreactor, que dificulta la formación de la red proteica. El k-carragenano se disolvió en la leche durante el tratamiento de inactivación térmica de *P. taetrolens* (Nguyen et al., 2017), no interfiriendo así en su proceso previo de oxidación. Estas fermentaciones se caracterizaron en cuanto a evolución del pH, UFC, lactosa, ácidos orgánicos, textura y grado de sinéresis.

Se realizaron ensayos adicionales añadiendo el texturizante k-carragenano directamente sobre los productos finales de la fermentación sin aditivos, en un rango de concentraciones del 1-2% (p/v), analizando a continuación la textura resultante.

3.9. Fermentación simultánea con biomasa encapsulada

3.9.1. Elaboración de las cápsulas

Las fermentaciones con biomasa encapsulada se llevaron a cabo ensayando tres hidrogeles encapsulantes de diferente composición (Tabla 3.2).

Tabla 3.2. Composición de los agentes encapsulantes.

Composición de los hidrogeles
2% (p/v) alginato de sodio (Acros Organics)
2% (p/v) alginato de sodio / 2% almidón (Panreac)
2% carboximetilcelulosa sódica (Sigma-Aldrich) / 1% k-carragenano (Sigma-Aldrich)

Se tomaron 40 mL de precultivos de *L. casei* y *P. taetrolens* en medio MRS y NB, respectivamente, conteniendo células en fase de crecimiento exponencial. Se centrifugaron a 12000 *x g* durante 10 minutos, separando el pellet de biomasa. Los polímeros, en las concentraciones indicadas, se disolvieron en 25 mL de agua destilada formando la solución polimérica. A continuación, la biomasa de cada microorganismo, por separado, se resuspendió en los 25 mL de solución polimérica. Como técnica encapsulante se seleccionó la extrusión, debido a su simplicidad y a que sus condiciones suaves de operación garantizan una alta supervivencia de los microorganismos. Se utilizó una bomba peristáltica para adicionar por goteo la mezcla de agente encapsulante y biomasa sobre una solución endurecedora de $CaCl_2$ 0.54 M (Alonso et al., 2015). Una vez formadas y endurecidas, las cápsulas se lavaron con tampón fosfato salino (PBS), a pH 7.4, estéril y filtrado por 0.22 µm (Tabla 3.3), para eliminar la solución gelificante residual. Después del lavado las cápsulas se recuperaron mediante filtración y se emplearon como inóculo en las fermentaciones.

Tabla 3.3. Composición del PBS empleado para el lavado de las cápsulas.

Composición del PBS
8 g/L NaCl
0.2 g/L KCl
1.44 g/L Na_2HPO_4
0.22 g/L KH_2PO_4

3.9.2. Diseño del bioproceso

La fermentación simultánea con biomasa encapsulada se ensayó a nivel de incubador orbital sin control de pH. La encapsulación se realizó de forma alternativa, con un microorganismo encapsulado y el otro libre en el medio en cada caso (Figura 3.4). Para cada microorganismo se ensayó la inmovilización en los diferentes materiales encapsulantes. Se introdujeron 400 mL de leche estéril en botellas de 2 L de capacidad y se inocularon con la biomasa libre procedente de 40 mL de precultivos en NB o MRS, y la biomasa encapsulada de 40 mL del precultivo de la otra bacteria. Las fermentaciones se desarrollaron bajo las condiciones ambientales más favorables para *P. taetrolens* (relación de volumen de trabajo: aire de 1:4, 30 ºC y 250 rpm) durante 72 horas.

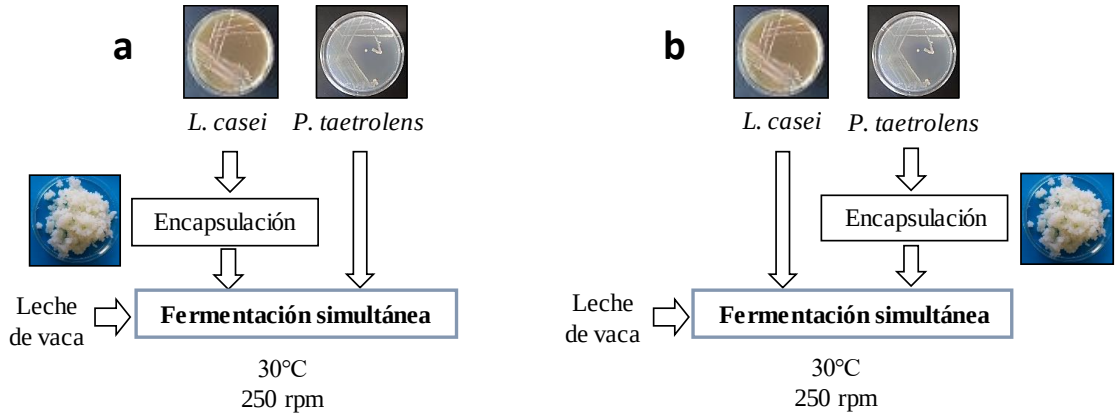

Figura 3.4. Fermentación simultánea con *L. casei* (a) o *P. taetrolens* (b) encapsulada.

Se llevó a cabo la caracterización textural de los agentes encapsulantes, así como el examen visual de las cápsulas al estereomicroscopio. Se determinaron la eficiencia de encapsulación y el perfil de liberación de las células encapsuladas en cada hidrogel. Durante las fermentaciones, se siguió la evolución del pH y las concentraciones de lactosa y ácidos orgánicos. La cuantificación de la biomasa, tanto libre como encapsulada, se llevó a cabo mediante el recuento de UFC. Para el seguimiento de la biomasa libre se tomaron muestras del medio de fermentación. En el caso de la biomasa encapsulada, se extrajo una cápsula y se resuspendió en 1 mL de citrato de sodio (1% v/v) para solubilizarla. Para favorecer la liberación de las células se efectuó un mezclado en el Vórtex y a continuación la muestra se sembró en placa para el recuento de UFC.

El sistema fermentativo que permitió obtener los mejores resultados (*L. casei* encapsulada en alginato/almidón y *P. taetrolens* libre en el medio) se escaló posteriormente a nivel de biorreactor con control de pH. Para ello, se inoculó 1 L de leche esterilizada con la biomasa libre procedente de 100 mL de precultivo de *P. taetrolens* en NB, y la biomasa encapsulada de 100 mL de precultivo de *L. casei* en MRS. Se aplicó el ajuste de pH en 6.5 durante la fase estacionaria, la adición de antiespumante y se ajustó la temperatura en 30 ºC. Para evitar la ruptura de las cápsulas no se aplicó agitación interna, pero se suministró una aireación de 1.5 Lpm para garantizar el aporte de oxígeno a *P. taetrolens*. Se tomaron muestras de forma periódica para cuantificar la lactosa, los ácidos orgánicos y la biomasa libre de *P. taetrolens* mediante el recuento de UFC/mL. El crecimiento de *L. casei* no se pudo monitorizar, debido a la imposibilidad de extraer las cápsulas del biorreactor manteniendo las condiciones de esterilidad.

3.9.3. Eficiencia de encapsulación y liberación de células encapsuladas

Se calculó la eficiencia de la encapsulación para cada agente encapsulante de acuerdo con la siguiente fórmula (Sandoval-Castilla et al., 2010):

$$Eficiencia = (A/B)x100$$

Donde:

A = UFC/mL de agente encapsulante después de la encapsulación.
B = UFC/mL de agente encapsulante antes de la encapsulación (10^8 UFC/mL en todos los casos).
Además de la eficiencia de encapsulación alcanzada, la idoneidad de cada agente encapsulante también se evaluó monitorizando la liberación de las células encapsuladas. Para ello, se tomaron 0.5 g de cápsulas, se suspendieron en 4.5 mL de la solución tampón fosfato salino y se incubaron durante 24 horas con agitación constante a 250 rpm. Se empleó la misma agitación fijada para el desarrollo de las fermentaciones, con el objetivo de simular el estrés mecánico experimentado por las cápsulas.

Se tomaron muestras del medio líquido a diferentes intervalos durante 24 horas para determinar la presencia de células fuera de la cápsula, realizando posteriormente el recuento de UFC sobre medio sólido.

3.10. Métodos analíticos

3.10.1. Recuento de células viables y cultivables en medio sólido

La concentración de células viables y cultivables se determinó mediante el recuento de UFC sobre placas de medio sólido (2% p/v de agar); MRS para el crecimiento de *L. casei* y NB para el crecimiento de *P. taetrolens*. Las muestras tomadas periódicamente a lo largo de las fermentaciones se centrifugaron (16000 x *g* durante 5 minutos), separando la biomasa del medio de fermentación. A continuación, la biomasa se resuspendió en un tampón de NaCl 0.7 % (p/v) y se sometió a un mezclado en el Vórtex para evitar la sedimentación de las células. Se prepararon diluciones seriadas en el tampón, y se sembraron tres diluciones. Tanto las placas para el recuento de células de *L. casei* como de *P. taetrolens* se incubaron a 30 ºC durante 48 horas. Una vez transcurrido el tiempo de incubación, se realizó el recuento de UFC

correspondientes a las diluciones estadísticamente significativas (20-200 colonias). El resultado promedio se expresó como UFC/mL de medio de cultivo.

3.10.2. Determinación de la densidad óptica

El seguimiento del crecimiento bacteriano en medio líquido como medida de la densidad óptica (DO) se llevó a cabo en las fermentaciones de suero de queso. La biomasa separada mediante centrifugación (16000 *x g* durante 5 minutos) se resuspendió en el tampón de NaCl 0.7 %, empleado a su vez como solución "blanco". Se midió la absorbancia de las muestras a 600 nm en un espectrofotómetro (modelo 1203, Shimazdu UV- Vis, Kioto, Japón).

En los experimentos de fermentación secuencial, el crecimiento de *L. casei* determinado mediante monitorización de la DO o las UFC/mL se convirtió a valores de peso seco, con el objetivo de obtener una medida exacta de la producción de biomasa de *L. casei* en un sustrato conteniendo ácido lactobiónico. Para realizar la conversión se partió de la curva de calibración previamente obtenida, expresando el resultado en g/L de biomasa.

3.10.3. Citometría de flujo

La citometría de flujo se empleó para monitorizar el estado fisiológico de *L. casei* y *P. taetrolens* a lo largo de las fermentaciones simultáneas en suero de queso, comparando los resultados con su condición fisiológica en cultivo puro. Se emplearon dos fluorocromos: ioduro de propidio (IP, Molecular Probes, ThermoFisher Scientific, Massachusetts, Estados Unidos), y ChemChrome V6 (CV6, Chemunex, Francia), llevando a cabo un procedimiento de tinción dual CV6/IP. El CV6 tiñe las células viables con actividad enzimática, mientras que el IP penetra en las células con la membrana permeabilizada, tiñendo por tanto las células dañadas y las células muertas.

La biomasa separada del medio de cultivo mediante centrifugación (16000 *x g* durante 5 minutos) se lavó dos veces con el tampón fosfato salino (PBS, pH 7.4, estéril y filtrado por 0.22 µm; Tabla 3.3). Se tomaron alícuotas de 200 µL de las suspensiones celulares, que se sometieron a ultrasonidos durante 2 segundos para evitar la agregación de las células antes de su tinción.

Para preparar la solución de trabajo del CV6, la solución comercial se diluyó 1:10 en agua destilada estéril. 8 µL de esta solución de trabajo se añadieron a los 200 µL de suspensión celular, incubándose 15 minutos en oscuridad a temperatura ambiente. En el caso del IP, la

solución stock (1 mg/mL) se diluyó en agua destilada estéril hasta alcanzar una concentración de 140 µg/mL en la solución de trabajo. A su vez, la solución de trabajo se añadió a los 200 µL de suspensión celular hasta una concentración de 5.25 µg/mL. La mezcla se incubó en oscuridad y temperatura ambiente durante 30 minutos.

Se prepararon tres controles positivos para establecer los patrones de tinción de cada microorganismo en función de su estado fisiológico: a) muestras conteniendo células muertas mediante incubación en alcohol-isopropilo al 70% durante 1 hora (Freire et al., 2015); b) muestras con células en fase exponencial temprana; y c) muestras conteniendo una mezcla 1:1 de células muertas y células en fase de crecimiento exponencial. Como control negativo se emplearon muestras sin teñir.

Los análisis se realizaron en un citómetro de flujo Cytomics FC 500 (Beckman Coulter, Nyon, Suiza) equipado con una fuente láser de iones (488 y 633 nm). La fluorescencia procedente de las células teñidas con CV6 se recogió en el canal FL1 (530 nm), mientras que la fluorescencia del IP se midió en el canal FL4 (675 nm). Los análisis se llevaron a cabo con una velocidad de 4000 eventos/s. Para la adquisición de datos se empleó Cytomics RXP y para su análisis Kaluza Analysis Software (Beckman Coulter).

Figura 3.5. Citómetro Cytomics FC 500.

3.10.4. Cromatografía líquida de alta eficacia

Los sobrenadantes libres de células se emplearon para realizar el seguimiento de la concentración de lactosa y los ácidos orgánicos mediante Cromatografía Líquida de Alta Eficacia (HPLC). Antes de su análisis, estos sobrenadantes se filtraron a través de filtros de PVDF de 0.45 µm.

Se empleó un cromatógrafo de líquidos Agilent (modelo serie 1200, California, Estados Unidos), equipado con una columna ICSep ICE-ION-300 (Transgenomic Inc., San José,

California, Estados Unidos) y acoplado a un detector de índice de refracción. Se llevó a cabo una elución isocrática, preparando como fase móvil una solución de H_2SO_4 (0.450 mM, pH 3.1). Se fijó un flujo de 0.3 mL/min y una temperatura de 75 ºC.

Las muestras se cuantificaron en base a patrones externos de lactosa, ácido láctico y ácido lactobiónico de grado HPLC. La adquisición y análisis de datos se llevó a cabo mediante el software Chemstation (Agilent).

Figura 3.6. Cromatógrafo Agilent serie 1200.

3.10.5. Análisis de propiedades texturales: índice de Bloom para determinar la resistencia de las cápsulas

Se analizaron las propiedades texturales de las cápsulas en las fermentaciones con biomasa inmovilizada, empleando un analizador de textura TA.XTplus (Stable Micro Systems, Reino Unido). Se llevó a cabo un ensayo Bloom, que permite determinar la fuerza Bloom de un gel de acuerdo con la norma UNE-EN ISO 9665. El parámetro Bloom es una medida de la fuerza que se debe aplicar para provocar una deformación en un gel. Los resultados se expresan en Grados Bloom, definidos como el peso en gramos que necesita la sonda para hundir la superficie del gel 4 mm sin romperlo. En general, se considera que la fuerza del gel es baja para valores Bloom inferiores a 120 gramos; media en el intervalo de 120-200 gramos; y alta para valores superiores a 200 gramos. Por otra parte, se ha determinado que valores Bloom más altos en un gel suponen una menor degradación y mayores eficiencias de encapsulación (Chou et al., 2016).

Para realizar el ensayo se emplearon 100 mL de cada hidrogel con biomasa en suspensión, guardando las mismas proporciones empleadas para la elaboración de las cápsulas. El Bloom

test se llevó a cabo a temperatura ambiente, una velocidad de 0.5 mm/s, distancia de penetración de 4 mm y tasa de adquisición de datos de 200 pps.

3.10.6. Análisis de propiedades texturales: firmeza y adhesividad de productos fermentados

El producto final de las fermentaciones secuenciales de leche de vaca se caracterizó texturalmente para evaluar el efecto espesante del ácido lactobiónico, mediante comparación con el producto final resultante de fermentaciones en cultivo puro de *L. casei*.

El análisis textural de los productos más firmes se llevó a cabo utilizando el texturómetro TA.XTplus (Stable Micro Systems). Se tomó una muestra de 50 mL y se sometió a un ensayo de penetración, a temperatura ambiente, empleando una sonda cilíndrica (P/0.55) y una altura de trabajo de 40 mm. Los resultados obtenidos se expresaron en términos de firmeza y adhesividad (gramos).

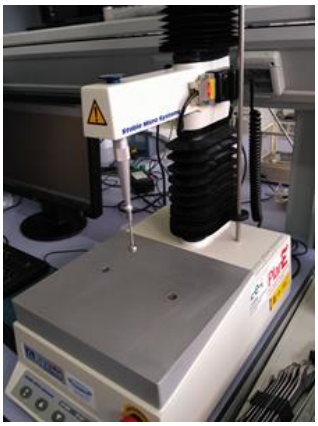

Figura 3.7. Texturómetro TA.XTplus.

3.10.7. Reología de productos fermentados

Las propiedades texturales de los productos fermentados con una consistencia más líquida, resultantes de las fermentaciones secuenciales de leche de vaca, se analizaron en un reómetro Haake Mars (Thermo Fisher Scientific) acoplado a un controlador de temperatura Peltier. Se empleó un intervalo de deformación de 0.01 – 10/s. Los resultados obtenidos se representaron mediante las curvas de flujo correspondientes a los valores de viscosidad (cP) y esfuerzo cortante (Pa) frente a velocidad cortante (1/s).

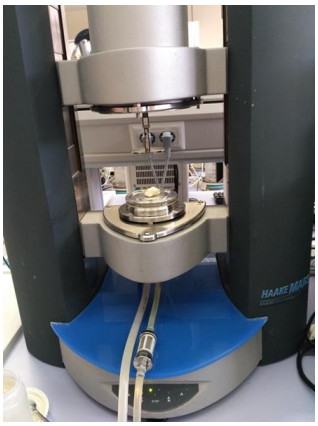

Figura 3.8. Reómetro Haake Mars.

3.10.8. Estereomicroscopía de fluorescencia: caracterización visual de las cápsulas

Las cápsulas elaboradas con los diferentes agentes encapsulantes se caracterizaron visualmente empleando un estereomicroscopio de fluorescencia LEICA M205FA (Leica Microsystems Inc., Heidelberg, Alemania). Las cápsulas no se sometieron a ningún tratamiento especial, colocándose directamente sobre el portaobjetos y aplicando una magnificación de 22x. Para el procesamiento de las imágenes obtenidas se empleó el software Leica Application Suite v4.0. Se determinaron las dimensiones de las cápsulas, calculando posteriormente el factor de esfericidad, de acuerdo con la siguiente ecuación (Córdoba et al., 2013):

$$Factor\ de\ esfericidad = \frac{d_{max} - d_{min}}{d_{max} + d_{min}}$$

Donde "d_{max}" es el diámetro mayor de la cápsula y "d_{min}" es el diámetro menor, perpendicular a "d_{max}". El factor de esfericidad varía desde un valor de 0, para una esfera perfecta, hasta 1 para un objeto de forma alargada.

La caracterización visual de las cápsulas, comparando los diferentes materiales encapsulantes, permite detectar la presencia de grietas y espacios en su superficie característicos de materiales porosos como el alginato, que pueden resultar determinantes para el mantenimiento de la integridad de las cápsulas durante la fermentación. Así mismo, la comparación de sus dimensiones puede poner de manifiesto fenómenos de hinchamiento, que reducen su capacidad para retener las células.

4. RESULTADOS

4. RESULTADOS

4.1. Estudio de la interacción entre *L. casei* y *P. taetrolens* en fermentaciones mixtas

En este apartado se recogen los resultados referentes al estudio de interacción entre *L. casei* y *P. taetrolens* sobre un sustrato de suero de queso. Como se ha señalado anteriormente, el empleo de *starters* mixtos es muy común en fermentaciones alimentarias, formando parte tanto de la manufactura de productos artesanales, como del diseño de nuevos alimentos con propiedades mejoradas (nutricionales, organolépticas, funcionales o tecnológicas). En esta memoria se propone el acoplamiento novedoso de la bioproducción de ácido lactobiónico por la bacteria *P. taetrolens*, al proceso fermentativo llevado a cabo por BAL como *L. casei*, utilizadas comúnmente como cultivos iniciadores. Como primer paso imprescindible se estudió la fermentación simultánea de *P. taetrolens* y *L. casei* sobre un sustrato lácteo desde el punto de vista de la viabilidad, crecimiento y capacidad productiva de ambos microorganismos, prestando especial atención a la biosíntesis del ácido lactobiónico. Se utilizó la citometría de flujo como una herramienta que permite monitorizar el estado fisiológico de los microorganismos, complementando la información aportada por el método tradicional de recuento de células viables. Teniendo en cuenta los diferentes requerimientos ambientales de *L. casei* y *P. taetrolens*, las fermentaciones mixtas se ensayaron en las condiciones más favorables a cada microorganismo, pudiendo estudiar así el efecto de la coexistencia de forma aislada. Esta investigación permitió establecer el tipo de relación que tiene lugar entre ambas bacterias cuando coexisten en el mismo nicho, sentando las bases para el futuro desarrollo de un proceso integrado de aplicación industrial, y poniendo de relieve la importancia general de los estudios de interacción microbiana en el diseño de nuevos *starters*.

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Microbial amensalism in *Lactobacillus casei* and *Pseudomonas taetrolens* mixed culture

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Abstract

Pseudomonas taetrolens has recently been revealed as an effective microbial producer of lactobionic acid from carbohydrates contained in dairy byproducts. In terms of food industrial applications, the implementation of lactobionic acid biosynthesis coupled with the classic bacterial production of lactic acid appears an important goal. This research paper studies the simultaneous fermentation of residual cheese whey by *P. taetrolens* and *Lactobacillus casei* to co-produce lactic and lactobionic acids. Experimental data showed the importance of the interactions established between the two microorganisms. Changes in physiology, viability, growth and productive capacity were tested experimentally. *Lactobacillus* was not seen to suffer any appreciable stress, but considerable variations were observed in the *Pseudomonas* behavior presumably owing to inhibitory lactic metabolites, interaction that can be classified as microbial amensalism. As to production, lactic acid remained without significant changes in mixed fermentations, whereas the production of lactobionic acid decreased sharply due to the competitive exclusion of *Pseudomonas*.

Keywords: Whey; *Pseudomonas taetrolens*; *Lactobacillus casei*; lactobionic acid; mixed fermentation; amensalism.

1. Introduction

In recent years, interest in the biotechnological production of chemical compounds has grown in numerous industrial sectors as a sustainable alternative to satisfy worldwide demand for such compounds. Among these compounds, traditional organic acids, such as lactic acid, whose industrial production through microbial fermentations is already in a mature state of implementation [1], are perhaps the better developed. The market is now paying growing attention to other emergent acids with unique physicochemical properties, such as lactobionic acid. This lactose derivative presents interesting industrial applications, including many in the dairy industry as a food additive, serving as an acidulant with sweet taste, firming agent, aroma preserver or flavor enhancer. Its addition enables the production of fermented dairy products with reduced lactose content. It is also considered to exert health benefits in functional foods, acting as a calcium carrier and potential prebiotic compound [2-4]. This plethora of applications and beneficial properties contributes to the interest in developing new processes to obtain dairy foods containing lactobionic acid.

Currently, this organic acid is exclusively produced industrially via chemical synthesis from refined lactose in a complex, costly and environmentally unfriendly process [3, 5]. However, research studies into the bioproduction of lactobionic acid by bacteria have shown the species *Pseudomonas*, and more specifically *Pseudomonas taetrolens* (a non-pathogenic, wild-type microorganism), to be the ideal microbial platform to oxidize lactose with 100% selectivity for lactobionic acid and high substrate conversion yields [4, 6]. Lactose-oxidizing activity has been observed in fermentations carried out with other bacterial species which have also exhibited good lactobionic acid productivities. However, resting cells and mutant or pathogenic strains have been employed in most approaches [3, 4, 7], which are not recommended for implementation at industrial level in either case [3]. Moreover, many of these producer microorganisms have been tested employing synthetic growth media [3, 4, 7, 8]. The high economic cost and complexity of these synthetic substrates have led to the search for industrial byproducts to be used as the raw material in fermentations, such as liquid wastes from dairy industry. In most countries there is a stringent legislation governing the disposal of this kind of effluents due to their high-polluting potential [1]. Their necessary management makes essential the development of valorization alternatives.

In this respect, efficient and sustainable bioprocesses have been developed to obtain lactobionic acid using cheese whey as a substrate for *Pseudomonas taetrolens* fermentations [3, 5, 10].

Cheese whey and other dairy byproducts (such as yoghourt whey) have also been employed as an inexpensive raw material for lactic acid bacteria growth and to produce lactic acid [1, 11, 12].

The development of these production methods raises the possibility of obtaining dairy products enriched with lactobionic acid, in which this acid is synthesized *in situ* from the lactose contained in dairy byproducts, thereby circumventing the need to incorporate it subsequently as an additive. *Pseudomonas* fermentation would be coupled to the usual fermentation processes carried out by lactic acid bacteria in fermented dairy products. If a probiotic lactic bacterium is employed as the starter, the final fermented product thus obtained could be considered a synbiotic, containing the prebiotic lactobionic acid as an additional ingredient [2]. *Lactobacillus casei* has acquired a recognized role in the development of functional foods thanks to its widespread ecological distribution and health-promoting properties, including its potential as a probiotic [13-16]. Its association with *P. taetrolens* for industrial purposes would necessitate a prior study of the potential interactions which may take place between the two, *a priori* antagonistic microorganisms, bearing in mind their respective anaerobic and aerobic metabolism. The facultative anaerobic character of *L. casei* [16] opens up the possibility of coexistence in which both bacterial species would compete for lactose as their source of carbon and energy.

Intensive research has been carried out in terms of comparing the ability of different microorganisms to produce metabolites of interest [17-19], though not always studying the interaction phenomena which would occur if they worked in microbial association. Nonetheless, mixed fermentations (occurring in liquid media) or co-cultures (in solid media) are nowadays receiving growing attention from the scientific community in several areas such as agriculture, human microbiome research, the pharmaceutical industry and the field of fermentations. In this last case, microbial consortiums are studied with the aim of understanding the dynamics of metabolite production or of improving the production yield of specific products [9, 20, 21]. Shedding light on the nature of these interactions thus becomes necessary, as the coexistence of microorganisms can affect their growth, morphology, development and adaptation patterns. These effects are frequently caused by the production of secondary metabolites in the context of defense or nutrient competition [22].

In this regard, the aim of the present research was to study the interactions established between the major lactic and lactobionic acid producers *Lactobacillus casei* CECT 475 and

Pseudomonas taetrolens LMG 2336, respectively, during mixed fermentations with sweet cheese whey as carbon source. Fermentations were evaluated in terms of cell growth, physiological status and productive capacity. Bacterial strains and bioprocessing factors were chosen on the basis of the productivity and efficiency maximization previously achieved by our research group with these microorganisms in pure culture [3, 5, 10, 11, 23]. The specific association between *Lactobacillus casei* and *Pseudomonas taetrolens* examined in this study has not been considered to date. The results will be useful to develop a bioprocess to obtain fermented dairy products enriched with sustainably-produced lactobionic acid, with industrial applications in the field of functional foods.

2. Materials and methods

2.1. Microorganisms

Lactobacillus casei CECT 475, obtained from the Spanish Type Culture Collection (Valencia, Spain), was maintained frozen (in 40% v/v solution at -20 °C) and subsequently subcultured on MRS (de Man Rogosa and Sharpe, Biokar Diagnostic, France) agar plates, incubated for 48 h at 30 °C and then stored at 4 °C.

Pseudomonas taetrolens LMG 2336 was obtained from the Belgian Coordinated Collection of Microorganisms (Gent, Belgium). The strain was conserved frozen in 40% (v/v) glycerol at -20 °C and subsequently subcultured on NB agar plates (Nutrient Broth, containing 1g L^{-1} meat extract, 2 g L^{-1} yeast extract, 5 g L^{-1} peptone and 5 g L^{-1} NaCl). The agar plates were incubated for 48 h at 30 °C and preserved at 4 °C.

2.2. Inocula preparation

L. casei was reactivated on MRS under microaerophilic conditions. A loopful from a MRS agar plate was used to inoculate a 250 mL storage media bottle containing 250 mL MRS broth. The culture was incubated in an orbital shaker (New Brunswick Scientific Co., model G25, USA) at 37 °C without agitation for 16 h. 10 mL of this culture were used to inoculate 90 mL of ultrafiltrated sweet whey (inoculum level 10% v/v), which were then incubated in the orbital shaker at 37 °C and 100 rpm for 20 h.

In the case of *P. taetrolens,* a 500 mL Erlenmeyer flask containing 100 mL of NB broth was inoculated with a loopful from a NB agar plate. The culture was incubated in the aforementioned orbital shaker at 250 rpm and 30 °C for 10 h and was then used as inoculum in 100 mL of ultrafiltrated sweet whey (inoculum level 10% v/v), subsequently incubated at 30 °C and 250 rpm for 12 h.

Actively growing cells from theses precultures in cheese whey were employed as inocula for the fermentations.

2.3. Sweet whey preparation

Permeate from sweet cheese whey ultrafiltration (provided by ILAS S.A., Asturias, Spain), with 200 g L^{-1} of lactose (18 °Bx) and small concentrations of mineral salts (12-15% of the total solids) [1, 24], was used as the substrate for bacterial fermentations. Protein content was determined by the Bradford Assay [25], confirming its insignificant presence after the ultrafiltration treatment. To achieve a suitable initial sugar concentration, whey permeate was diluted with distilled water until obtaining a lactose concentration of 40 g L^{-1} and adjusted to pH 6.5 (by adding 6 m mol^{-1} NaOH). It was then sterilized using a tangential microfiltration device equipped with a 0.22 μm pore size PVDF membrane-cassette (Millipore, Massachusetts, USA).

2.4. Culture and fermentation conditions

To evaluate the interaction between *L. casei* and *P. taetrolens* and lactic and lactobionic acid co-production from sweet whey, both competitive and pure culture batch fermentations were carried out in orbital shakers without pH control. Competitive fermentations were performed under two types of operating conditions: those which were more favorable to *L. casei,* and those more favorable to *P. taetrolens*, employing the same conditions as for fermentations with pure cultures of *L. casei* and *P. taetrolens,* respectively. In the case of fermentations under the conditions favorable to *L. casei*, these were: 100 rpm agitation rate, 37 °C and final working volume of 500 mL in 500 mL storage media bottle. In the case of *P. taetrolens*, the conditions were: 250 rpm, 30 °C and final working volume of 400 mL in 2 L storage media bottle. A 10% (v/v) inoculation level was used in all fermentations, employing actively growing cells from precultures developed in sweet whey. Samples were periodically taken from the fermentation growth media to determine bacterial growth and to monitor the physiological status of the

microorganisms via multiparametric flow cytometry. Cell-free culture samples were stored (at -20 °C) for the chemical analysis of substrate consumption and organic acid production. Fermentations were carried out in duplicate as independent experiments and the reported results correspond to the mean value of at least three measurements. Positive and negative error values are shown as error bars in the figures.

2.5. Analytical methods

Bacterial growth was determined spectrophotometrically as a measure of the optical density at 600 nm (Shimazdu, UV 1203 model), after centrifuging culture samples at 16000 *x g* for 5 min. The viable cell count was performed via the spread plate method in MRS and NB agar for *L. casei* and *P. taetrolens*, respectively. Colony Forming Units (CFU) were counted after incubating the agar plates for 48 h at 30 °C in both cases. Results are expressed as CFU mL^{-1}.

Lactose, lactic acid and lactobionic acid concentrations from cell-free culture samples were measured via High Performance Liquid Chromatography (HPLC). The liquid chromatography system used for the analysis (Agilent 1200, Agilent Technologies Inc., CA, USA) was equipped with an ICSep ICE-ION-300 column (Transgenomic Inc., CA, USA) coupled to a refractive index detector. The mobile phase was a sulfuric acid solution (0.450 mmol L^{-1}, pH 3.1), employing a 0.3 mL min^{-1} flow rate and a column temperature of 75 °C. Data acquisition and analysis were performed using ChemStation software (Agilent).

2.6. Staining procedures and multiparametric flow cytometry

Multiparametric flow cytometry (FCM) was used to determine the physiological status of the microorganisms in bioprocesses with the aim of distinguishing between healthy, damaged and dead subpopulations. Culture samples were harvested by centrifugation at 16000 *x g* for 5 min and then cells were washed twice in phosphate-buffered saline (PBS, pH 7.4, sterile and filtered at 0.22 μm). Before staining, samples were held in a sonication bath for 2 s to prevent bacterial aggregation. Propidium iodide (PI, Molecular Probes) and ChemChrome V6 (CV6, Chemunex, France) were used as fluorescent dyes in a dual staining procedure (CV6/PI). Metabolic activity and membrane integrity were assessed via staining with CV6 and PI, respectively. 200 μL of cellular suspension were added to the staining solutions prepared as previously reported by Alonso et al. [23]. To establish gates in the FCM density dot plots, control samples were prepared as follows: dead cells by incubation in 70% isopropyl alcohol for 1 h [26]; early

exponentially growing cells; and mixtures containing exponential growth and dead cells (1:1). Unstained cells were used as control. FCM measurements were implemented using a Cytomics FC 500 flow cytometer (Beckman Coulter) with a 488- and 633-nm excitation light source from an argon ion laser. Green fluorescence, corresponding to CV6-stained cells, was collected on the FL1 channel (530 nm), while PI fluorescence was measured on the FL4 channel (675 nm). Analyses were carried out at a low flow rate setting (4000 events s^{-1}). Data acquisition and analysis were implemented using Cytomics RXP and Kaluza Analysis software (Beckman Coulter), respectively.

3. Results and discussion

3.1. Growth and pH evolution, substrate consumption and product synthesis throughout L. casei *and* P. taetrolens *in pure culture fermentations*

In order to study the influence on the two microorganisms of their coexistence in the same niche, batch pure culture fermentations were performed to compare their sole behavior with bacteria in consortium. Operating conditions were chosen in line with previous studies to maximize the production of lactic and lactobionic acids for each microorganism [10, 11]. Table 1 summarizes the main fermentation parameters performed in the four systems studied.

Fig. 1 shows bacterial growth (as an optical density measurement), pH, lactose and organic acids evolution during pure culture fermentations of *L. casei* (Fig.1a and b) and *P. taetrolens* (Fig. 1c and d). In this study, ultrafiltrated sweet cheese whey with 40 g L^{-1} initial lactose content was used as substrate for all fermentations. In pure culture, *L. casei* CECT 475 consumed 18% of the initial carbohydrates to produce a final concentration of 8.9 g L^{-1} lactic acid after 72 h of incubation, with a lactic acid productivity of 0.10 g L^{-1} h^{-1} and a lactic acid yield on substrate of 0.87 g g^{-1}. These results confirmed the ability of this particular strain of *L. casei* to exploit the available carbohydrates contained in dairy byproducts as a carbon and energy source to produce lactic acid, as previously reported by other authors [1, 11]. In the case of *P. taetrolens,* 34.6% of the initial lactose content was consumed to produce 11.1 g L^{-1} lactobionic acid after 72 h of incubation, with a lactobionic acid productivity of 0.15 g L^{-1} h^{-1} and a lactobionic acid yield on substrate of 0.8 g g^{-1}.

3.2. Effect of coexistence with P. taetrolens *on* L. casei *growth*

Mixed fermentations were evaluated in terms of bacterial growth and productivity. Fig. 2 shows a comparison of the optical density measurements corresponding to the four culture systems studied. Mixed fermentations developed under the most favorable conditions to *L. casei* resulted in a similar curve to that obtained for *L. casei* in pure culture, reflecting the low contribution of *P. taetrolens* to the global biomass. When operating environment was favorable to *P. taetrolens*, only a low increase in the optical density was observed throughout the cultures, indicating a poor development of both microorganisms. A CFU mL^{-1} count was performed to study the development of *L. casei* and *P. taetrolens* separately. This type of analysis was feasible because each microorganism from a mixed culture sample only grew on its specific solid media (*L. casei* colonies on MRS and *P. taetrolens* on NB agar).

Fig.3a shows the *L. casei* CFU mL^{-1} count throughout pure culture and mixed fermentations performed under the two sets of operating conditions studied here. The results showed a similar curve for *L. casei* when it developed in pure culture, compared to when it grew in the presence of *P. taetrolens,* whenever the physical environment parameters were favorable to it (temperature of 37 °C and microaerophilic conditions). Moreover, no morphologic changes were observed in *L. casei* colonies. When mixed fermentations were developed under the most favorable operating conditions for *P. taetrolens*, which were totally the opposite (30 °C and highly aerobic conditions), colonies also maintained their characteristic appearance, although their count decreased to a value of 0 CFU m L^{-1} after 6 h of incubation. The bacteria became non-culturable. Previous studies have reported that, when fermentation starters are brought under different kinds of sublethal stress, lactic acid bacteria may be harmed and unable to grow on a medium which is adequate for growth of unstressed cells [27].

3.3. Effect of coexistence with L. casei *on* P. taetrolens *growth*

Fig. 3b shows the growth curves corresponding to the *P. taetrolens* CFU mL^{-1} count throughout pure culture and mixed fermentations with *L. casei*. Experimental results showed that coexistence with *L. casei* is especially harmful to *P. taetrolens,* as *Pseudomonas* was not able to grow on the NB agar plates after 8 h of incubation, not even under its most favorable growth conditions. When mixed fermentations were performed under the optimum conditions for *L. casei,* no growth was registered on the agar plates right from the beginning of the process.

The liberation of harmful substances from *L. casei* to the culture medium could be considered as a plausible hypothesis explaining the impossibility of *P. taetrolens* to develop colonies on the solid media. Previous studies have reported the inhibitory effect of probiotic cultures, and more specifically of *L. casei*, against spoilage and pathogenic bacteria, probably induced by the production of an antimicrobial substance, such as a bacteriocin [13, 28]. Bacteriocins are protein metabolites which are ribosomally synthesized and extracellularly released by lactic acid bacteria (LAB). They have bactericidal or bacteriostatic effects, generally over bacteria belonging to closely related species [29]. However, they have been seen to be able to exert their inhibitory effect over species from different genera [30]. Several studies report that LAB bacteriocins can even be active against Gram-negative, pathogenic or food spoilage bacteria, such as some species belonging to *Pseudomonas spp*. [31], and even fungi [32]. Jamuna and Jeevaratnam [31] detected bacteriocin production at the end of the exponential growth phase, which reached the highest concentration in the middle of the stationary phase. As can be observed in Figs. 1a and 3a, both the OD and the CFU mL^{-1} curves indicate that *L. casei* exponential growth finishes after approximately 10 h of incubation and is followed by the stationary phase. No *P. taetrolens* colony growth was registered after 8 h of incubation, when *L. casei* could have begun to release the inhibitory bacteriocins. From this moment onwards, cells were no longer culturable. The lactic acid bacteria could already have released their inhibitory metabolites into the precultures employed as inocula for fermentations, so a certain amount of bacteriocins could have been present in the culture medium from the beginning of the process. However, previous studies on growth dynamics in mixed cultures, in which one microorganism excretes toxins that harm the other, have found that a minimum concentration of toxin is required before the onset of the inhibitory effect [33]. This necessary period of accumulation would explain the 6 h lag phase during which *P. taetrolens* was still able to develop colonies on the agar plates.

3.4. Atypical colony growth of P. taetrolens *in the presence of* L. casei

Photographs corresponding to *P. taetrolens* colonies developed on NB agar plates are shown in Fig.4. During mixed fermentation performed under the most favorable growth conditions for *Pseudomonas,* bacteria still maintained their ability to grow on agar plates after 6 h of incubation, as previously reported. However, their colonies showed atypical growth with significant differences from their characteristic morphology. As can be seen in Fig. 4 a1 and a2, typical *P. taetrolens* colonies present a circular form, slightly raised, with a smooth surface,

entire edge and an amorphous internal surface [34]. When *P. taetrolens* grew in the presence of *L. casei*, the colonies had achieved a smaller size and appeared less compact, with a more subdued color after 6 h of culture (Fig. 4 b1 and b2). This anomalous growth of colonies could be attributed to the harmful effect of bacteriocins [31]. The inhibitory metabolites could be present in small amounts from the start of cultivation, enabling colony growth until 6 h of incubation, but harming their normal development.

3.5. Physiologic status monitoring via multiparameter flow cytometry

Fig. 5 shows the density dot plots obtained from a pure culture of *L. casei* (a) and those corresponding to the microbial consortium of the lactic bacteria and *Pseudomonas taetrolens* under favorable environmental parameters to *L. casei* (b), combining CV6 and PI staining dyes. The upper left quadrant (F1) of Fig. 5a and b shows viable cells with enzymatic activity (CV6-positive cells). Damaged cells (corresponding to CV6 (+)/PI (+)) are shown in the upper right quadrant (F2), while dead cells (PI-positive) appear in the lower right quadrant (F4). The red/green/blue/violet hot spots represent the density gradient inside each population, from highest to lowest cell concentrations, respectively.

As can be observed in these cytograms, almost all the *L. casei* cells in pure culture (Fig. 5a) preserved their metabolic activity (more than 95% of cells from 10 h of incubation to the end of fermentation, as shown in Fig. 5a). Previous studies reported the capacity of *L. casei* cells to remain metabolically active throughout dairy byproduct fermentations, despite the environmental acidic conditions when they are implemented without pH control [23]. It seems that lactobacilli are capable of developing an acid tolerance response in order to survive under acid stress conditions, maintaining their cell membrane functionality [15, 35].

In mixed fermentations with *P. taetrolens*, however, the appearance of a small damaged subpopulation can be observed after 48 h of incubation, which became larger at 72 h (Fig. 5b). At this time, the metabolically-active subpopulation decreased from 94% at 10 h to 37 % and more than 15% of cells were dead (Fig. 6b). This decline in viability could be attributed to the presence of *P. taetrolens*, which would be affected by environmental conditions so far from its optimum and the inhibitory substances produced by *L. casei*. The estimation of CFU mL^{-1} for *P. taetrolens* in this mixed fermentation showed the inability of *P. taetrolens* to develop colonies from the beginning of the bioprocess. Nevertheless, damaged and dead cells were registered only from 48 h of incubation onwards. Before this time, only one metabolically-active population was obtained in the cytometric analysis, meaning neither *L. casei* nor *P.*

taetrolens cells were dead until this time. Consequently, after 48 h of culture, *P. taetrolens* cells would be viable, but non-culturable (VBNC).

It has been seen that unfavorable environmental conditions can induce this physiological state in *Pseudomonas* species, in which cells are able to maintain their membrane integrity and take up oxygen to respire, but are unable to form colonies on solid media [36-38]. The entry of *P. taetrolens* into this VBNC state in mixed fermentations could be caused by the bacteriostatic effect of *L. casei* bacteriocins, which prevents bacteria from reproducing, and the adverse environment when the bioprocess was implemented under lactic acid bacteria operating conditions. From 48 h onwards, cells would begin to suffer greater damage and die.

This VBNC subpopulation can be detected if the culture method is used in combination with flow cytometry, highlighting the usefulness of this technique to assess the competitive exclusion on mixed cultures in conjunction with traditional microbiological analysis via CFU counting [39, 40]. Although previous studies identifying bacterial subpopulations reported similar results using plate counting and flow cytometry as complementary methods in pure cultures, they found significantly differences in mixed populations [39, 27].

3.6. Substrate consumption

Fig. 7 shows the evolution of lactose content and organic acids in the pure culture and mixed fermentations carried out. The highest lactose consumption was observed in *P. taetrolens* pure cultures (Fig. 7a). In keeping with the results obtained through growth monitoring, coexistence with *L. casei* leads to the competitive exclusion of *P. taetrolens*. As a result of this negative effect, only 20.5% of the initial carbohydrate content was consumed in mixed fermentations carried out under optimal operating conditions for *P. taetrolens*, compared to the 34.6% degraded by *P. taetrolens* in pure culture. Moreover, part of this 20.5 % could be attributed to the fermenting activity of *L. casei*. Although the lactic bacteria were unable to form colonies after 6 h of incubation (Fig. 3), they were able to preserve their metabolic activity. Environmental stress, predominantly the highly aeration favorable to *P. taetrolens*, would induce *L. casei* to enter into this VBNC state. Besides the acid tolerance response, other authors have reported adaptations in lactic acid bacteria to other sources of stress, such as an oxidative stress resistance, which allows cells to remain viable. A Mn-containing pseudocatalase has been detected in some *L. casei* strains, with the function of detoxifying harmful reactive oxygen species and whose transcription would be induced by aeration [16, 35].

In contrast to *P. taetrolens*, *L. casei* cells in the VBNC state would preserve their productive capacity. It has been seen that non-culturable starters which preserve their metabolic capacity may contribute to fermentation activity [27, 41]. The enzymes present may be active and cells could even have the ability to synthesize new enzymes. Consequently, metabolic routes may be functional and *L. casei* could oxidize lactose to produce lactic acid. This behavior becomes especially important in the case of probiotic bacteria, as non-culturable cells could preserve their healthy effects. Bacteria need to be active, though not necessarily culturable, not just to convert lactose, but also to produce antibacterial compounds or antioxidant effects [27]. Several *Lactobacillus* strains have even been found to exert their probiotic effects in a nonviable state [14]. Thus, in mixed fermentations under stressful conditions of highly aeration, from 6 hours of incubation onwards, *L. casei* became nonculturable, but it would have preserved its capacity to degrade carbohydrate and produce inhibitory bacteriocins.

3.7. Product generation: lactic and lactobionic acids

The study of lactic acid production during experiments is shown in Fig. 7b. As can be observed, the final concentration in the mixed fermentation under the optimal conditions for *L. casei* barely differs from that obtained in the *L. casei* pure culture (8.3 and 8.9 g L^{-1}, respectively). Lactic acid productivity was 0.09 g L^{-1} h^{-1} and lactic acid yield on substrate, 0.82 g g^{-1}, relatively close values to those obtained in the pure culture experiments. The final lactic acid concentration does not differ greatly from that obtained by other authors in dairy byproduct fermentations using *L. casei* in pure culture under similar operating conditions [11]. The results obtained in this work support the conclusion that if its growth conditions are maintained, the productive capacity of *L. casei* is not affected by coexistence with *P. taetrolens*. Lactic acid production decreased when mixed fermentations were implemented under the optimal physical parameters for *P. taetrolens*, which are very far from the optimal conditions for lactic acid bacteria In this last case, lactic acid final concentration decreased by 48.3 % (4.6 g L^{-1} versus 8.9 g L^{-1} in the *L. casei* pure culture) with a productivity of 0.03 g L^{-1} h^{-1}. Despite this decrease, *L. casei* was able to synthesize lactic acid, which means it preserved its metabolic activity, as previously reported.

In the case of lactobionic acid production, important differences were found when *P. taetrolens* grew in the presence of *L. casei* compared with the final concentration reached in pure cultures, as can be observed in Fig. 7c. Production decreased from 11.1 g L^{-1} in the pure culture to around

1.4 g L^{-1} in the mixed fermentations (productivity values of 0.15 and 0.02 g L^{-1} h^{-1}, respectively) in spite of *P. taetrolens* developing under its optimal growth conditions. This decrease in the final concentration of lactobionic acid evidences that the coexistence of the two microorganisms leads to an antagonistic interaction, with the inhibition of *P. taetrolens* growth, regardless of the optimal experimental conditions for its growth. In contrast, *L. casei* is able to preserve its viability and productivity even when the environment is not favorable to it. Thus, *L. casei* was not damaged by the interaction; it was only affected by experimental conditions. The predominance of lactic acid bacteria could be supported by their production of inhibitory compounds, such as bacteriocins. In terms of ecological typing, this microbial interaction can be considered a form of amensalism [33, 42], in which the growth of one of the microorganisms (*P. taetrolens*) is restrained by the coexistence of the two species, while the other (*L. casei*) remains unaffected. Amensalism can even be strong enough to cause the death of the sensitive strain [33].

Despite the fact that *P. taetrolens* was unable to grow on the agar plates, the small amount of lactobionic acid produced in mixed fermentations under the favorable growth conditions of this microorganism would imply that some cells, though non-culturable, would be viable and metabolically active. In mixed fermentations under the optimal conditions for *L. casei*, no lactobionic acid production was observed.

4. Conclusion

The results of this research study show that shedding light on the interactions established between bacteria when they coexist in a microbial consortium becomes indispensable for their exploitation for industrial purposes. Considering the amensalistic nature of the *L. casei* and *P. taetrolens* association, the simultaneous production of lactic and lactobionic acids is not feasible either in terms of production or viability. Major interest in the co-production of lactic and lactobionic acids, within the context of a synbiotic product with food applications, will motivate the development of sequential cultures.

Conflict of interest

The authors declare that they have no conflict of interest.

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Table 1 - Summary of the main operating conditions for fermentations

Fermentation parameters	*L. casei* pure cultures	*P. taetrolens* pure cultures	Mixed fermentations favorable to *L. casei*	Mixed fermentations favorable to *P. taetrolens*
Inoculum level (%)	10	10	10 *L. casei* + 10 *P. taetrolens*	10 *L. casei* + 10 *P. taetrolens*
Working volume: air ratio	1:0	1:4	1:0	1:4
Agitation rate (rpm)	100	250	100	250
Temperature (ºC)	37	30	37	30

Figure captions

Fig. 1 –Optical density (OD) and pH (a) and lactose and lactic acid evolution (b) during pure culture fermentations of *L. casei*. OD and pH (c) and lactose and lactobionic acid evolution (d) during pure culture fermentations of *P. taetrolens*

Fig. 2 – OD evolution in the four culture systems studied: *L. casei* pure cultures, mixed fermentations under the optimal growth conditions of *L. casei*, *P. taetrolens* pure cultures and mixed fermentations under the optimal growth conditions of *P. taetrolens*

Fig. 3 – Comparison of the bacterial growth evolution as CFU mL^{-1} in mixed fermentations developed under the optimal conditions for both microorganisms against pure cultures of *L. casei* (a) and *P. taetrolens* (b). There was no growth of *P. taetrolens* in mixed fermentations under *L. casei* more favorable conditions

Fig. 4 – (a.1) and (a.2) *P. taetrolens* colonies in NB agar corresponding to a pure culture fermentation. (b.1) and (b.2) *P. taetrolens* colonies in NB agar corresponding to a mixed fermentation with *L. casei*. Scale bars = 1 cm

Fig. 5 – Cytograms representing physiological subpopulations of *L. casei* during pure culture fermentations (a) and *L. casei* with *P. taetrolens* during mixed fermentations (b)

Fig. 6 – Percentages of (a) *L. casei* pure culture and (b) mixed fermentations subpopulations of metabolically active, damaged and dead cells

Fig. 7 – Lactose consumption (a), lactic acid (b) and lactobionic acid (c) generation in the four culture systems studied: *L. casei* pure cultures, mixed fermentations under the optimal growth conditions of *L. casei*, *P. taetrolens* pure cultures and mixed fermentations under the optimal growth conditions of *P. taetrolens*

Figure 1

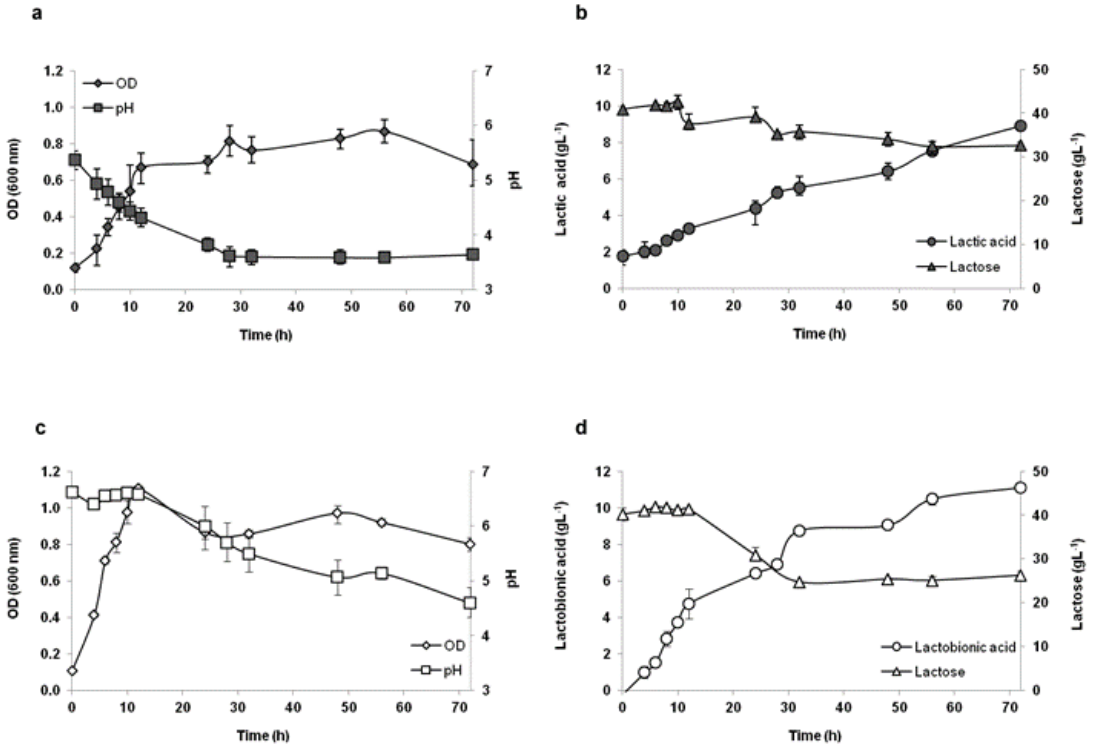

a
OD
pH
OD (600 nm)
pH
Time (h)
b
Lactic acid
Lactose
Lactic acid (gL⁻¹)
Lactose (gL⁻¹)
Time (h)
c
OD
pH
OD (600 nm)
pH
Time (h)
d
Lactobionic acid
Lactose
Lactobionic acid (gL⁻¹)
Lactose (gL⁻¹)
Time (h)

Figure 2

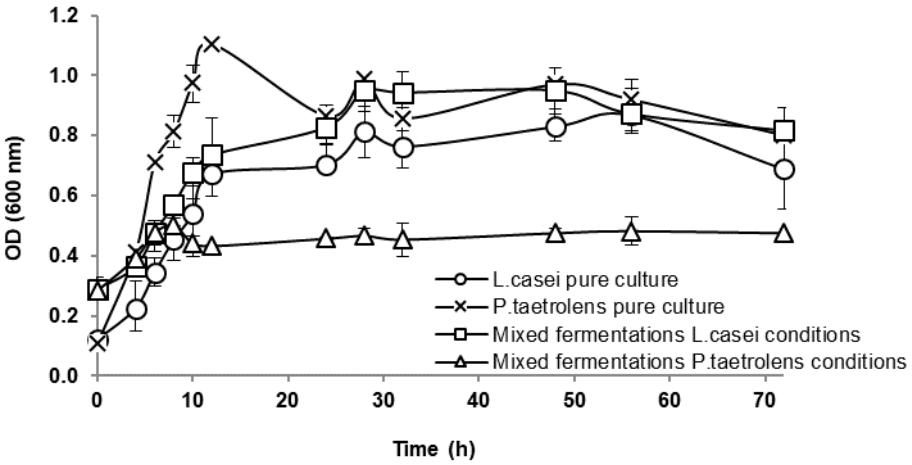

1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD (600 nm)
0
10
20
30
40
50
60
70
Time (h)
L.casei pure culture
P.taetrolens pure culture
Mixed fermentations L.casei conditions
Mixed fermentations P.taetrolens conditions

Figure 3

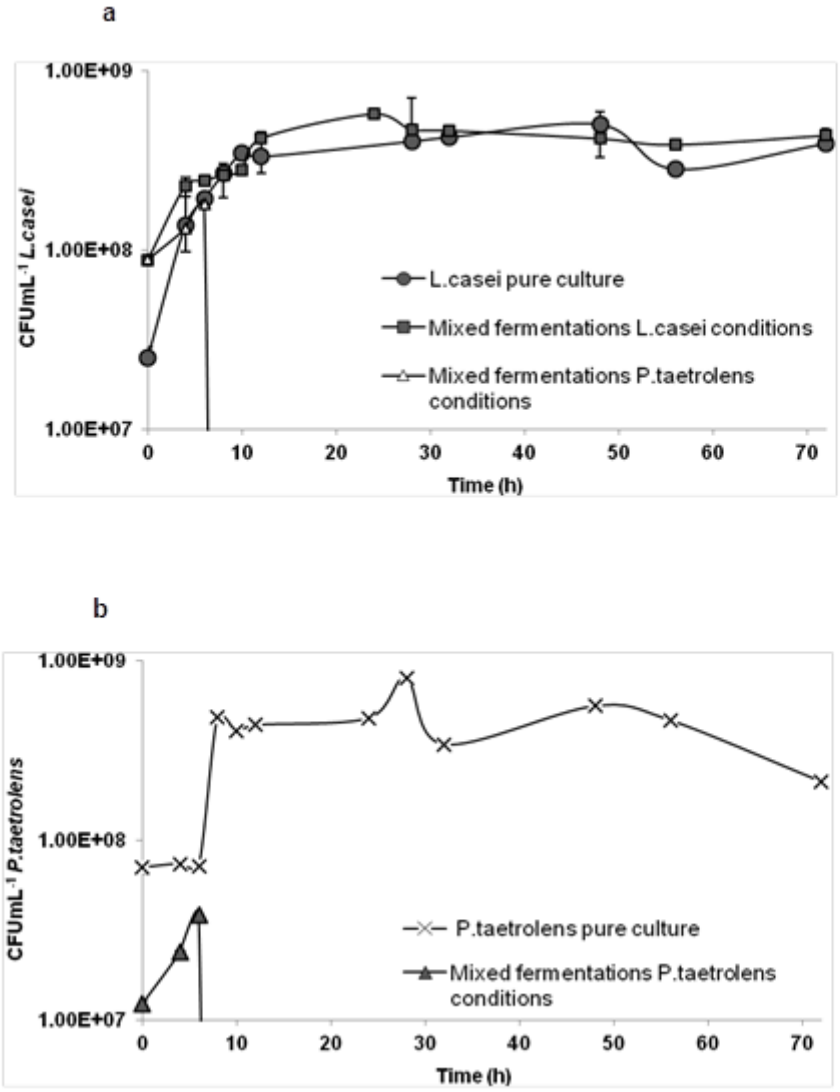

a
1.00E+09
1.00E+08
1.00E+07
CFUmL-1 L.casei
0
10
20
30
40
50
60
70
Time (h)
L.casei pure culture
Mixed fermentations L.casei conditions
Mixed fermentations P.taetrolens conditions
b
1.00E+09
1.00E+08
1.00E+07
CFUmL-1 P.taetrolens
0
10
20
30
40
50
60
70
Time (h)
P.taetrolens pure culture
Mixed fermentations P.taetrolens conditions

Figure 4

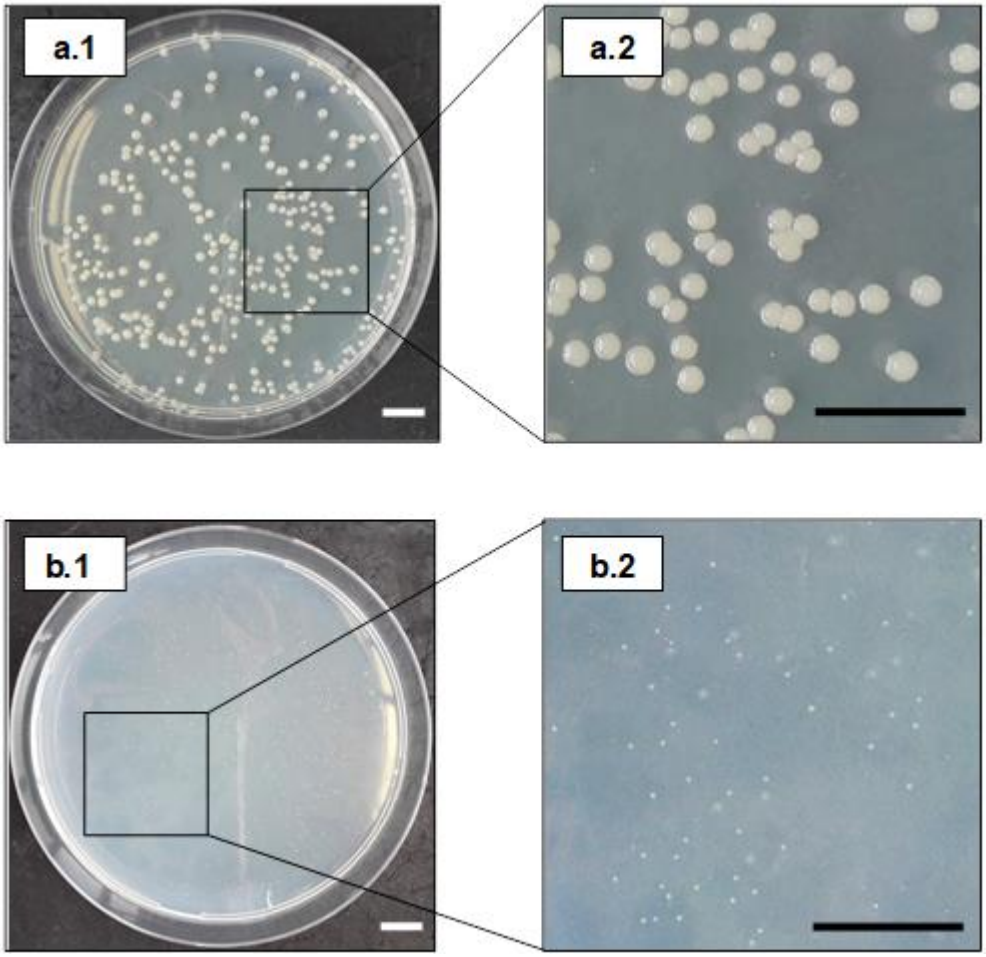


Figure 5

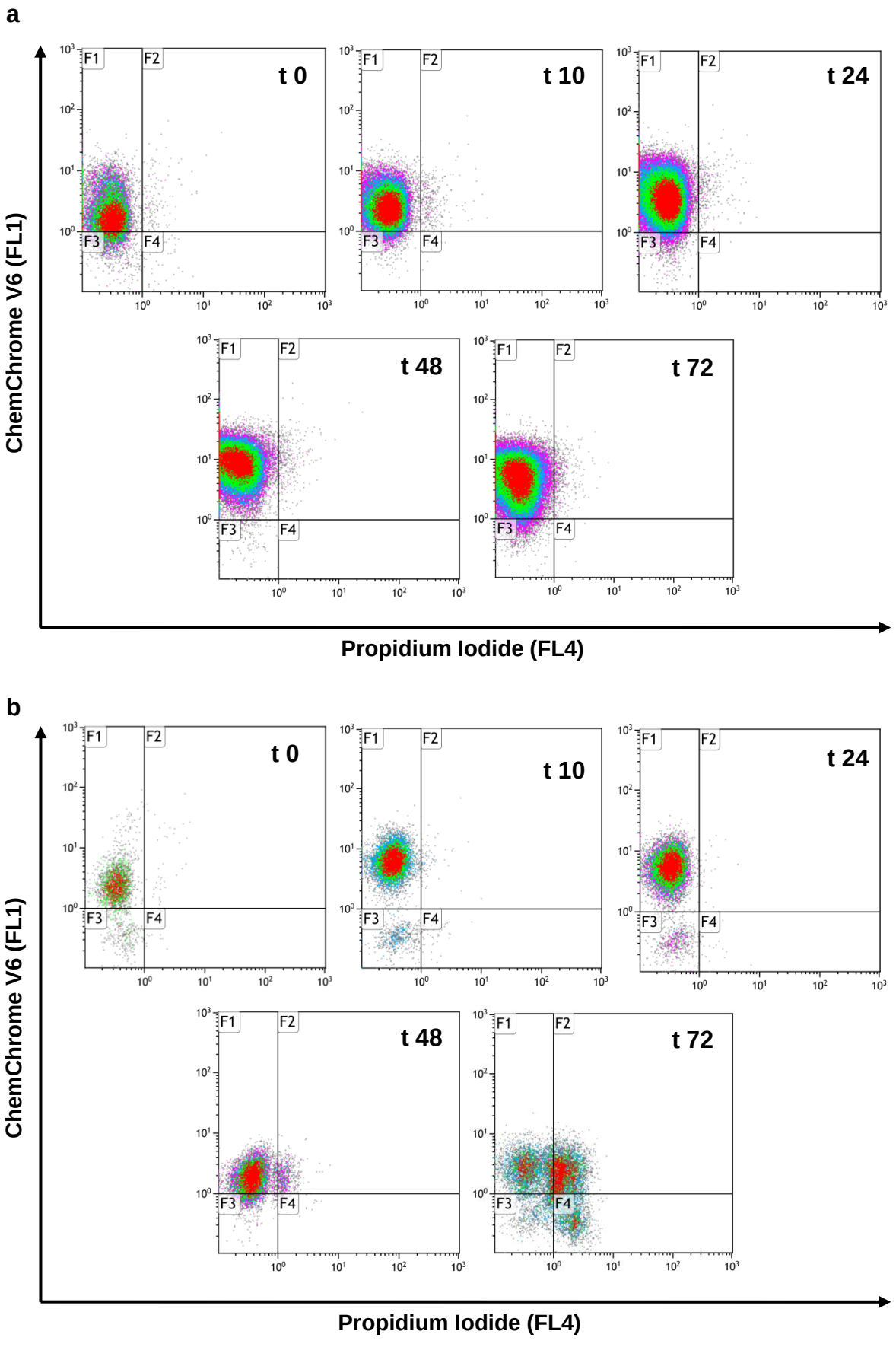

a
t 0
t 10
t 24
t 48
t 72
F1
F2
F3
F4
ChemChrome V6 (FL1)
Propidium Iodide (FL4)
b
t 0
t 10
t 24
t 48
t 72
F1
F2
F3
F4
ChemChrome V6 (FL1)
Propidium Iodide (FL4)

Figure 6

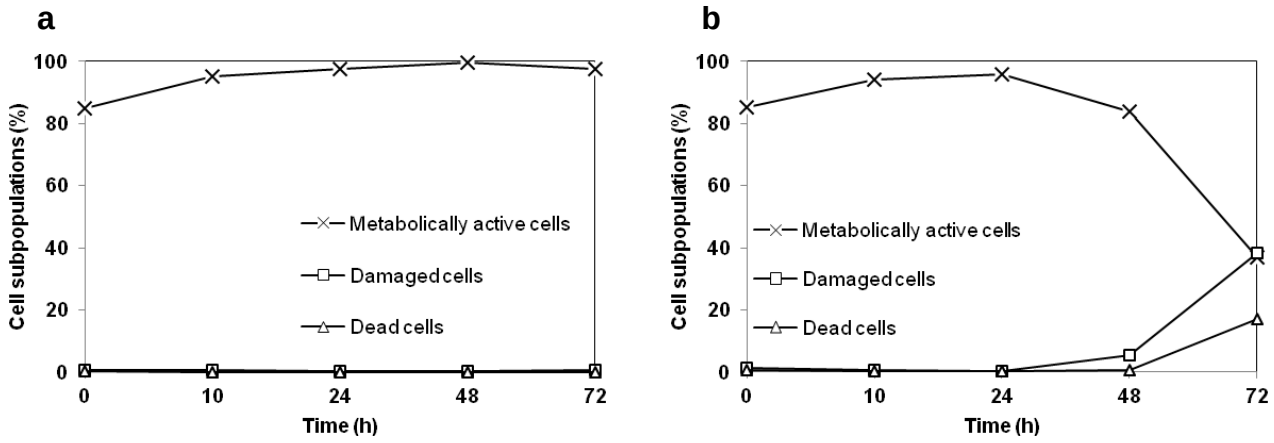
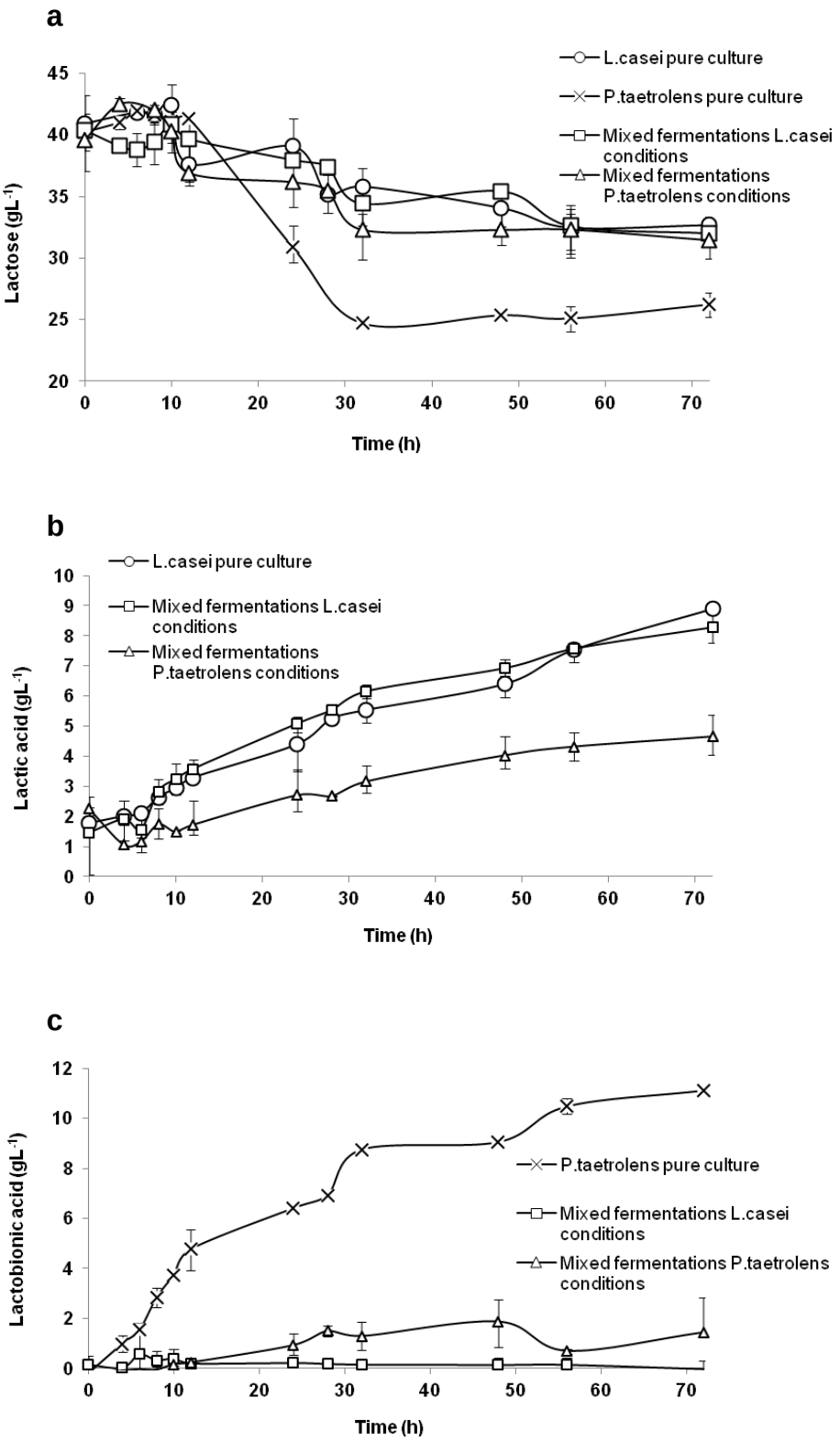

a
b
Cell subpopulations (%)
Time (h)
Metabolically active cells
Damaged cells
Dead cells
0
10
24
48
72
20
40
60
80
100

Figure 7


a
L.casei pure culture
P.taetrolens pure culture
Mixed fermentations L.casei conditions
Mixed fermentations P.taetrolens conditions
Lactose (gL-1)
Time (h)
b
L.casei pure culture
Mixed fermentations L.casei conditions
Mixed fermentations P.taetrolens conditions
Lactic acid (gL-1)
Time (h)
c
P.taetrolens pure culture
Mixed fermentations L.casei conditions
Mixed fermentations P.taetrolens conditions
Lactobionic acid (gL-1)
Time (h)

4.2. Sistema de fermentación secuencial para la co-producción de los ácidos láctico y lactobiónico

La inoculación secuencial es habitualmente utilizada en procesos industriales en los que intervienen BAL, como en la vinificación. Esta estrategia permite al microorganismo introducido en primer lugar desarrollar libremente su metabolismo, pero además posibilita establecer etapas diferenciadas durante las cuales pueden aplicarse las condiciones operacionales más favorables a cada microorganismo. La interacción de tipo amensalista detectada entre *P. taetrolens* y *L. casei* en fermentaciones simultáneas llevó al planteamiento del sistema secuencial, inoculando a *P. taetrolens* en primer lugar para evitar el efecto inhibitorio de los compuestos antimicrobianos liberados por *L. casei*. En la presente investigación se diseñó el esquema fermentativo, estableciéndose el pH como el parámetro determinante para establecer la duración del intervalo entre la primera y segunda inoculación. Durante ambas etapas se monitorizaron el crecimiento de los microorganismos y su capacidad productiva, con el objetivo de analizar la viabilidad y eficacia del proceso diseñado. Además del acoplamiento de la biosíntesis de ácido lactobiónico a la fermentación ácido-láctica, se pretende que el producto final resultante presente propiedades simbióticas por su contenido en el prebiótico lactobiónico y el probiótico *L. casei*. En la actualidad se comercializa una gran variedad de complementos alimenticios a los que se atribuyen propiedades simbióticas, pero a veces las evidencias de que el componente prebiótico favorezca el crecimiento del microorganismo probiótico, dando lugar a un efecto realmente sinérgico, son limitadas (Adebola et al., 2014). En este trabajo se analizó la capacidad de la cepa seleccionada de *L.casei* (CECT 475) para aprovechar el ácido lactobiónico en ausencia de otra fuente de carbono. Esta propiedad es esencial para que el producto ejerza su papel funcional en el intestino grueso, donde la presencia de glucosa es limitada.

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Synbiotic fermentation for the co-production of lactic and lactobionic acids from residual dairy whey

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Abstract

Besides its properties as an antioxidant, stabilizer or acidifier, lactobionic acid has emerged as a potential prebiotic compound, raising the possibility of being included together with the probiotic microorganism *Lactobacillus casei* in novel functional fermented foods with synbiotic characteristics. Their manufacturing strategy could benefit from the recently implemented microbial synthesis of lactobionic acid by the strong producer *Pseudomonas taetrolens*, employing residual dairy whey as raw material. The phenomenon of amensalism established between *Pseudomonas* and *Lactobacillus* makes simultaneous fermentation unfeasible. A novel sequential process has been developed in which *L. casei* is inoculated in a second step. Its ability to utilize lactobionic acid as a carbon and energy source was previously tested. Experimental results showed the capacity of *L. casei* to work efficiently on the residual substrate fermented by *P. taetrolens*, producing lactic acid by degrading the remaining lactose, with a lactic acid yield on substrate and productivity of 0.95 g g^{-1} and 0.20 g L^{-1} h^{-1}, respectively. Lactobionic acid was barely consumed in this complex growth medium, thus ensuring its presence in the resulting fermented product.

Keywords: synbiotic; *Lactobacillus casei*; *Pseudomonas taetrolens*; lactobionic acid; residual whey.

1. Introduction

The current fight against diseases such as obesity or cancer and global concern over the acquisition of healthy eating habits have led to the development of functional foods with an added positive health benefit besides their basic nutritional functions.[1,2] "Synbiotics" can be included within this group, i.e. synergistic combinations of probiotics and prebiotics which are co-administrated with the idea that the prebiotics will improve the survival and growth of the probiotics and their beneficial metabolic effect.[3,4]

A variety of compounds are currently marketed as synbiotic products. However, previous research has reported that not all synbiotic combinations are effective, as probiotic bacteria may be unable to utilize the prebiotic as a source of carbon. Optimal growth is only achieved with combinations of probiotics and prebiotics which are specific at the strain level.[4,5]

Most of the probiotics currently in use belong to *Lactobacillus* and *Bifidobacterium* species, while numerous indigestible carbohydrates are used as prebiotics.[4,6] A certain amount of these prebiotics are lactose derivatives,[7] such as lactobionic acid.[4,8,9] This policarboxylic acid has been a subject of major interest in recent years due to its numerous industrial applications. Within the food industry, in addition to its potential as a prebiotic, it has been found to enhance calcium absorption and to present antioxidant or gelling properties,[8,10] thereby raising interest in its role as a food additive.[11] The industrial production of lactobionic acid is currently carried out via chemical synthesis. However, the high cost of the processes involved and their environmental drawbacks have led to the development of microbial biosynthesis mechanisms.[8,12-14] In this respect, efficient bioprocesses have been implemented to produce lactobionic acid via fermentation of an inexpensive feedstock such as residual cheese whey, utilizing *Pseudomonas taetrolens* bacteria as the fermenter microorganism.[8,15] The suitability of dairy waste as a substrate for the synthesis of traditional organic acids, such as lactic acid, and for producing fermented foods or beverages in which microorganisms are directly inoculated into the dairy substrates to obtain the final fermented product has been proved in previous studies.[16-19] These biotechnological approaches provide an opportunity to valorize whey, an important industrial byproduct whose content in lactose and other essential nutrients make it suitable for microbial growth.[17,20] Otherwise, a large volume of residual dairy whey has to be managed, resulting in a major environmental problem.[17,21]

As regards the major industrial interest in lactobionic acid, its efficient production by microbial fermentation of dairy whey opens up the possibility of obtaining fermented dairy products enriched with a lactobionic acid produced "in situ", eliminating the need to add it later in the process. If probiotic bacteria with the ability to utilize lactobionic acid are used as lactic acid starters, the final fermented product could be considered a synbiotic. The specificity of probiotic and prebiotic combinations to obtain a synbiotic with real, positive, synergic properties necessitates the study of the probiotic capacity to benefit from the particular prebiotic.

In this research study, the ability of *Lactobacillus casei* CECT 475, a probiotic strain widely used in the food industry,[22] to utilize the prebiotic lactobionic acid effectively as a source of carbon to grow and synthesize lactic acid was proved. Sequential fermentations of *L. casei* CECT and *Pseudomonas taetrolens* LMG 2336 were carried out with the aim of developing a biotechnological process which enables a fermented product with synbiotic properties containing *L. casei* CECT 475 and lactobionic acid to be obtained. This *Pseudomonas* strain was selected due to its proven selectivity for lactose conversion with high conversion yields in bioprocesses previously developed with bacteria in pure culture and cheese whey as substrate.[8,15] The sequential fermentation system was chosen over a simultaneous fermentation process to avoid the harmful effect exerted by the lactic acid bacteria antimicrobials on the survival and growth of *Pseudomonas*.[23] Residual sweet cheese whey was employed as the source of carbon, making the addition of a synthetic substrate unnecessary and providing an alternative use to valorize this waste with a high pollutant potential.

2. Materials and methods

2.1. Microorganisms

Lactobacillus casei CECT 475, obtained from the Spanish Type Culture Collection (Valencia, Spain), was maintained frozen (in a 40% v/v solution at -20 °C) and subsequently subcultured on MRS (de Man Rogosa and Sharpe, Biokar Diagnostic, France) agar plates, incubated for 48 h at 30 °C and then preserved at 4 °C.

Pseudomonas taetrolens LMG 2336, obtained from the Belgian Coordinated Collection of Microorganisms (Gent, Belgium), was maintained frozen in 40% (v/v) glycerol at -20 °C. This strain was subsequently subcultured on NB agar plates (Nutrient Broth, containing 1g L^{-1} meat

extract, 2 g L^{-1} yeast extract, 5 g L^{-1} peptone, and 5 g L^{-1} NaCl), incubated for 48 h at 30 °C and then preserved at 4°C.

2.2. Sweet whey preparation

Permeate from sweet cheese whey ultrafiltration (kindly supplied by ILAS S.A., Asturias, Spain) containing 200 g L^{-1} of initial lactose (18 °Bx) was diluted in distilled water until obtaining an initial lactose concentration of 40 g L^{-1}. The pH was then adjusted to 6.5 (by adding 6 M NaOH) prior to sterilization using a tangential microfiltration device equipped with a 0.22 μm pore size PVDF membrane-cassette (Millipore, Massachusetts, USA).

2.3. L. casei *CECT 475 growth in the presence of synthetic lactobionic acid*

With the aim of determining the capacity of the *Lactobacillus casei* CECT 475 strain to utilize lactobionic acid as a source of carbon and energy, the following experiment was conducted according to the procedure described by Adebola et al.[4] A basal MRS broth[6] with the following composition (g L^{-1}) was prepared: peptone (10.0), yeast (5.0), sodium acetate dihydrate ($C_2H_3NaO_2$ x $2H_2O$) (5.0), dipotassium phosphate trihydrate (K_2HPO_4 x $3H_2O$) (2.0), ammonium citrate dihydrate ($(NH_4)_3C_6H_5O_7$ x $2H_2O$) (2.0), magnesium sulfate heptahydrate ($MgSO_4$ x $7H_2O$) (0.2), manganese sulfate tetrahydrate ($MnSO_4$ x $4H_2O$) (0.05), and Tween 80 (1 mL). The pH was adjusted to 6.2 with NaOH 6M and the medium was sterilized at 120 °C for 15 min. This basal MRS was supplemented with 0.5, 1, 2.5 and 5% lactobionic acid (Sigma-Aldrich, Steinheim, Germany) or glucose from Panreac (Barcelona, Spain), the latter being used as positive control. A basal MRS medium without any source of carbon was used as negative control. 250 mL storage media bottles containing 250 mL of basal or supplemented MRS broth (medium volume to air ratio of 1:0) were inoculated with a loopful from an MRS agar plate and then incubated in an orbital shaker (Infors HT, model FLyer Aerotron, Switzerland) at 37 °C without agitation for 52 h. Samples were periodically taken from the fermentation media to determine bacterial growth via the spectrophotometric measurement of the optical density at 600 nm (Shimazdu, UV 1203 model). Cell-free culture samples were stored (at -20 °C) for subsequent analyses of lactobionic acid and glucose consumption and lactic acid production.

2.4. L. casei *CECT 475 growth on cheese whey fermented by* P. taetrolens *LMG 2336*

Sequential fermentations were performed in an orbital shaker without pH control to study the capacity of *L. casei* to grow on sweet cheese whey previously fermented by *P. taetrolens,* resulting in a synbiotic final product containing both *L. casei* and lactobionic acid.

The ultrafiltration permeate of cheese whey prepared as previously described was inoculated first with *P. taetrolens* LMG 2336. In a prior step, *P. taetrolens* was reactivated as follows: a loopful from a NB agar plate was used to inoculate a 500 mL Erlenmeyer flask containing 100 mL of NB broth (medium volume to air ratio of 1:4), which was incubated in the orbital shaker at 250 rpm and 30 °C for 10 h. 10% (v/v) of this culture was used as inoculum in 100 mL sweet whey, subsequently incubated at 30 °C and 250 rpm for 12 h. Actively growing cells from this precultures were used to inoculate 400 mL sweet cheese whey (medium volume to air ratio of 1:4), with a 10% level of inoculum, incubating this culture employing an agitation rate of 250 rpm at 30 °C. Samples were periodically taken from the fermentation medium to determine pH and bacterial growth, storing cell-free culture samples (at -20 °C) for subsequent analyses. Fermentation was carried out until obtaining a medium pH of 5, at which point the *P. taetrolens* biomass was removed via centrifugation at 12000 *x g* for 10 min.

After removing the *P. taetrolens* biomass, the fermented medium was inoculated with *L. casei* CECT 475. Previously, *L. casei* was reactivated in MRS broth under microaerophilic conditions. A loopful from an MRS agar plate was used to inoculate a 250 mL storage media bottle containing 250 mL of MRS broth (medium volume to air ratio of 1:0), which was subsequently incubated in an orbital shaker at 37 °C without agitation for 16 h. 10% (v/v) of this culture was used to inoculate 90 mL sweet whey, which were then incubated in the orbital shaker at 37 °C and 100 rpm for 20 h. Actively growing cells from theses precultures were employed as inoculum for the residual whey previously fermented by *P. taetrolens* (medium volume to air ratio of 1:0), which was then incubated at an agitation rate of 100 rpm at 37 °C. Samples were periodically taken from the fermentation medium to determine pH and bacterial growth and to perform subsequent analyses of lactose and lactobionic acid consumption and lactic acid generation. *L. casei* fermentations were carried out for 48 h.

2.5. Analytical methods

Bacterial growth was measured spectrophotometrically as optical density (OD) at 600 nm (Shimazdu, UV 1203 model) after centrifuging culture samples at 16000 *x g* for 5 min. Optical density values were converted to dry cell weight through the calibration curve previously obtained and results were expressed as g L^{-1}. Moreover, the viable cell count was performed via the spread plate method in MRS and NB agar for *L. casei* and *P. taetrolens*, respectively. Colony forming units (CFU) were counted after incubating agar plates for 48 h at 30 °C in both cases. Results were expressed as CFU mL^{-1}.

Lactose, lactic acid and lactobionic acid concentrations from cell-free culture samples were measured via high performance liquid chromatography (HPLC). The liquid chromatographic system used for the analysis (Agilent 1200, Agilent Technologies Inc., CA, USA) was equipped with an ICSep ICE-ION-300 column (Transgenomic Inc, CA, USA) coupled to a refractive index detector. Sulphuric acid (0.450 mM, pH 3.1) was employed as the mobile phase at a flow rate of 0.3 mL/min, setting the column temperature at 75 °C. Data acquisition and analysis were performed with ChemStation software (Agilent).

Fermentations were carried out in duplicate as independent experiments. The reported data correspond to the medium values of at least three measurements. Positive and negative error values are presented as error bars in the figures.

3. Results and discussion

3.1. Ability of L. casei *CECT 475 to utilize synthetic lactobionic acid*

Bacterial growth of the probiotic strain *L. casei* CECT 475 in the presence of a synthetic lactobionic acid prebiotic substrate, as a measurement of optical density, and the medium pH evolution, are compared in Figure 1 with those registered in the presence of glucose (positive control) and in the absence of a carbon source (negative control). *L. casei* CECT 475 growth from 12 h of culture onwards in the presence of lactobionic acid was significantly greater than that achieved without a carbon source, for initial lactobionic acid concentrations in the growth medium of 0.5% (Figure 1A) and 1% (Figure 1B). As Table 1 shows, at a lactobionic acid concentration of 0.5%, the bacterial growth curve was comparable to that obtained with the same concentration of glucose (with maximum dry cell weights of 2.36 and 2.47 g L^{-1},

respectively). At the 1% concentration, lower bacterial growth was registered (2.74 g L^{-1} compared with 3.44 g L^{-1} in presence of glucose) and the phase lag length was longer. Employing an initial concentration of lactobionic acid greater than 1%, an increase in the *L. casei* biomass was not observed from the beginning of the culture (Figures 1C and D).

Our results concur with those reported by Adebola et al.[4] These authors tested the ability of five probiotic *Lactobacilli* strains to utilize lactobionic acid as a carbon source. Of these, only *L. reuteri* NCIMB and *L. acidophilus* NCFM were able to grow in this prebiotic substrate. A low preference for lactobionic acid has also been reported by other authors.[6,24] In our study, the *L. casei* CECT 475 strain proved capable of utilizing the lactobionic acid as a carbon source to grow, only as long as the acid concentration did not reduce the initial pH to below 5 (Figures 1A and 1B), which was also in line with the results obtained by Adebola et al..[4] This inhibition of growth is explained by the environmental requirements of lactic acid bacteria (LAB), which are neutrophiles with an optimal pH for growth ranging between 5 and 9.[25] The loss in viability in LAB mainly caused by the accumulation of organic acids resulting from the fermentation and the consequent decrease in the pH of the medium has been previously reported.[4,25] This loss in viability is a major drawback particularly in the case of probiotic bacteria, which have to cope with a stressful environment throughout industrial manufacturing processes and which must also survive acid stress in the gastrointestinal tract.[27] LAB have been shown to be capable of developing an acid response tolerance. However, this adaptation has been found to occurs mainly in two physiological phases: during exponential growth and, more specifically, after entering the stationary phase,[25,28] though no acid adaptation has been reported at the onset of the lag phase. The acidic environment at the beginning of the fermentation limits growth, thereby making the lag phase interminable.[29] As Figures 1C and 1D show, the low initial pH prevented *L. casei* CECT 475 from accomplishing its normal development cycle.

Figure 2 shows the results corresponding to substrate consumption (lactobionic acid) and lactic acid production, which are consistent with the previously reported evolution of bacterial growth. Thus, for initial concentrations of lactobionic acid of 0.5% and 1%, *L. casei* CECT 475 was capable of consuming the entire prebiotic substrate, using it as source of carbon to produce lactic acid. For an initial concentration of 0.5%, 4.92 g L^{-1} lactic acid were obtained, with a lactic acid yield on substrate and molar percentage yield of 0.98 g g^{-1} and 97.90%, respectively. For the 1% lactobionic acid concentration, 7.30 g L^{-1} were synthesized after 52 h of culture, achieving a lactic acid yield on substrate of 0.73 g g $^{-1}$ and 72.63% molar percentage yield

(Table 1). For higher concentrations, as there was almost no growth of the strain, practically no substrate consumption or lactic acid production was observed.

3.2. L. casei *CECT 475 growth on residual whey fermented by* P. taetrolens *LMG 2336*

The previously reported results proved the ability of *L. casei* CECT 475 to utilize synthetic lactobionic acid effectively in the absence of another source of carbon in its growth medium. The capacity of this strain to grow and utilize lactobionic acid when developed in a substrate which contains other sources of carbon was evaluated by carrying out sequential fermentations in which residual cheese whey was first inoculated with *P. taetrolens*. Figure 3 shows the evolution of *P. taetrolens* growth and the variation in medium pH (A), lactose consumption and lactobionic acid synthesis (B). After 32 h of culture, a lactobionic acid concentration of 6.30 g L^{-1} was obtained (Table 2).

The development of a sequential fermentation, inoculating first with *Pseudomonas*, prevents its inhibition by *L.casei* antimicrobials such as bacteriocins, which are bacterial proteins and metabolites with bactericidal or bacteriostatic effects active against Gram-negative, pathogenic or food spoilage bacteria such as species of the genus *Pseudomonas*.[23] The inhibitory effect of the lactic acid bacteria on the lactobionic acid producer makes the coexistence of the two microorganisms for production purposes inviable. At this respect, sequential fermentations are being implemented in the food industry to obtain products with enhanced properties. This strategy allows the microorganism inoculated first to exert its metabolism without the influence of the subsequent microorganism.[30,31] Ciani et al.[31] reported the duration of the interval between first and second inoculations as a key parameter to achieve the desired results. In our case, the length of this interval determines the value that the medium pH will have reached. After 32 h of fermentation, the pH was 5.2. Based on the results reported by Adebola et al.[4] and data obtained with the synthetic lactobionic acid substrate, this value was considered the acceptable level of acidity to inoculate *L. casei* CECT 475 into the fermentation medium.

Figures 4A and 4B show the growth of *L. casei* and the decrease in pH throughout the lactic fermentation, as well as lactose consumption and lactic acid production, respectively. The lactose remaining after *P. taetrolens* fermentation supposed an initial lactose concentration available for *L. casei* of 15.23 g L^{-1} (Table 2). The results showed the survival and growth capacity of *L. casei* CECT 475 in this medium previously fermented by *P. taetrolens*, which, in addition to the usual compounds present in residual cheese whey, contains 6.30 g L^{-1}

lactobionic acid. As can be seen in Figure 4A, the OD curve reached a maximum value of 2.73 OD units, which corresponds to 3.0 g L^{-1} of dry biomass (Table 2). This OD measurement is even higher than the maximum achieved by lactic bacteria when cultivated in synthetic lactobionic acid concentrations of 5 g L^{-1} (1.53 OD units and 2.36 g L^{-1}) or 10 g L^{-1} (2.24 OD units and 2.74 g L^{-1}).

The OD values obtained in the bacterial growth curve were also higher than those obtained in previous studies with *L. casei* in pure culture employing substrates with similar characteristics,[32] despite the lower concentration of initial lactose and the presence of lactobionic acid. Hence, lactobionic acid and other metabolites in the medium fermented by *P. taetrolens* were not harmful to the survival or growth of *L. casei* CECT 475, as long as the initial pH was above 5.

As to bacterial production capacity, a lactic acid concentration of 9.52 g L^{-1} was obtained 48 h after inoculation with *L. casei*, with a lactic acid productivity of 0.20 g L^{-1} h^{-1}. Lactic acid yield on substrate and molar percentage yield of 0.95 g g^{-1} and 91.79%, respectively, were achieved. These values reflect an efficient use of the substrate, but low lactic acid productivity. It should be borne in mind that *L. casei* is considered a nonstarter lactic acid bacterium, with slow growth in milk and requiring a longer fermentation time than the traditional yoghurt starters *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*.[33] In these experiments, permeate from the ultrafiltration of residual cheese whey, containing water, lactose and small amounts of other soluble components,[32] was employed as substrate for fermentations. Other authors have proved the ability of *L. casei* CECT 475 to utilize the carbohydrates contained in dairy byproducts effectively as source of carbon,[16,17] but this bacterium has been seen to show preference for other sugars, such as glucose and sucrose, over lactose.[16] Moreover, previous studies have reported that fermentation of this dairy permeate by *L. casei* requires supplementation with extra nutrients, such as specific minerals, amino-acids and nitrogenous bases, to achieve the complete conversion of lactose and obtain high lactic acid yields in a reasonable fermentation time.[32] The addition of supplements would, however, involve increased costs.

Analysis of the evolution of lactobionic acid throughout the sequential fermentation (Figure 4B) reveals that its concentration decreased in a very low proportion from *L. casei* inoculation until the end of the fermentation (Table 2). Nevertheless, this decline proves that lactic acid bacteria were capable of consuming a small amount of lactobionic acid, despite the substantial

concentration of lactose remaining in the fermentation medium. The consumption results confirm the ability of *L. casei* to utilize lactobionic acid as a source of carbon and energy, supporting the possibility of coupling in a product with synbiotic properties. Nevertheless, considering their very low degradation, it can be stated that lactic bacteria displayed a preference for lactose over lactobionic acid, which constitutes an important advantage, allowing the presence of the acid in the final fermented product. Thus, to be able to refer to the product reaching the consumer as a synbiotic, the prebiotic compound must be present in the product to enhance the survival and proliferation of the probiotic when ingested.[4] Probiotic bacteria encounter several stress sources, not only during industrial processes, but also in the gastrointestinal tract when they are ingested,[27] where they must survive to colonize the colon. The prebiotic, which must have be remained mainly undigested until this point, will be metabolized there by probiotic bacteria.[4]

4. Conclusions

Sequential fermentation of permeate from residual cheese whey by *Pseudomonas taetrolens* and *Lactobacillus casei* has been shown to be a feasible strategy to achieve the biotechnological co-production of lactic and lactobionic acids. The *L. casei* CECT 475 strain proved capable of growing in a fermented medium containing lactobionic acid, with the initial pH as a limiting factor. The ability of *L. casei* CECT 475 to utilize lactobionic acid as a source of carbon, though in a second place after lactose in its hierarchy of carbohydrate utilization, means that the resulting product containing the probiotic *L. casei* strain and lactobionic acid as a prebiotic may be considered a synbiotic with functional properties.

Conflict of interest

No conflict of interest exists.

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Table 1 - Summary of the productivity values obtained in the experiments with *L. casei* CECT 475 degrading synthetic lactobionic acid

Initial lactobionic acid (g L^{-1})	**5.00**	**10.00**	**25.00**	**50.00**
Final lactobionic acid (g L^{-1})	0.00 ± 0.00	0.00 ± 0.00	23.97 ± 0.15	49.77 ± 0.27
Lactic acid produced (g L^{-1})	4.92 ± 0.19	7.30 ± 0.15	0.47 ± 0.02	0.23 ± 0.00
Lactic acid yield on substrate (g g^{-1})	0.98 ± 0.04	0.73 ± 0.02	0.46 ± 0.07	1.00 ± 0.52
Lactic acid molar yield (%)	97.90 ± 0.04	72.63 ± 0.02	4.53 ± 0.07	99.93 ± 0.51
Lactic acid productivity (g L^{-1} h^{-1})	0.09 ± 0.00	0.14 ± 0.00	0.01 ± 0.00	0.00 ± 0.00
Maximum dry cell weight (g L^{-1})	2.36 ± 0.01	2.74 ± 0.00	1.73 ± 0.00	1.57 ± 0.00

Table 2 - Summary of the productivity values obtained in the experiments with *L. casei* CECT 475 degrading the residual cheese whey after the *Pseudomonas* fermentation

Initial lactose (g L^{-1})	15.23 ± 0.97
Final lactose (g L^{-1})	6.38 ± 0.91
Initial lactobionic acid (g L^{-1})	6.30 ± 0.26
Final lactobionic acid (g L^{-1})	5.16 ± 0.14
Lactic acid produced (g L^{-1})	9.56 ± 0.51
Lactic acid yield on substrate (g g^{-1})	0.95 ± 0.09
Lactic acid molar yield (%)	91.79 ± 0.09
Lactic acid productivity (g L^{-1} h^{-1})	0.20 ± 0.01
Maximum *L. casei* dry cell weight (g L^{-1})	3.00 ± 0.01

Figure captions

Fig. 1 – Evolution of the culture medium pH and the *L. casei* CECT 475 growth exposed to the following glucose (□) and lactobionic acid (Δ) concentrations: (A) 0.5%, (B) 1%, (C) 2.5% y (D) 5%. As negative control a basal growth medium without carbon source was employed (×)

Fig. 2 – Evolution of the lactobionic acid consumption (Δ) and the lactic acid production (◊) by *L. casei* CECT from the following initial concentrations of lactobionic acid: (A) 0.5%, (B) 1%, (C) 2.5% and (D) 5%, and in absence of other source of carbon

Fig. 3 – (A) Evolution of the pH (□) and OD (o) and (B) evolution of the lactose consumption (×) and the lactobionic acid production (◊) throughout the *P. taetrolens* culture during the first part of the sequential fermentation

Fig. 4 – (A) Evolution of the pH (□) and OD (o) and (B) evolution of the lactose (×) and lactobionic acid consumption (Δ) and the lactic acid production (◊) throughout the *L.casei* culture during the second part of the sequential fermentation

Figure 1

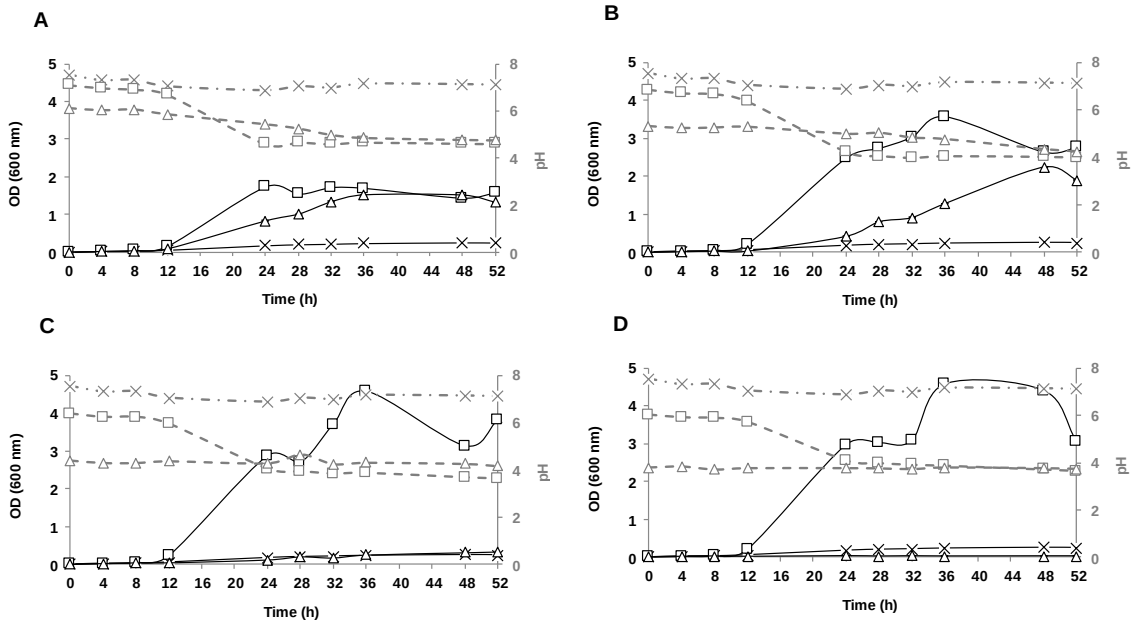

A
B
C
D
OD (600 nm)
pH
Time (h)
0 4 8 12 16 20 24 28 32 36 40 44 48 52
0 1 2 3 4 5
0 2 4 6 8

Figure 2

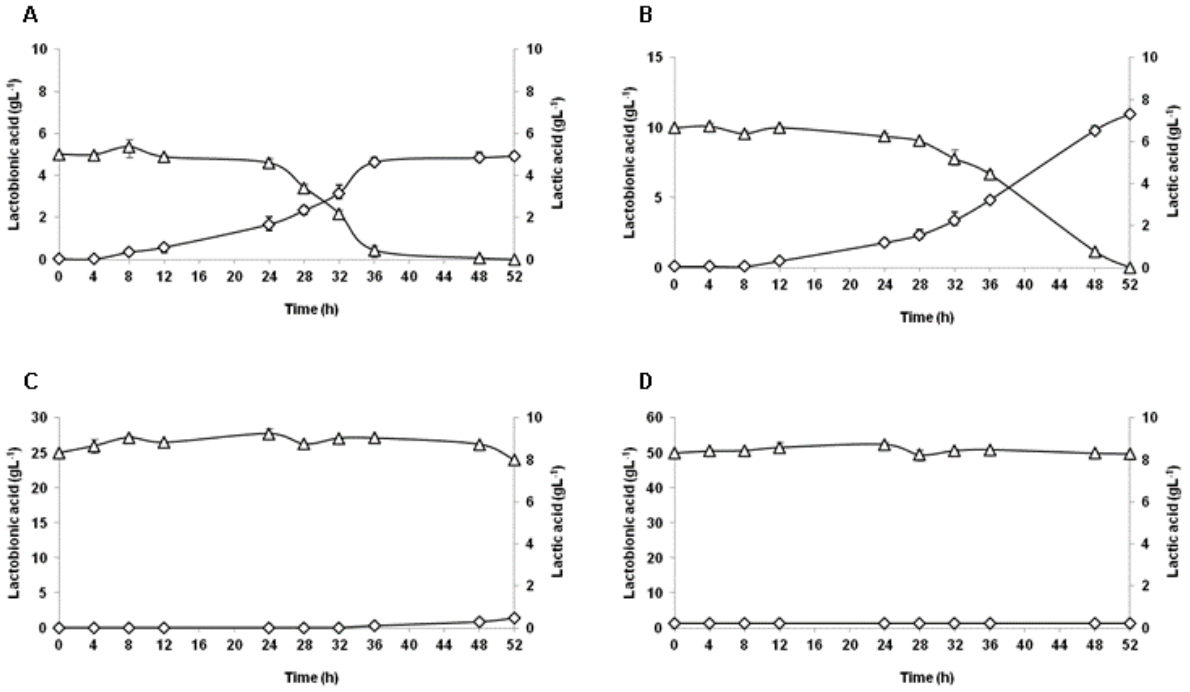


Figure 3

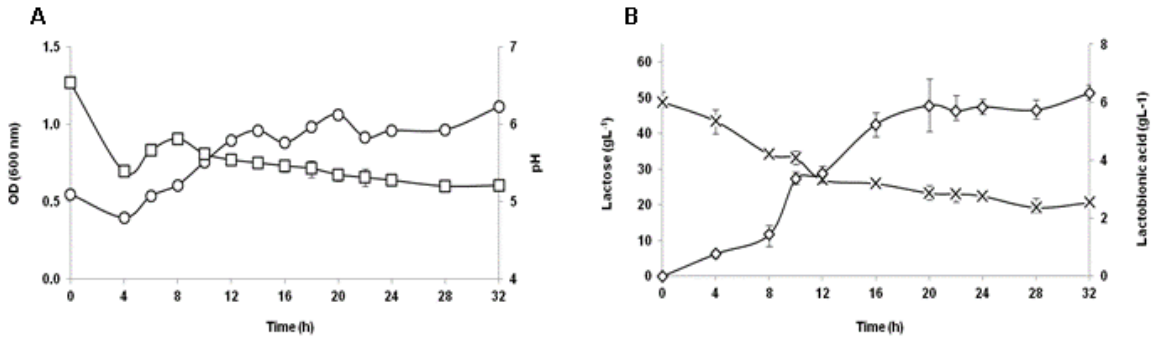
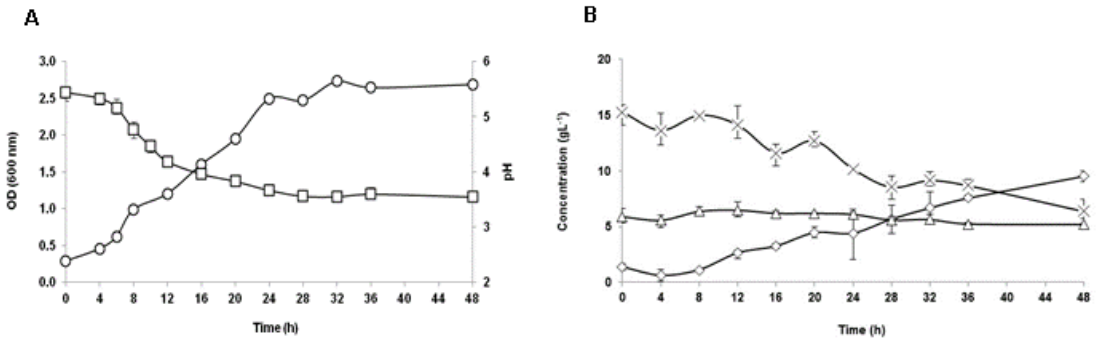


Figure 4



4.3. Desarrollo de un nuevo producto lácteo simbiótico

En este subcapítulo el sistema de fermentación secuencial diseñado previamente se ensayó sobre un sustrato de leche de vaca, con el objetivo de hacer una aproximación a su aplicación industrial para la obtención de un alimento lácteo fermentado funcional. Con este mismo fin se escaló el proceso a nivel de biorreactor con sistema digital de control, pudiendo implementar una estrategia de ajuste automático del pH que permite optimizar la bioconversión de lactosa en ácido lactobiónico (Alonso, 2011). La idoneidad de la leche como sustrato para el bioproceso secuencial se determinó cuantificando el crecimiento microbiano, el consumo del sustrato y la producción de ácidos orgánicos durante la fermentación. Las concentraciones alcanzadas en el producto final permitieron valorar su carácter funcional simbiótico. Así, se evaluó su contenido en *L. casei* y ácido lactobiónico, atendiendo a las concentraciones de probiótico a las que se atribuyen propiedades terapéuticas en alimentos procesados, y a la dosis mínima de prebiótico con efectos bifidogénicos. Además, debido a la creciente preocupación de los consumidores sobre la incidencia de intolerancias alimentarias, se clasificó el producto fermentado final en base a su nivel de lactosa. La demanda actual de alimentos funcionales va acompañada de un mayor interés en el consumo de productos "naturales". El concepto "natural" se asocia de forma común con la ausencia de aditivos sintetizados químicamente. Por ello, en este subcapítulo se evaluó también el efecto texturizante aportado por el ácido lactobiónico, comparando el análisis reológico del producto final de la fermentación secuencial de *L. casei* y *P. taetrolens* con los valores obtenidos fermentando la leche con *L. casei* en cultivo puro. De forma adicional se ensayó la suplementación con un espesante tradicionalmente utilizado como es el k-carragenano. Los resultados de esta investigación ponen de manifiesto los beneficios de integrar la bioproducción del ácido lactobiónico en la manufactura de lácteos fermentados, modificando *"in situ"* sus propiedades funcionales y tecnológicas.

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A new synbiotic dairy food containing lactobionic acid and *Lactobacillus casei*

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Abbreviated running headline: New synbiotic food with lactobionic acid

Abstract

Besides its beneficial properties for health, the incorporation of the prebiotic lactobionic acid (LBA) in fermented dairy products can provide a technological advantage due to its gelling capacities. This study aimed to develop a new functional dairy product containing LBA synthesized within the process itself by the bacteria *Pseudomonas taetrolens*. The probiotic *Lactobacillus casei* was introduced through a sequential fermentation system. After incubation, in the case of the most effective experimental procedure, a synbiotic fermented milk with 30 g L^{-1} of prebiotic LBA was obtained, together with an active *L. casei* population of 10^9 CFU mL^{-1} and <1% lactose content.

Keywords: Functional dairy food; synbiotic; lactobionic acid; *Pseudomonas taetrolens*; *Lactobacillus casei*; sequential fermentation.

1. Introduction

The growing interest of consumers in the acquisition of healthy eating habits has promoted the widespread development of functional foods, such as dairy products with probiotic and prebiotic properties (Ferrão *et al.* 2016; Balthazar *et al*., 2018; Silva *et al.* 2018; Sperry *et al*., 2018). Currently, different indigestible carbohydrates are used as prebiotics, many of them lactose derivatives (Seki and Saito 2012). This group includes lactobionic acid (LBA), a product of lactose oxidation potentially prebiotic (Gutiérrez *et al.* 2012; Alonso et al. 2013). Besides its

healthy properties, the commercial interest in LBA within the food field is also due to its antioxidant, stabilizing, gelling and acidifying properties (Alonso *et al*. 2013; Ribeiro *et al.* 2016).

Because of this set of characteristics, the introduction of LBA in the manufacturing processes of fermented dairy products would lead to lower processing times and cost savings. Until now, most methodologies investigated for the synthesis of LBA for industrial purposes consist of chemical, electrochemical, biocatalytic and heterogeneous catalytic oxidations of lactose (Gutiérrez *et al.* 2012), implying in many cases high economic and energy costs. Nevertheless, the bio-based production has been revealed as a sustainable alternative, and an efficient process has been developed employing the bacteria *Pseudomonas taetrolens* as the producer microorganism, acting on an inexpensive substrate of residual cheese whey (Alonso *et al.* 2012b). *P. taetrolens*, a non-pathogenic specie, presents high lactose bioconversion abilities without the strict requirements of other bacterial platforms investigated using synthetic substrates (Malvessi *et al.* 2013). The biosynthesis of LBA carried out *in situ* by *P. taetrolens* from a dairy substrate would allow fermented dairy products enriched in LBA to be obtained within the production process itself, thus avoiding its subsequent synthetic production and addition.

In this regard, the relation established between *P. taetrolens* and lactic acid bacteria when both coexist in the same niche has been studied with the goal of designing an integrated bioprocess. The use of a probiotic lactic acid bacteria would result in products with synbiotic properties. In addition, the synergistic contribution of both microorganisms to degrading the dairy substrate would also generate food products with lower lactose concentrations than those found in traditional yogurts.

In previous studies, the ability of the probiotic lactic strain *Lactobacillus casei* CECT 475 to utilize LBA as a substrate in the absence of another carbon source was proved (García *et al.* 2017b). This is an indispensable requirement for a combination of probiotic microorganisms and prebiotic compounds to be classified as synbiotic (Adebola *et al.* 2014). A sequential fermentation system involving *P. taetrolens* and *L. casei* was proposed, in view of the amensalistic interaction discovered between *P. taetrolens* and *L. casei* (García et al. 2017a), which makes a simultaneous fermentation unfeasible.

The objectives of the present research work were to test the ability of *P. taetrolens* to use effectively milk as substrate and, subsequently, to develop a new functional dairy food

containing LBA as a bioactive ingredient and enough presence of active *L. casei* cells to present probiotic properties. Sequential fermentations of *P. taetrolens* and *L. casei* were carried out, characterizing processes and products and evaluating the gelling effect of LBA.

2. Materials and methods

2.1. Microorganisms

Lactobacillus casei CECT 475 was obtained from the Spanish Type Culture Collection (Valencia, Spain) and maintained frozen (in 40% v/v solution at -20 °C). After that, the strain was incubated on MRS (de Man, Rogosa and Sharpe, Biokar Diagnostic, France) agar plates, cultured for 48 h at 37 °C and then maintained at 4 °C.

Pseudomonas taetrolens LMG 2336 was obtained from the Belgian Coordinated Collection of Microorganisms (Ghent, Belgium) and conserved frozen in 40% (v/v) glycerol at -20 °C. Then it was subcultured on NB agar plates (Nutrient Broth, containing 1g L^{-1} meat extract, 2 g L^{-1} yeast extract, 5 g L^{-1} peptone and 5 g L^{-1} NaCl), which were incubated for 48 h at 30 °C and preserved at 4 °C.

2.2. Inocula and substrate preparation

P. taetrolens is a highly aerobic microorganism. It was reactivated by inoculating a loopful from an NB agar plate in a 500 mL Erlenmeyer flask containing 100 mL of NB broth (medium volume to air ratio of 1:4). The culture was incubated in an orbital shaker (New Brunswick Scientific Co., model G25, USA) at 250 rpm and 30 °C for 10 h.

On the contrary, *L. casei* is a facultatively anaerobic strain. It was reactivated on MRS under microaerophilic conditions, from a loopful of an MRS agar plate which was used to inoculate a 250 mL storage media bottle containing 250 mL MRS broth (medium volume to air ratio of 1:0). The culture was incubated at 37 °C without agitation for 16 h.

Skimmed bovine milk was heated in a water bath at 90ºC for 10 minutes for sterilization (Yousseef *et al.* 2016) and subsequently used as substrate in fermentations.

2.3. Pure culture fermentations

Pure culture fermentations of *L. casei* and *P. taetrolens* were developed to obtain information about the fermentative behaviour of microorganisms when they grow in milk. Samples were periodically taken from the fermentation medium to determine pH and bacterial growth and to perform subsequent analyses of lactose consumption and lactic and LBA generation. The textural properties of the final fermented products were characterized. All fermentations were carried out in duplicate as independent experiments and the reported results correspond to the mean value of at least three measurements. Positive and negative error values are shown as error bars in the figures and the standard deviation values are presented in tables for the parameters of final fermented products.

2.3.1. Without pH control

Fermentations during which the pH was not controlled were performed in the orbital shaker. *P. taetrolens* was cultured in 2 L storage media bottles with 400 mL of milk (medium volume to air ratio of 1:4). 40 mL from NB cultures were used as inoculum (inoculum level of 10% v/v). Cultures were incubated at 30ºC and 250 rpm agitation. *L. casei* fermentations were carried out in 500 mL storage media bottles containing 400 mL of sterilized skimmed milk (medium volume to air ratio of 4:1), again employing a 10% v/v inoculum level, at 37ºC and 100 rpm of agitation (according to Alonso *et al.* 2010).

2.3.2. With pH control

Pure cultures of *L. casei* and *P. taetrolens* were scaled at the level of a 2-L bioreactor (Bioflo 110, New Brunswick Scientific, NJ, USA) with mechanical agitation and 1 L of working volume, employing in both cases a 10% v/v inoculum level.

In the case of *P. taetrolens* cultures, the following operating parameters were used: 30ºC, 350 rpm and 1 Lpm aeration, according to the conditions optimized by Alonso et al (2012b) for maximizing LBA production. Excessive foam formation was prevented by automatic addition of diluted (1:10) Y-30 emulsion (Sigma–Aldrich, Steinheim, Germany). The bioreactor was equipped with a pHmeter (Mettler Toledo, Switzerland) and a two-stage pH-shifted strategy was implemented, leaving pH uncontrolled during the exponential growth phase and then

maintained at 6.5 by means of the automatic addition of 2M NaOH. This strategy was established by Alonso *et al.* (2012b) as the most favourable for maximizing LBA synthesis.

Pure *L. casei* cultures were grown at 37ºC and 50 rpm to keep the fermentation medium mixed (Alonso *et al.* 2010) and without aeration. In this case, the pH was left uncontrolled during the process, with the objective of developing the typical conditions of acidity found in traditional fermented dairy products.

2.4. Sequential fermentations

Sequential fermentations were performed according to the system described by García *et al.* (2017b), in which *P. taetrolens* is inoculated first (Fig. 1) to avoid the inhibitory effect exerted by *L. casei* on the LBA producer. Modifications were introduced to adapt the protocol to the new dairy substrate. As in pure cultures, samples were periodically taken during the two stages of the process to determine pH, bacterial growth, lactose, LBA and lactic acid concentrations and the texture of the final products was characterized. The final concentration of *L. casei* cells, determined by counting colony forming units (CFU), was converted to dry cell weight through the calibration curve previously obtained, and results were expressed as g L^{-1}.

2.4.1. Without pH control

40 mL from the NB cultures with *P. taetrolens* were used to inoculate 2 L storage media bottles containing 400 mL of sterilized skimmed milk (10% inoculum level). Cultures were incubated in the orbital shaker with an agitation rate of 250 rpm at 30 °C, just as in the pure *P. taetrolens* cultures, until a medium pH of 5 was obtained, when the process was halted. A pH value of 5 was determined in previous studies (Adebola *et al.* 2014; García *et al.* 2017b) as the acceptable initial level of acidity to inoculate *L. casei* CECT 475 into the fermentation medium.

Since *P. taetrolens* is not a Generally Recognized as Safe Microorganism (GRAS) for food application, *P. taetrolens* biomass was inactivated in the water bath at 90ºC for 10 minutes (Yousseef *et al.* 2016).

After heat treatment, the oxidized medium was inoculated with *L. casei* CECT 475 from the MRS cultures, employing an inoculation level of 10% (v/v) and incubated in 500 mL storage media bottles at 37 °C and 100 rpm for 24 h.

2.4.2. With pH control

The sequential fermentation system was scaled at the level of the 2-L bioreactor with 1 L of working volume. Following the scheme employed in fermentations without pH control (Fig. 1), *P. taetrolens* was inoculated first and oxidation took place under the same operating conditions as those implemented in the pure cultures of *P. taetrolens* (30 ºC, 350 rpm, 1 Lpm and the two-stage pH-shifted strategy). Having eliminated the possibility of an inhibition of *L. casei* due to an initial pH that was too acidic, the oxidation carried out by *P. taetrolens* was stopped when there was still lactose in the medium, in view of the results obtained previously in fermentations with *P. taetrolens* in pure culture. Oxidized medium containing *P. taetrolens* was pumped out of the bioreactor, sterilized in the water bath (90ºC for 10 min), cooled and inoculated with *L. casei* (10% v/v) and reintroduced again into the bioreactor whilst preserving the conditions of sterility.

L. casei was incubated at 37 ºC, 50 rpm and without aeration. pH was left uncontrolled in the same way as in the pure cultures.

2.5. Texturizer addition

Sequential cultivations were carried out with a gelling agent added to the fermentation medium. The objective was to evaluate the possible improvement in the textural properties of the final fermented products containing LBA, due to the gelling effect of the acid itself. Different concentrations of k-carrageenan were tested in the search for the most appropriate proportion to obtain a final fermented product with a suitable consistency. k-carrageenan (Sigma Aldrich) was added during the thermal inactivation treatment of the *P. taetrolens* before the *L. casei* inoculation, dissolving it in the skimmed milk (Nguyen *et al.* 2017). 0.1% and 0.5% concentrations were tested in fermentations without and with pH control, respectively. In fermentations under pH control the concentration tested was higher in order to counteract the effect of the internal agitation in the bioreactor hindering the formation of the protein network.

Additional tests were also conducted by adding the texturizer directly to the final fermented products, with concentrations ranging from 1% to 2%.

2.6. Textural characterization of final fermented products

Textural properties of fermented products were analysed to evaluate the influence of the LBA concentrations on the consistency of the gels, compared to that obtained in dairy gels produced by pure cultures of lactic acid bacteria from skimmed milk.

The texture of the fermented products with the firmest consistency was determined using a TA.XTplus Texture Analyzer (Stable Systems). A 50 mL of sample was subjected to a penetration test, at room temperature, employing a spherical probe P/0.55 and a working height of 40mm. Results are expressed in terms of firmness and stickiness values (grams).

The textural properties of the final fermented products with a more liquid consistency were evaluated employing a Haake Mars Rheometer (Termo Scientific), associated with a temperature controller (Peltier) using a speed of deformation interval of 0.01-10 s^{-1}. Results are represented with the corresponding flow curves determined by the viscosity values (cP) and shear stress (Pa) versus shear rate (s^{-1}).

Experiments were duplicated and reported results correspond to the mean value.

2.7. Syneresis determination

The level of spontaneous whey separation in fermented products was determined by the siphon method, according to Amatayakul *et al.* (2006). Samples of 50 mL of each final product were preserved in cup at 4 ºC. Subsequently, they were weighed and maintained at an angle of 45º to collect the whey at the side of the cup. A syringe was used to syphon the whey from the surface of the sample and after that cups were weighed again. Syneresis was calculated as the percent weight of the whey over the initial weight of the sample.

2.8. Substrate and products analysis

Lactose and organic acid concentrations were measured via High Performance Liquid Chromatography (HPLC). A liquid chromatography system (Agilent 1200, Agilent Technologies Inc., CA, USA) was used for the analysis, employing an ICSep ICE-ION-300 column (Transgenomic Inc., CA, USA) coupled to a refractive index detector. A mobile phase consisting of a sulphuric acid solution (0.450 mmol L^{-1}, pH 3.1) was used at 0.3 mL min^{-1} flow

rate and with a column temperature of 75ºC. Data acquisition and analysis were performed using ChemStation software (Agilent).

3. Results and discussion

3.1. Suitability of bovine milk as substrate for the biosynthesis of LBA by P. taetrolens

Fig. 2 shows the pH curve and the increase in biomass (from an inoculum of 10^8 CFU mL^{-1} of *P. taetrolens*), for experiments without pH control (2a.1) and with pH control (2a.2). In experiments carried out in the orbital shaker, pH varied freely until a value of 4.97 at 72 h due to the accumulation of LBA in the fermentation medium (Fig. 2a.1). A change in the physiological status from metabolically active to damaged cells has been reported for *P. taetrolens* at pH values lower than 6.0, with loss of cell membrane integrity (Alonso *et al.* 2012b). The lack of an adaptive response from the *P. taetrolens* cells makes the medium pH a key factor for LBA biosynthesis (Alonso *et al*. 2012b, 2013). Thus, despite the lower increase in biomass (Fig. 2a.2), a higher lactose consumption and LBA production were achieved in fermentations implemented under pH control (Fig. 2b.2). The pH control allowed an increase in the final LBA concentration from 28.59 to 41.15 g L^{-1}, achieving a productivity and a yield on substrate of 0.57 g L h^{-1} and 0.92 g g^{-1}, respectively, compared to the 0.4 g L h^{-1} and 0.83 g g^{-1} values obtained in fermentations conducted without pH control (Table 1).

3.2. Sequential fermentation system: process and product characterization

Figs. 3 and 4 show the results corresponding to the characterization of sequential fermentation processes, carried out without pH control (Fig. 3) and under pH control (Fig. 4). As stated above, in experiments without pH control, *P. taetrolens* oxidation was stopped when the pH reached a value slightly higher than 5 (Fig. 3a), whereas under conditions of controlled pH, the determining factor was the concentration of lactose. Thus, in this last case, the highest initial pH (6.5 compared to 5 in fermentations without pH control) allowed a greater increase in the biomass of *L. casei* and a shorter lag phase, reaching maximum growth at 8 hours of incubation (Figs. 3b and 4b).

In both systems, the second stage continued for 24 hours (Figs. 3b and 4b), reaching a pH lower than that found in commercial yogurts: 4.5 or slightly lower (Lee and Lucey 2010; Shah 2000). Lactic acid bacteria are tolerant of acid stress, being capable of surviving in the acidic

environment generated by the accumulation of their fermentation products. However, growth cessation has been reported for probiotic bacteria below pH 4.0 (Shah 2000). In the present study, the acidity caused by the generation of lactic acid in the second stage is added to that previously produced by LBA.

3.2.1. Prebiotic synthesis

The control of the pH allowed the production of LBA to increase, despite the oxidation of *P. taetrolens* being stopped at 48 hours. Thus, 32.62 gL^{-1} were produced compared to 19.21 g L^{-1} in 58 h in fermentations without pH control (Table 2). Consistent with the LBA synthesis, the lactose degradation by *P. taetrolens* was more pronounced when pH control was applied. Nevertheless, despite the low initial concentration of lactose in the medium, the control of the pH also allowed the production of lactic acid by *L. casei* to be increased from 4.89 g L^{-1} in experiments with free pH to 9.63 g L^{-1} (Table 2). As can be observed in Figs. 3 and 4, the lactic acid was synthesized by *L. casei* employing lactose as a source of carbon, no significant reduction in the concentration of LBA being observed. The preference of *L. casei* CECT 475 for lactose over LBA had been stated in previous studies (García *et al.* 2017b), as well as its ability to degrade LBA in the absence of another source of carbon. This behaviour is essential to ensure the synbiotic nature of the product: the LBA will not be consumed during the manufacturing process, thus reaching the lower intestine undegraded, where other sources of carbon are scarce and the LBA can be fermented by the probiotic bacteria (Adebola et al., 2014).

As reported above, dairy fermented products developed in this study through an optimized process would have a content of around 30 g L^{-1} of LBA, which would mean 3.75 g in a typical ration of fermented product of 125 mL. The common recommendation for supplementation of inulin-type prebiotics (inulin, oligofructose and fructooligosaccharides) to obtain an increase in healthy microbiota is a daily dose of 2.5 g. It has been seen that 2.5-5 g would be the minimal dose with bifidogenic effects, 10 g being considered the optimal daily dose (Kelly 2008). Nevertheless, in previous studies, the ability of *L. casei* CECT 475 to take advantage of a substrate containing 5-10 g/L of LBA as the only carbon source was proved (García *et al.* 2017b). Thus, the daily intake of a 125 mL portion of the fermented product containing 30 g L^{-1} would be enough to exert its beneficial effects on the microbiota. Furthermore, the effect on human health of exposure to the continued consumption of LBA has been investigated, concluding that amounts of up 24 g of LBA per day were well tolerated without producing adverse effects (Schaafsma 2008).

3.2.2. Lactose content

The different kinds of mammalian milk contain up to 5% lactose and during yogurt manufacture, lactic acid bacteria reduce this lactose content by one third (Cutrim *et al.* 2016), resulting in concentrations of around 3%. In the present research, the final product obtained by fermentations without pH control had a concentration of lactose of 13.53 g L^{-1}, which decreased to 7.31 g L^{-1} under pH control (Table 2). These values would mean lactose levels of 1.35% and 0.73%, respectively, lower than those found in traditional yogurts due to the synergistic action of *P. taetrolens* and *L. casei* degrading lactose. Commercial food products with ≤1% lactose can be declared as low in lactose, whereas the lactose-free level means ≤0.01% of lactose and its degradation products (Morlock *et al.* 2014). Therefore, optimization of the sequential process by pH control provides "low lactose" fermented products with <1% lactose, which could be consumed by individuals with low tolerance to lactose.

3.2.3. Probiotic quantification

Besides its high content in LBA and low lactose, the final fermented product obtained has a *L. casei* concentration in the range of 10^9 CFU mL^{-1}, corresponding to 3.71 and 4.93 g L^{-1} of the probiotic strain in fermentations without and with pH control, respectively (Table 2). In processed foods, populations of 10^6-10^7 CFU mL^{-1} in final products are considered therapeutic quantities, provided by a daily consumption of 100 mL or 100 g of food (Cruz *et al.* 2009). This amount must be present at the expiry date, so at the time of sale higher concentrations of 10^7-10^8 CFU mL^{-1} are recommended. In the present study, the spread plate method was used for quantifying the biomass of *L. casei*, assuring that in the final fermented product the probiotic bacteria were not only alive, but they were also culturable, preserving their metabolic activity intact.

3.2.4. Textural properties

Fig. 5 shows the rheological analysis corresponding to the product of *P. taetrolens* and *L. casei* sequential fermentation compared to that obtained in pure cultures of *L. casei*, in both cases without pH control. As can be observed, they are pseudoplastic fluids, with a decreasing viscosity when the shear rate increases (Fig. 5a). This is the typical behaviour found in yogurts and other semi-solid dairy desserts, which can be classified as pseudoplastic materials (Lee and

Lucey 2010). A significative difference, however, can be observed in the initial viscosity, with a higher value in the product resulting from the sequential fermentation than in that generated only by *L. casei* (11840 compared to 4571 cP, respectively). In consonance, higher values of shear stress were recorded in the sequential fermentation product (Fig. 5b). This increase in apparent viscosity and shear stress can be attributed to the presence of LBA, given its properties as a gelling and firming agent (Gutiérrez *et al.* 2012; Alonso *et al.* 2013), along with the increase in the solid content. This phenomenon has also been reported for yogurts to which other prebiotics had been added. Cruz *et al.* (2013) observed an increased apparent viscosity and shear stress in yogurts with added olifructose compared to yogurts without prebiotic addition, together with a greater stability in these values during storage. Therefore, a positive effect in product consistency can result from the integration of LBA synthesis in the manufacturing processes of dairy foods.

In the dairy industry, therefore, the implementation of this integrated biotechnological process would allow to obtain fermented products with improved properties, not only from the nutritional and healthy point of view (low in lactose, probiotic and prebiotic) but also technological due to the gelling capacity of lactobionic acid. Greater efficiencies could be achieved while satisfying the consumer demand for natural health-promoting ingredients, instead of synthetic additives.

The rheological analysis of products obtained by implementing the pH control strategy revealed a great difference in the textural properties. Thus, while products generated in bottles with orbital agitation (no pH control) underwent a gelation process after the *L. casei* fermentation, those produced in a bioreactor with mechanical agitation (under pH control) showed a more liquid consistency. In this last case, no significant differences were found in viscosity values between the product resulting from the sequential system incorporating *P. taetrolens* and that generated only by the activity of *L. casei* (4.65 and 5.03 cP, respectively). During the first stage in the sequential process, rapid agitation of 350 rpm was implemented in the bioreactor to ensure the aeration needed by *P. taetrolens*. The rotation of the impeller produced an effect in the fermentation medium that could be equated to that generated in the manufacturing process of stirred yogurts. In these yogurts, mixing causes a decrease in viscosity, which will be only partially restored after shearing is stopped (Lee and Lucey 2010). Thus, the destabilization of the casein network by the mechanical agitation counteracted the gelling effect of LBA in the bioreactor.

3.3. Addition of the stabilizer k-carrageenan

3.3.1. Process characterization

In the present study, skimmed bovine milk was chosen as the raw material for obtaining the fermented products. Some authors have found firm, consistent and viscous textures, as well as improved flavour and palatability, in yogurts produced from non-fat milk (Soukoulis *et al.* 2007). Nevertheless, high syneresis and reduction in smoothness and creaminess are usually reported due to the omission of fat (Nguyen *et al.* 2017). Stabilizers are used, especially in the manufacture of skimmed and stirred yogurts, to achieve the desirable textural properties and prevent syneresis.

Two different concentrations of k-carrageenan were tested in sequential processes: 0.1% in cultures with free pH and 0.5% in fermentations under pH control. In the bioreactor with mechanical agitation the concentration was increased due to the more liquid consistency of fermented products obtained by this system. In both cases, texturizer was added during the heat treatment prior to the *L. casei* inoculation (Fig. 1), thus not interfering in the growth of *P. taetrolens* in the first stage. Results obtained are summarized in Table 3. In the case of fermentations under orbital agitation (no pH control), a pH of 3.47 was reached at 24 h, accompanied by a higher production of lactic acid than that registered in cultures without texturizer addition (22.02 compared to 4.89 g L^{-1}, respectively). Not unexpectedly, there was a greater increase in the *L. casei* biomass, from $1.06x10^9$ to $5.58x10^9$ CFU mL^{-1}. These results support the findings reported by other authors, in terms of a shorter incubation time due to the addition of stabilizer, with the end point pH being reached sooner. Specifically, shorter culture times have been found for milks containing k-carrageenan than those with xantan, guar and pectin (Soukoulis *et al.* 2007). In view of this result, it can be concluded than in the case of fermentations without pH control and without texturizer addition, an incubation time longer than 24 hours could have led to a greater production of lactic acid and a lower pH value.

In experiments in a bioreactor with pH control and mechanical agitation, the addition of k-carrageenan did not coincide with a reduction in the incubation time. On the contrary, the *L. casei* fermentative behaviour was impaired, reaching lower biomass, lower lactic acid production and consequently, a final pH higher than that reached without k-carrageenan (Tables 2 and 3). It seems that the interaction of hydrocolloids such as k-carrageenan with proteins affects the ion equilibrium and casein precipitation, shortening the incubation time. But in the

bioreactor with mechanical agitation, this effect is counteracted by the gel rupture due to rotation.

3.3.2. Firmness and stickiness of products

The products resulting from the addition of k-carrageenan showed a consistency which was not suitable for the determination of the flow curve in the rheometer, so a texture analyser was used, thus obtaining the values of firmness and stickiness reflected in Table 3. As can be observed, despite increasing the concentration of texturizer to 0.5% in the case of products obtained in a bioreactor, lower values of firmness were registered (101.05 g compared to 189.93 g in products from fermentations in an orbital shaker with 0.1% of k-carrageenan). A concentration of 0.1-0.5% is described as the typical usage level of carrageenan in yogurts. In skimmed yogurts, an increase in the water holding capacity, enhancing the retention of serum in the yogurt gel structure, has been reported for the addition of 0.3% carrageenan (Gyawali and Ibrahim 2016). In the present study, the concentration range of 0.1-0.5% was insufficient to prevent the release of serum, which was around 25% in all cases. Nevertheless, adding such an amount of k-carrageenan resulted in an excessive increase in consistency with >100g of firmness compared to the 50-60g typically found in yogurts without any supplement (Ozturkoglu-Budak *et al.* 2016). The gelling effect of k-carrageenan could act synergistically with LBA, producing an undesirable effect known as over-stabilization. This is a typical textural defect in dairy products and desserts that is related to the addition of stabilizers (Lee and Lucey, 2010).

The additional tests which involved adding the carrageenan directly to the final fermented products, employing higher concentrations (1-2%), avoided serum separation, but increased the negative effect of over-stabilization.

4. Conclusions

In this study, a feasible and efficient process to obtain dairy fermented products enriched in LBA has been developed. Bovine milk was revealed as a suitable raw material for the productive activity of *P. taetrolens*, allowing high concentrations of LBA to be reached. The product resulting from the sequential system could be marketed as synbiotic, containing enough concentration of the prebiotic LBA and the probiotic bacteria *L. casei*. This product has higher viscosity than traditional fermented milks produced by the sole action of lactic acid bacteria, due to the gelling effect of LBA. Avoiding acidification during the first oxidative stage in the

sequential process generates products with a higher concentration of LBA. Nevertheless, the internal mechanical agitation in the bioreactor hinders curd formation, attenuating the firming capacity of LBA. Further experiments would be necessary to evaluate the proper concentration of an additional stabilizer to improve the product's textural properties.

Conflict of interest

There is no conflict of interest.

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Table 1 - Productivity values in pure cultures of *P. taetrolens* in skimmed bovine milk

	Without pH control	With pH control
Lactose consumed (g L^{-1})	34.61 ± 2.21	44.64 ± 3.64
LBA produced (g L^{-1})	28.59 ± 2.83	41.15 ± 2.22
LBA yield on substrate (g g^{-1})	0.83 ± 0.05	0.92 ± 0.11
LBA productivity (g L^{-1} h^{-1})	0.40 ± 0.04	0.57 ± 0.05

Table 2 - Values of parameters in final fermented products obtained through sequential fermentation with and without pH control

	Without pH control	With pH control
Final pH	3.62 ± 0.01	3.56 ± 0.00
Final lactic acid concentration (g L^{-1})	4.89 ± 1.40	9.62 ± 0.99
Final LBA concentration (g L^{-1})	18.77 ± 1.70	34.08 ± 1.42
Final lactose concentration (g L^{-1})	13.53 ± 2.02	7.31 ± 0.38
Final CFU mL^{-1} *L. casei*	$1.06x10^9$ ± 0.00	$1.67x10^9$ ± 0.00
Final *L. casei* dry cell weight (g L^{-1})	3.71 ± 0.00	4.93 ± 0.00

Table - 3 Values of parameters in final fermented products obtained through sequential fermentation with and without pH control, with the texturizer addition

	Without pH control 0.1% k-carr	With pH control 0.5% k-carr
Final pH	3.47 ± 0.01	3.85 ± 0.00
Final lactic acid concentration (g L^{-1})	22.02 ± 1.55	4.53 ± 0.18
Final CFU mL^{-1} *L. casei*	$5.58x10^{9}$ ± 0.00	$5.63x10^{8}$ ± 0.00
Final *L. casei* dry cell weight (g L^{-1})	12.75 ± 0.00	2.72 ± 0.00
Firmness (g)	189.93 ± 30.26	101.05 ± 8.53
Stickiness (g)	-33.87 ± 7.33	-3.73 ± 1.48

Figure captions

Figure 1 - Diagram of the sequential fermentation process, integrating the *P. taetrolens* lactose oxidation (first stage) and the subsequent *L. casei* fermentation (second stage)

Figure 2 - Bacterial growth (△) and pH (×) evolution (a) and lactose (□) and LBA (○) concentrations (b) in pure cultures of P. *taetrolens* on skimmed milk without pH control (1) and with pH control (2)

Figure 3 - *P. taetrolens* growth (△) and pH (×) (a) and lactose (□) and LBA (○) concentrations (c) during the first stage in the sequential fermentation without pH control. *L. casei* growth (△) and pH (×) (b) and lactose (□), LBA (○), and lactic acid (✻) concentrations (d) during the second stage

Figure 4 - *P. taetrolens* growth (△) and pH (×) (a) and lactose (□) and LBA (○) concentrations (c) during the first stage in the sequential fermentation under pH control. *L. casei* growth (△) and pH (×) (b) and lactose (□), LBA (○), and lactic acid (✻) concentrations (d) during the second stage

Figure 5 - Rheological analysis corresponding to the final fermented product obtained through sequential fermentation of *P. taetrolens* and *L. casei* without pH control (✻), compared to that obtained from a pure culture of *L. casei* (○). (a) viscosity curve; (b) flow curve

Figure 1

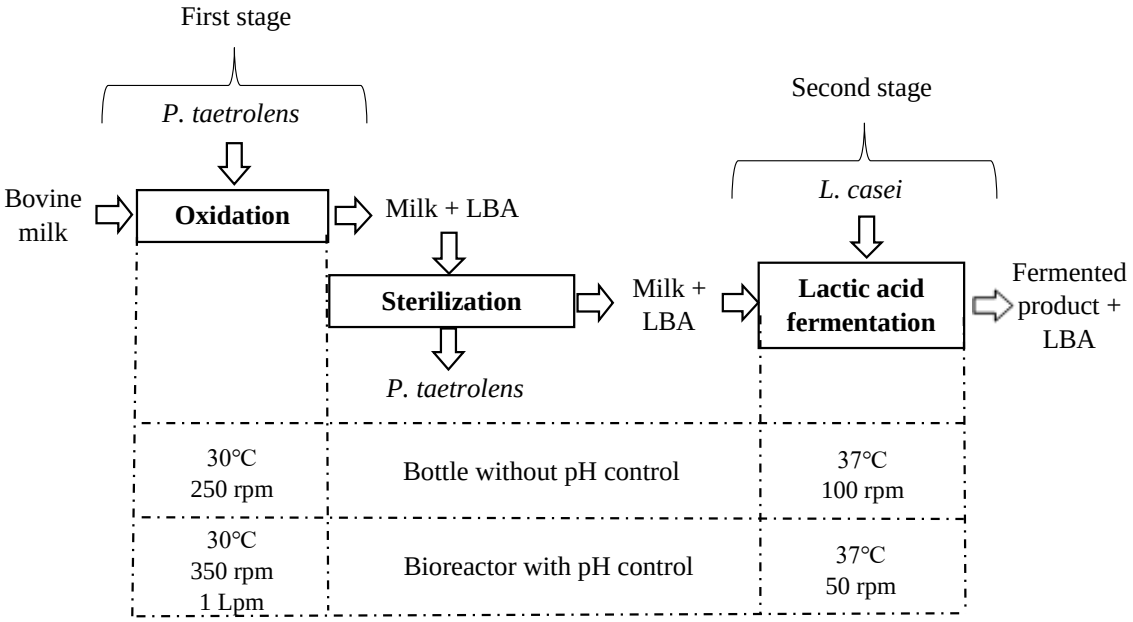

First stage
P. taetrolens
Second stage
L. casei
Bovine milk
Oxidation
Milk + LBA
Sterilization
Milk + LBA
Lactic acid fermentation
Fermented product + LBA
P. taetrolens
30°C
250 rpm
Bottle without pH control
37°C
100 rpm
30°C
350 rpm
1 Lpm
Bioreactor with pH control
37°C
50 rpm

Figure 2

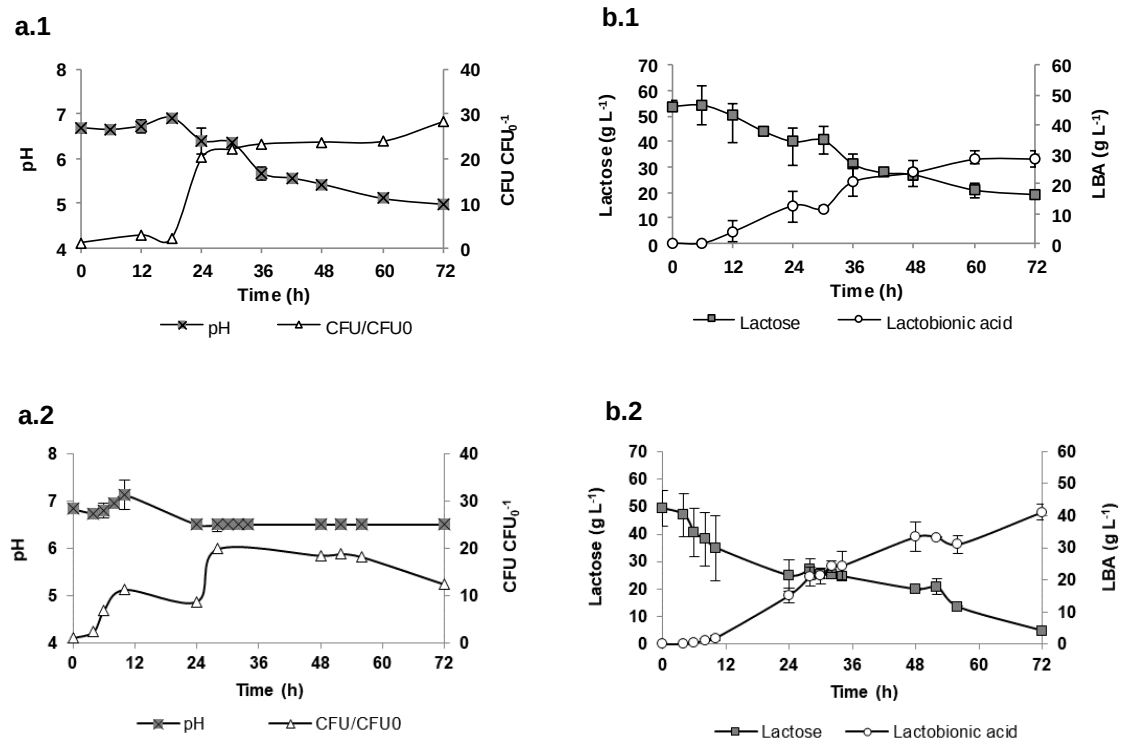

a.1
pH
CFU CFU$_0^{-1}$
Time (h)
pH
CFU/CFU0
b.1
Lactose (g L^{-1})
LBA (g L^{-1})
Time (h)
Lactose
Lactobionic acid
a.2
pH
CFU CFU$_0^{-1}$
Time (h)
pH
CFU/CFU0
b.2
Lactose (g L^{-1})
LBA (g L^{-1})
Time (h)
Lactose
Lactobionic acid

Figure 3

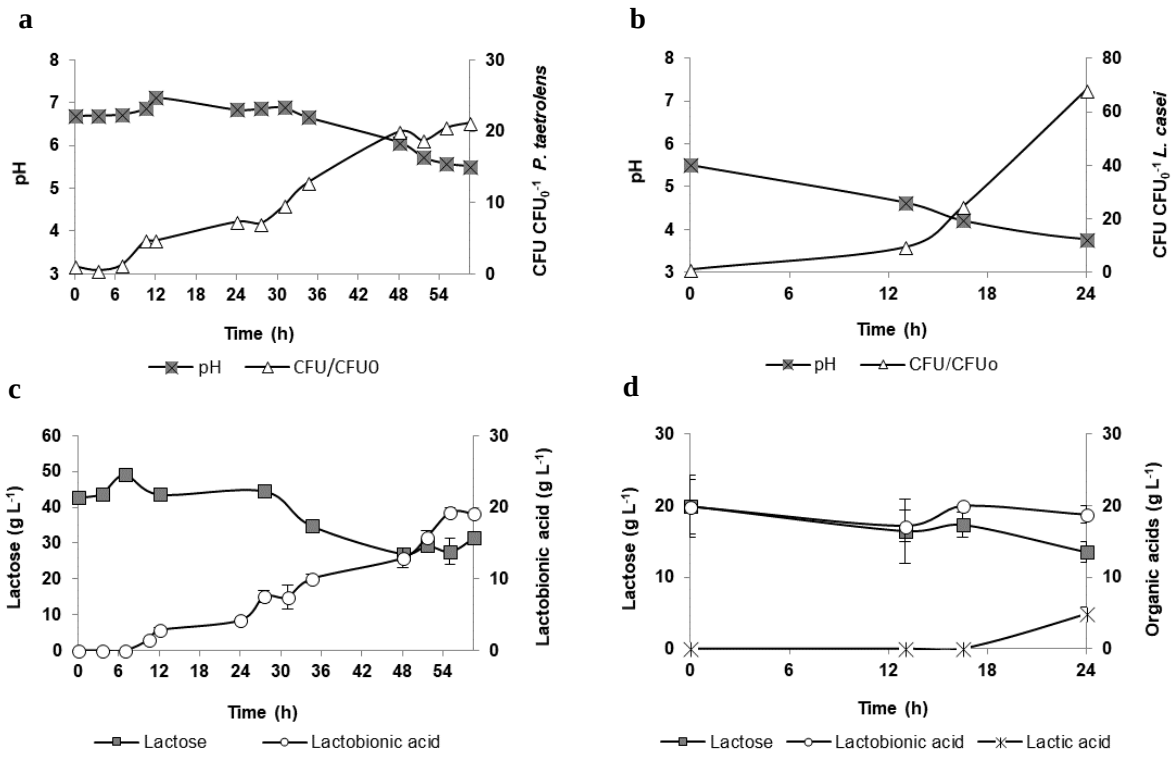

a
pH
CFU CFU$_0^{-1}$ *P. taetrolens*
Time (h)
pH
CFU/CFU0
b
pH
CFU CFU$_0^{-1}$ *L. casei*
Time (h)
pH
CFU/CFUo
c
Lactose (g L^{-1})
Lactobionic acid (g L^{-1})
Time (h)
Lactose
Lactobionic acid
d
Lactose (g L^{-1})
Organic acids (g L^{-1})
Time (h)
Lactose
Lactobionic acid
Lactic acid

Figure 4

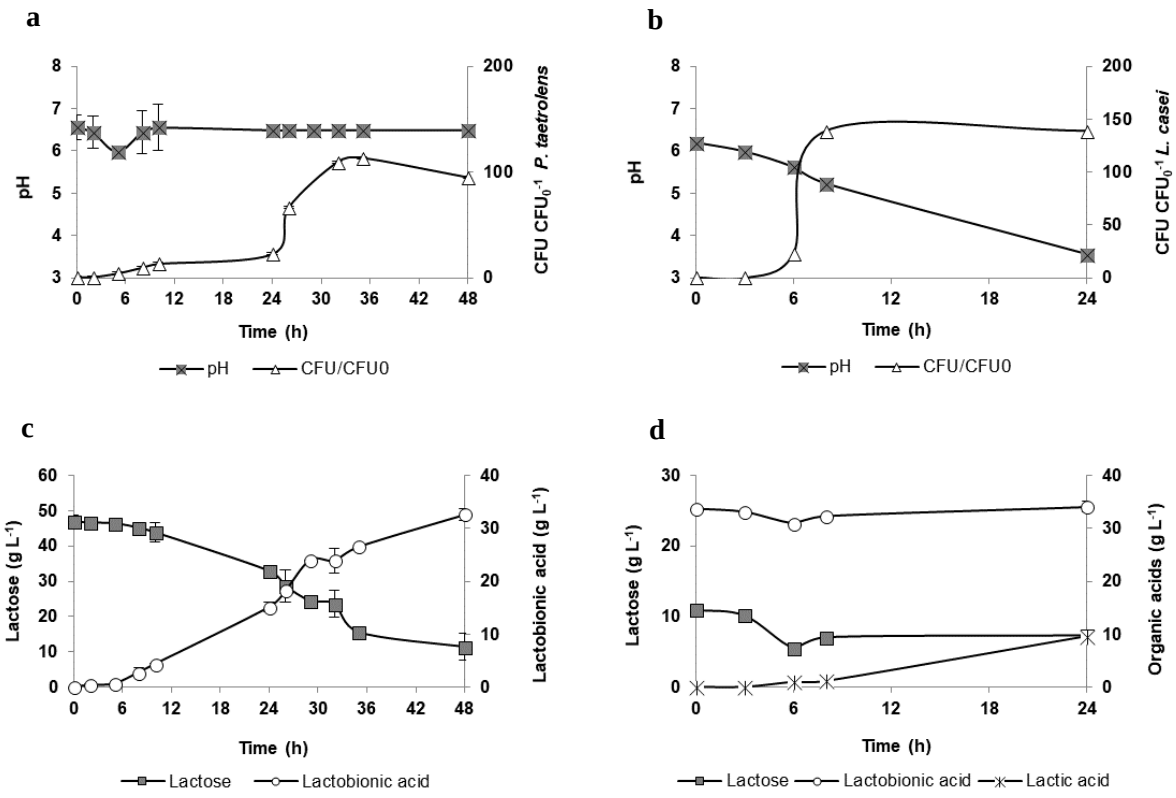
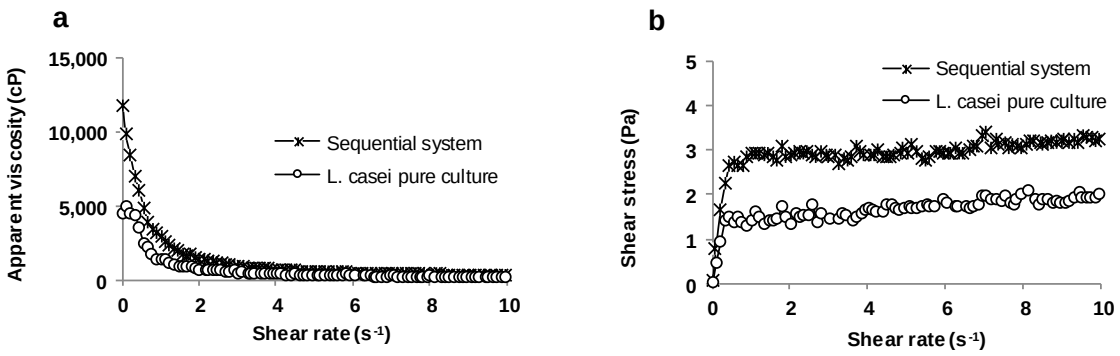

a
pH
CFU CFU$_0^{-1}$ *P. taetrolens*
Time (h)
pH
CFU/CFU0
b
pH
CFU CFU$_0^{-1}$ *L. casei*
Time (h)
pH
CFU/CFU0
c
Lactose (g L^{-1})
Lactobionic acid (g L^{-1})
Time (h)
Lactose
Lactobionic acid
d
Lactose (g L^{-1})
Organic acids (g L^{-1})
Time (h)
Lactose
Lactobionic acid
Lactic acid

Figure 5


a
15,000
10,000
5,000
0
Apparent viscosity (cP)
0
2
4
6
8
10
Shear rate (s-1)
Sequential system
L. casei pure culture
b
5
4
3
2
1
0
Shear stress (Pa)
0
2
4
6
8
10
Shear rate (s-1)
Sequential system
L. casei pure culture

4.4. Evaluación de la encapsulación como estrategia para evitar el amensalismo entre *L. casei* y *P. taetrolens*

Además del sistema de fermentación secuencial, otra estrategia propuesta en esta memoria para minimizar la inhibición ejercida por *L. casei* sobre *P. taetrolens* es la inmovilización bacteriana. Esta técnica ha sido ampliamente estudiada en el contexto de la encapsulación de bacterias probióticas para asegurar su viabilidad en un ambiente desfavorable. La capacidad de la encapsulación para limitar la difusión de sustancias antimicrobianas a través de la matriz encapsulante o el aumento de la tolerancia al estrés de las células inmovilizadas, haciéndolas más resistentes a los inhibidores (Heidebach et al., 2012; Westman et al., 2012), puede suponer una alternativa para minimizar la exclusión competitiva en cultivos antagónicos. En el presente subcapítulo se ensayaron fermentaciones mixtas con *L. casei* y *P. taetrolens* encapsuladas de forma alternativa. Se evaluó la idoneidad de tres hidrogeles de aplicación alimentaria (alginato, alginato/almidón y CMC/k-carragenano), comparando la eficiencia de encapsulación alcanzada, su resistencia mecánica y su capacidad para retener las células encapsuladas. Puesto que el objetivo principal era mejorar el comportamiento fermentativo de la cepa sensible, en este trabajo se hace hincapié en la capacidad de crecimiento de *P. taetrolens* y la producción de ácido lactobiónico, valorando la efectividad de mantener inmovilizada una u otra cepa. No obstante, se estudió también la viabilidad y capacidad productiva de *L. casei*, ya que se pretende posibilitar la coexistencia de ambas cepas preservando también la actividad metabólica de la BAL. Este trabajo muestra el potencial de la encapsulación bacteriana para controlar el desarrollo de consorcios microbianos competitivos. Los resultados obtenidos mediante la inmovilización alternativa de las cepas sensible y dominante revelan, además, que la protección puede resultar efectiva no solo sobre las células encapsuladas, sino también sobre las células que permanecen libres en el medio.

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Exploring encapsulation strategies as a protective mechanism to avoid amensalism in mixed populations of *Pseudomonas taetrolens* and *Lactobacillus casei*

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Abstract

Pseudomonas taetrolens constitutes an efficient platform for the biosynthesis of lactobionic acid, a potentially prebiotic compound. Unfortunately, an amensalistic interaction has been demonstrated between *P. taetrolens* and lactic acid bacteria (LAB), characterised by the competitive exclusion of *P. taetrolens*, hindering the *in situ* production of fermented dairy products with synbiotic properties. In the present research, encapsulation was explored as a barrier to the diffusion of the antimicrobial metabolites generated by LAB. Mixed fermentations involving *P. taetrolens* LMG 2336 and *Lactobacillus casei* CECT 475 were cultivated, entrapping both microorganisms alternately. Alginate, alginate/starch and carboxymethyl cellulose/k-carrageenan were tested as encapsulating agents. The immobilization of *L. casei* in 2% alginate/2% starch beads was found to be the best strategy, improving the production of lactobionic acid by 182% with respect to co-cultures with free cells. This study proves the potential of LAB encapsulation for the protection of sensitive strains in mixed food fermentations.

Keywords: Microbial encapsulation; mixed fermentations; *Pseudomonas taetrolens*; *Lactobacillus casei*; lactobionic acid.

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1. Introduction

Microbial immobilization through encapsulation is seen as a promising technique, especially with probiotic microorganisms, in order to provide them with a protective environment during the manufacturing process and storage of probiotic products, and their passage through the gastrointestinal tract [6,8,30]. Different hydrogels used in food applications have been tested for encapsulation purposes. Alginate remains the most commonly used, due to its non-toxicity, the simplicity of its use and low cost [9]. It has been reported that alginate produces a hydrogel barrier in solution which retards the permeation of acid fluid [25]. However, alginate is susceptible to damage in harsh environments and has high permeability owing to its porous and hydrophilic nature. The addition of other polymers as fillers, such as starch, allows the formation of matrices with improved structural properties [24]. Carboxymethyl cellulose (CMC) is the most widely used cellulose ether, employed in many food applications as a viscosity modifier or thickener. Blends of CMC and k-carrageenan (CMC/k-carr) have been studied for probiotic encapsulation with k-carrageenan as a coating material [12,19].

A few studies have employed encapsulation to control the strain ratios and to provide physical and chemical protection to microorganisms in mixed fermentations [10,14,17]. Microbial associations are present in most food fermentation processes, providing the final product with the desired characteristics. But obtaining stable mixed cultures is a complex task due to the different nutritional requirements, optimal growth conditions and growth rate of each population [33]. In traditional and novel fermented dairy products it is common to find a complex microbiota [32], normally including LAB which produce a wide range of inhibitory compounds, such as organic acids, ethanol, diacetyl, hydrogen peroxide or bacteriocins [13]. In this context, cell immobilization could be employed to exercise some control over mixed cultures containing LAB and sensitive species, making use of the limited diffusion of such inhibitory substances through the wall of the capsules [17, 25, 33].

Lactobionic acid, an aldonic acid derived from the oxidation of lactose, has become a subject of major interest as an additive in dairy products. It possesses valuable technological properties, but also provides health benefits as an agent promoting calcium absorption and it is potentially prebiotic [2,18]. An efficient and sustainable bioprocess has been optimized to obtain lactobionic acid from dairy substrates, employing the bacterium *P. taetrolens* as the producer microorganism [3,4,5]. The coupling of *P. taetrolens* lactose oxidation to traditional fermentation carried out by probiotic LAB would make it possible to obtain functional synbiotic

products, containing the probiotic bacteria and the prebiotic lactobionic acid. But an amensalistic association was found between LAB such as *L. casei* and *P. taetrolens,* in which the release of antimicrobial substances by the LAB caused the inhibition of *P. taetrolens* growth and productive capacity. This antagonistic interaction makes the simultaneous production of lactic and lactobionic acids for commercial purposes unfeasible [15].

In the present study, the effect of encapsulation on the interaction of *L. casei* and the sensitive strain *P. taetrolens* was studied by employing combinations of alginate, starch, CMC and k-carrageenan as encapsulating agents. Mixed fermentations of *P. taetrolens* and *L. casei* were carried out, alternately encapsulating one or other of the two microorganisms in the different hydrogels. Operating conditions were chosen with reference to the optimum conditions for *P. taetrolens* in pure culture, as determined previously by Alonso et al. [3,4,5]. A dairy substrate based on skimmed milk was employed, in consideration of the interest of this study for the dairy food sector.

2. Materials and methods

2.1. Microorganisms

Lactobacillus casei CECT 475, obtained from the Spanish Type Culture Collection (Valencia, Spain), was maintained frozen (in 40% v/v solution at -20 °C) and subsequently incubated on MRS (de Man Rogosa and Sharpe, Biokar Diagnostic, France) agar plates, cultured for 48 h at 30 °C and then stored at 4 °C.

Pseudomonas taetrolens LMG 2336 was obtained from the Belgian Coordinated Collection of Microorganisms (Ghent, Belgium). The strain was conserved frozen in 40% (v/v) glycerol at -20 °C and subsequently subcultured on NB agar plates (Nutrient Broth, containing 1g L^{-1} meat extract, 2 g L^{-1} yeast extract, 5 g L^{-1} peptone and 5 g L^{-1} NaCl). The agar plates were incubated for 48 h at 30 °C and preserved at 4 °C.

2.2. Inocula and substrate preparation

L. casei was reactivated on MRS under microaerophilic conditions. A loopful from an MRS agar plate was used to inoculate a 250 mL storage media bottle containing 250 mL MRS broth.

The culture was incubated in an orbital shaker (New Brunswick Scientific Co., model G25, USA) at 37 °C without agitation for 16 h.
In the case of *P. taetrolens* the culture method was adapted to its aerobic metabolism. A 500 mL Erlenmeyer flask containing 100 mL of NB broth was inoculated with a loopful from an NB agar plate. The culture was incubated at 250 rpm and 30 °C for 10 h.

Skimmed cow's milk was heated in a water bath at 90 ºC for 10 minutes for sterilization [36] and subsequently used as substrate in fermentations.

2.3. Bead-forming procedure

Three different hydrogel formulations were prepared by dissolving the corresponding polymeric mixture in distilled water: 2% [w/v] sodium alginate (Acros Organics); 2% sodium alginate/2% starch (Panreac); and 2% sodium carboxymethyl cellulose (Sigma Aldrich)/1% k-carrageenan (Sigma Aldrich). The choice of these proportions was based on the information obtained from hardening studies carried out with different concentrations of hydrogels and on the results previously reported by other authors [12, 34].

Lactobacillus casei and *Pseudomonas taetrolens* were alternately encapsulated, whilst leaving the other species free in the fermentation medium. In each case, 40 mL from the MRS or NB inoculum cultures containing actively growing cells were centrifuged at 12,000 *x g* for 10 min. The resulting pellet was used for immobilization, by the extrusion methodology described by Alonso et al. [6], with modifications. The biomass was re-suspended in 25 mL of the hydrogel solutions. A peristaltic pump was used to transfer the solutions dropwise into 400 mL of $CaCl_2$ 0.54 M as a gelling solution. The resulting beads were collected, washed in phosphate-buffered saline (PBS, pH 7.4 sterile and filtered at 0.22 µm), filtered and subsequently used as inoculum for fermentations.

2.4. Culture conditions and fermentation experiments

The biomass from 40 mL of MRS or NB precultures was introduced in free suspension, together with the encapsulated biomass, into 2 L storage media bottles containing 400 mL of skimmed milk (10% v/v inoculum level). Thus, fermentations were carried out employing a working volume to air ratio of 1:4, with agitation at 250 rpm and at 30 ºC for 72 hours. These operating conditions, favourable to *Pseudomonas taetrolens*, were chosen in order to avoid it undergoing

environmental stress and maximize lactobionic acid production. Pure cultures of *Pseudomonas taetrolens* and mixed fermentations with both microorganisms in free suspension, under the same operating conditions, were used as controls. Samples were periodically taken to determine bacterial growth, pH and for the chemical analysis of substrate consumption and the production of organic acids. All fermentations were carried out in duplicate as independent experiments and the reported results correspond to the mean value of at least three measurements. Positive and negative error values are shown as error bars in the figures. The experimental data obtained were fitted to the Gompertz kinetic model.

2.5. Quantification of L. casei *and* P. taetrolens *cells*

Growth of free and encapsulated bacteria was determined by means of the spread plate method in MRS and NB agar for *L. casei* and *P. taetrolens,* respectively. In the case of the immobilized biomass, beads were solubilized and encapsulated cells were released by suspending one bead in 1 mL of sodium citrate 1% (v/v). Colony Forming Units (CFU) were counted after incubating the agar plates for 48 h at 30 ºC in all cases. Results are expressed as the increase in the number of CFU during fermentations with respect to initial concentration (CFU_0), according to the formula CFU mL^{-1}/CFU_0 for free bacteria and CFU $bead^{-1}/CFU_0$ for encapsulated bacteria.

2.6. Hydrogels and bead characterization

The textural properties of the different encapsulating hydrogels were studied using a TA.XTplus Texture Analyzer (Stable Micro Systems). The Bloom test, for the determination of bloom strength of gelatin according to the International Standard ISO 9665, was implemented. The Bloom test measures the weight in grams needed by a specific plunger to depress the surface of the gel by 4 mm without breaking it and the result is expressed as the Bloom number. A higher Bloom number indicates higher gel strength. Measurements were carried out using 100 mL samples of each hydrogel suspension, mixed with the biomass and preserving the same proportion used for the encapsulation. The Bloom test was conducted at room temperature at a speed of 0.5 mm s^{-1}, a penetration distance of 4 mm and a data acquisition rate of 200 pps. Measurements were carried out in triplicate for each material.

A visual characterization of the different types of beads was carried out at time 0, with a LEICA M205FA fluorescence stereo microscope (Leica Microsystems Inc., Heidelberg, Germany), without giving the beads any special treatment. A magnification of 22x was employed and

image processing was performed with the Leica Application Suite v4.0 software platform, in order to determine the bead size. The shape of the beads was characterized using the sphericity factor (SF), calculated according to the following equation [23]:

$$SF = \frac{d_{max} - d_{min}}{d_{max} + d_{min}}$$

Where d_{max} is the largest diameter and d_{min} is the smallest diameter perpendicular to d_{max}. The SF varies from 0 for a perfect sphere to 1 for an elongated object.

2.7. Encapsulation efficiency (EE) and cell leakage profiles

Entrapment efficiency was calculated for the different encapsulating hydrogels according to Sandoval et al. [29] by the following formula:

$$Efficiency = (A/B)x100$$

Where A = CFU of bacteria mL^{-1} of hydrogel solution after encapsulation; and B = CFU of bacteria mL^{-1} of hydrogel solution before encapsulation (10^8 CFU mL^{-1} in all cases).

To study the cell leakage phenomenon in entrapped cells, 0.5 g of beads were suspended in 4.5 mL of buffer solution and incubated for 24 h with constant agitation at 250 rpm. Samples were taken from the surrounding medium to quantify the bacterial growth outside the capsule. The counting of viable cells was carried out by the spread plate method as previously described for free bacteria.

2.8. Substrate and product analysis

Lactose, lactic acid, and lactobionic acid concentrations were measured by High Performance Liquid Chromatography (HPLC). The liquid chromatography system used for the analysis (Agilent 1200, Agilent Technologies Inc., CA, USA) was equipped with an ICSep ICE-ION-300 column (Transgenomic Inc., CA, USA) coupled to a refractive index detector. The mobile phase was a sulphuric acid solution (0.450 mmol L^{-1}, pH 3.1), employing a 0.3 mL min^{-1} flow rate and a column temperature of 75 ºC. Data acquisition and analysis were performed using ChemStation software (Agilent).

3. Results

3.1. Hydrogel strength and encapsulation efficiency

The strength of the encapsulating hydrogels was measured according to the Bloom test. A decrease in degradation and higher encapsulation efficiencies have been reported for gels when the Bloom value increases [11,31]. As can be observed in Table 1, in the present study the highest Bloom value was obtained for the gelling blend composed of alginate/starch, corresponding to the highest encapsulation efficiency (53.3 and 83.50%, respectively). The lowest encapsulation efficiency (63.98%) was obtained when only alginate was employed as the encapsulating material. Reduction in encapsulation efficiency is mainly attributed to the cell damage caused by detrimental conditions during the encapsulation process itself, in addition to the loss of cells into the hardening solution [20]. The loss of cells during the preparation of the beads, favoured by their high porosity, constitutes the major limitation in alginate solutions [23]. The addition of starch to alginate solutions leads not only to stronger composites, but also to an increase in the encapsulation efficiency [21,23,34] by promoting the stabilization of the alginate matrix [25].

Table 1 – Bloom values (g) and encapsulation efficiencies (%) for the different encapsulating hydrogels tested.

Hydrogel composite	Bloom value (g)	Encapsulation efficiency (%)
Alginate 2%	43.73	63.98
Alginate 2% + starch 2%	53.30	83.50
CMC 2% + k-carr 1%	37.03	76.82

3.2. Optical characterization of beads

Photographs and stereo microscope images corresponding to the three types of beads are shown in Fig. 1. The surface of the alginate/starch beads appears smoother (b.2), without the cracks that can be observed in the alginate and CMC/k-carr beads (b.1 and b.3). This smoothing effect is caused by the starch, which acts as a filler, occupying the interstitial space in the alginate matrix [23].

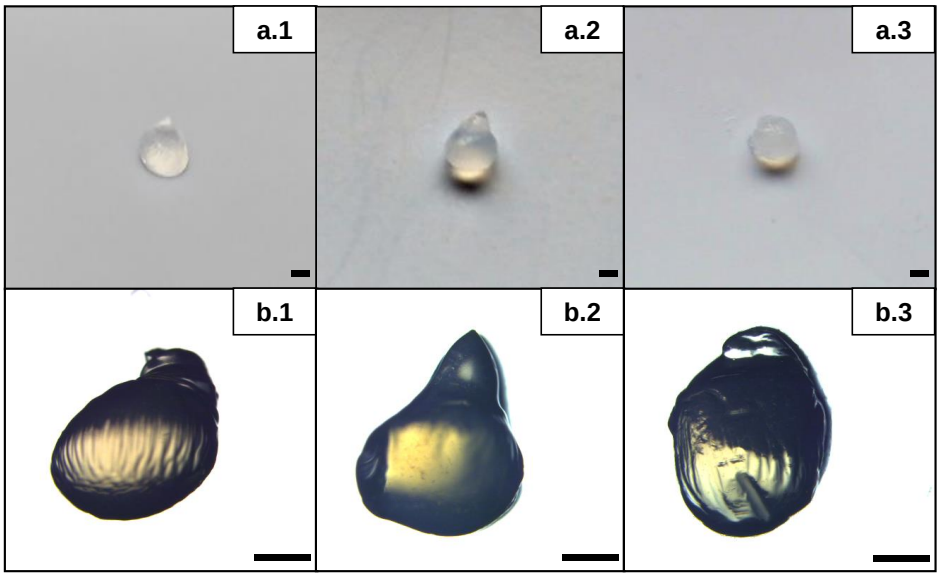

Fig. 1 – Photographs of (a.1) alginate, (a.2) alginate/starch and (a.3) CMC/k-carr beads at time 0 of cultivation; stereo microscope images of (b.1) alginate, (b.2) alginate/starch and (b.3) CMC/k-carr beads at time 0 of cultivation. Scale bars = 1 mm.

Regarding the size, all beads had an approximate diameter of 2.5-3.5 mm (Table 2).

Table 2 – Largest diameter (mm), smallest diameter (mm), sphericity factor and weight (g) for the different encapsulating hydrogels tested.

Hydrogel composite	d_{max} (mm)	d_{min} (mm)	SF	Weight (g)
Alginate 2%	3.139	2.906	0.04	0.013
Alginate 2% + starch 2%	3.673	2.965	0.11	0.016
CMC 2% + k-carr 1%	3.38	2.477	0.15	0.016

The "tail" in the alginate capsules can be explained by the surface tension which is generated when the droplets are extruded. Hydrogel mixtures containing starch become more viscous and the droplets tend to be retained longer before falling into the gelling solution, generating longer "tails" (a.2 and b.2). For this reason, the sphericity factor (SF) shows an increase from 0.04 in alginate beads to 0.11 in alginate/starch beads (Table 2), indicative of an elongation in the bead shape. Systems with SF < 0.05 can be considered spherical [23]. Because of the absence of elongation in the CMC/k-carr beads, they are larger in terms of volume. This difference in size may be partly due to their greater swelling capacity, caused by the strong electrostatic repulsion between the sulphate groups of the k-carrageenan [12].

3.3. Cell leakage

The same cell loading conditions were used in experiments with the different entrapment materials (10^8 $CFUml^{-1}$). After encapsulation, bacterial growth in the liquid phase was monitored with the aim of determining the degree of cell leakage from the beads. Fig. 2 compares the increase in the number of free cells in the liquid medium for each encapsulating hydrogel, represented as CFU/CFU_0 of *L. casei*. As can be observed, the largest increase in free cells occurred in the case of the alginate beads, especially during the first hours of incubation, revealing the low mechanical stability that has been reported by other authors [28]. Similarly, a significant degree of cell leakage was observed in previous studies employing alginate beads at 250 rpm of agitation [6]. In addition to the mechanical factors, alginate presents low stability in the presence of chelating agents, which share affinity for calcium and destabilize the gel. Therefore, problems are encountered during lactic fermentations [22] and these could be exacerbated in the mixed fermentation of *L. casei* and *P. taetrolens*, due to the presence in the medium of the lactobionic acid, another calcium chelating agent [7].

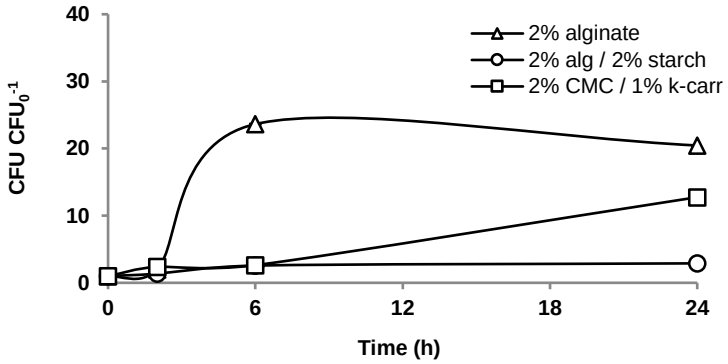

Fig. 2 – Increase in cell leakage during the first 24 h of incubation for the three types of beads tested.

Mixing with starch produces an improvement in the stability of the beads, resulting in better retention of encapsulated microbial cells [22, 28]. As can be observed in Fig. 2., the addition of 2% starch to the alginate matrix led to a reduction in cell leakage. Beads prepared with CMC/k-carr showed an intermediate cell leakage profile, influenced by the swelling capacity of the hydrogel mixture. The swelling phenomenon influences their retention capacity, leading to greater porosity and facilitating the release of the entrapped molecules [27]. A high degree of swelling implies high water uptake and the consequent solubilization of the hydrogel matrix

[26]. This disintegration would involve the progressive release of cells observed in Fig. 2 for CMC/k-carr beads.

3.4. Mixed fermentations with immobilized L. casei *and free* P. taetrolens

Bearing in mind the efficient productivity achieved by the encapsulation of LAB [1,16,38] and the low production of lactobionic acid reported in previous studies with encapsulated *P. taetrolens* cells [6], tests with *L. casei* immobilized and *P. taetrolens* free in suspension were carried out first.

3.4.1. *L. casei* growth

Fig. 3a shows the growth of *L. casei* inside the three types of beads (expressed as CFU/CFU_0). The lowest increase in biomass was registered when only alginate was used as the encapsulating material. The curve corresponding to *L. casei* encapsulated in alginate/starch beads shows a large increase in biomass, reflecting the improved retention of the entrapped cells due to starch addition. In the case of the CMC/k-carr, the growth curve shows that the density of *L. casei* cells did not increase significantly until 32 hours of incubation.

3.4.2. Lactic acid production

Regarding the productive capacity, the immobilization of *L. casei* did not imply a reduction in the lactic acid synthesized in the case of alginate and alginate/starch beads (Fig. 3b). In fact, the encapsulation of *L. casei* with alginate/starch led to an increase in the final lactic acid concentration with respect to the mixed fermentations employing free cells (from 8.15 gL^{-1} to 10.68 gL^{-1}), as can be observed in Table 3. In previous studies with mixed free cultures under the same operating conditions (30 ºC and highly aerobic environment), very different from the optimum for *L. casei* (37 ºC and microaerophilic conditions), it was found that the LAB could survive but their productive capacity was harmed [15]. Immobilization of *L. casei* by encapsulation would improve lactic acid productivity by protecting cells exposed to these harsh environmental conditions [28]. This preservation of the healthy status of *L. casei* is important, given the significance of this study in contributing to the development of a synbiotic product containing probiotic active cells.

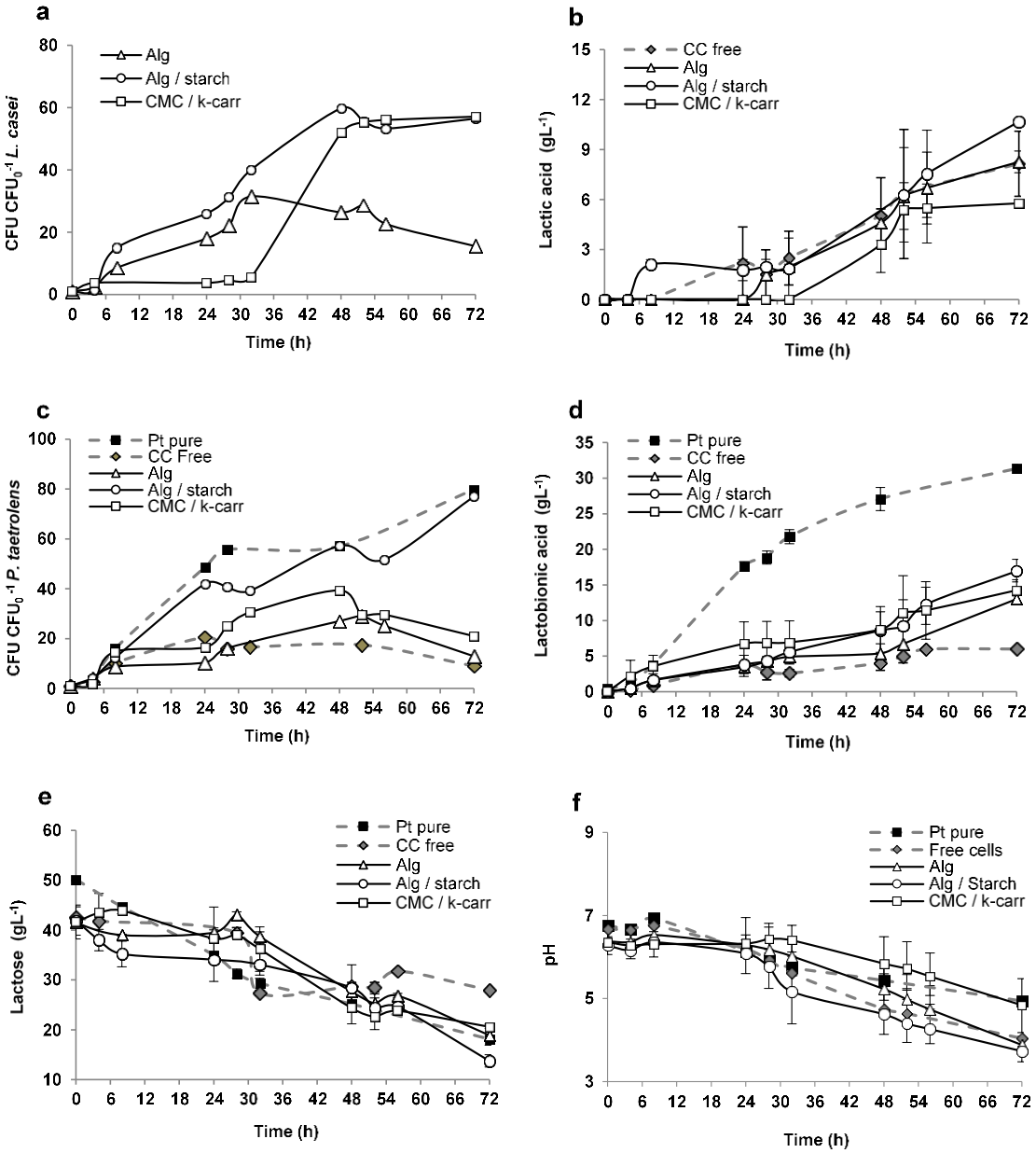

Figure 3 - Evolution of *L. casei* growth inside beads (a), lactic acid production (b), *P. taetrolens* growth in the free medium (c), lactobionic acid production (d), lactose consumption (e) and pH (f) in mixed fermentations with *L. casei* encapsulated in alginate, alginate/starch and CMC/k-carr beads. Pure cultures of *P. taetrolens* and mixed fermentations with both microorganisms free in the medium are used as controls.

In the case of CMC/k-carr beads, according to the registered growth, no lactic acid production was obtained until 32 hours of incubation. Consequently, the final concentration of lactic acid for the CMC/k-carr beads was only 5.77 gL^{-1} (Table 3).

Table 3 - Summary of the final values obtained in the different fermentation systems tested.

	Controls		***L. casei* cap**			***P. taetrolens* cap**	
Fermentation system	Pt pure	Free cells	Alginate	Alg/ starch	CMC/ k-carr	Alginate	Alg/ starch
Lactose (gL^{-1})	18.14	27.96	18.89	13.76	20.61	18.30	24.60
Lactic acid (gL^{-1})	-	8.15	8.26	10.68	5.77	10.30	9.99
Lactobionic acid (gL^{-1})	31.32	5.99	13.02	16.93	14.24	6.80	8.10
Lactobionic acid productivity ($gL^{-1}h^{-1}$)	0.43	0.08	0.18	0.23	0.20	0.09	0.11

3.4.3. *P. taetrolens* growth

In Fig. 3c, the effect of *L. casei* encapsulation on the growth capacity of *P. taetrolens* when both coexist in mixed fermentations can be observed. In the mixed fermentations with free cells used as control, the CFU count showed a very low increase compared to that obtained for *P. taetrolens* in pure culture, starting from 10^8 $CFUmL^{-1}$ at time 0 in all cases (CFU_0). This limited growth constitutes a clear sign of the inhibition exerted by *L. casei* on *P. taetrolens* growth. The increase in the number of CFUs followed a similar curve in the case of mixed fermentations with *L. casei* entrapped in the alginate and CMC/k-carr beads. Nevertheless, in mixed fermentations with *L. casei* encapsulated in alginate/starch, the growth curve of *P. taetrolens* reached levels comparable to those obtained with *P. taetrolens* in pure culture (Fig. 3c).

3.4.4. Lactobionic acid production

The above results are consistent with the lactobionic acid concentration registered in the cultures (Fig. 3d). A quantity of 5.99 gL^{-1} of lactobionic acid was obtained in mixed fermentations with free cells, compared to the 31.32 gL^{-1} synthesized by *P. taetrolens* in pure culture. The encapsulation of *L. casei* increased the final concentrations of lactobionic acid to 13.02, 14.24 and 16.93 gL^{-1} for alginate, CMC/k-carr and alginate/starch beads, respectively (Table 3). The entrapment of *L. casei* in the alginate/starch beads resulted in the greatest increase in lactobionic acid productivity, from 0.08 $gL^{-1}h^{-1}$ for mixed fermentations with both microorganisms free, to 0.23 $gL^{-1}h^{-1}$.

It has been reported that encapsulation allows mass transfer between the bead core and the external environment to be limited by the shell material acting as a physical barrier [8,20]. Although some previous studies have determined that encapsulation may not affect the diffusion of certain antimicrobial substances towards the external medium, it has been seen that the diffusion capacity is related to the size of the bead. Therefore, in smaller capsules the release of encapsulated compounds is faster due to the greater surface to volume ratios, while in large capsules the diffusion path length increases and most of the release starts when the hydrogel matrix begins to degrade [20,37]. In the present study, the lowest release profile, coinciding with the greatest concentration of lactobionic acid found in the medium, was achieved by encapsulating *L. casei* in the alginate/starch beads (Figs. 2 and 3). These beads would constitute an impediment to the diffusion of the inhibitory compounds generated by *L. casei* towards the medium in which *P. taetrolens* was free. This result corresponds with those reported by other authors, according to which the blend of alginate and starch slows the release of antimicrobial substances such as the bacteriocin nisin [21].

3.4.5. Lactose and pH evolution

The lactose concentration and the pH varied during the cultures in agreement with the production results. The greatest decrease in lactose during the first 24 h was registered in mixed fermentations with *L. casei* encapsulated in alginate/starch (Fig. 3e), which also achieved the lowest final concentration (13.76 gL^{-1}, as can be observed in Table 3). The higher production of both lactic and lactobionic acids also resulted in the lowest final pH in fermentations with *L. casei* encapsulated in alginate/starch (Fig. 3f).

3.4.6. Kinetic modelling

The modified Gompertz model was used to describe the fermentative behaviour of *L. casei* and *P. taetrolens* in experiments with *L. casei* encapsulated in the three encapsulating materials. The Gompertz kinetic model defines the asymmetrical sigmoid curve of microbial growth composed of the initial lag phase, the exponential growth phase and the stationary period [39]. The kinetics of the bacterial population growth is given by the following equation:

$$Y = A\exp\left\{-\exp\left[\frac{\mu_m e}{A}(\lambda - t) + 1\right]\right\}$$

Where Y is the logarithm of the relative population size [$Y= \log(N/N_0)$], A is the maximum potential growth [$A= \log(N_\infty/N_0)$], μ_m is the maximum specific growth rate (h^{-1}) and λ is the lag time (h).

The relationship between biomass, organic acid production and substrate degradation was determined using the following equations:

$$r_{p1} = Y_{\frac{p1}{x1}} \mu_{m1} x_1$$

$$r_{P2} = Y_{\frac{p2}{x2}} \mu_{m2} x_2$$

$$r_s = -\frac{1}{Y_{\frac{S}{x1}}} \mu_{m1} x_1 - \frac{1}{Y_{\frac{S}{x2}}} \mu_{m2} x_2$$

Where r_s is the substrate consumption rate ($gL^{-1}h^{-1}$), r_p is the product formation rate of lactic and lactobionic acids ($gL^{-1}h^{-1}$), $Y_{p/x}$ is the product yield/biomass (gg^{-1}) and $Y_{s/x}$ is the lactose yield/biomass (gg^{-1}).

Fitting of experimental data to the kinetic model is shown in Fig. 4, corresponding to the lag, exponential growth and stationary phases, until 48 hours of incubation, before the appearance of the cell death phase.

The presence of growth from time 0 in all the cultivations resulted in $\lambda=0$ in all cases.

In accordance with results previously mentioned, the highest maximum specific growth rate and the maximum potential growth of *L. casei* were found when alginate/starch was used as encapsulating material (μ_m=0.21 h^{-1} and A=1.95, compared to μ_m=0.07 h^{-1} and A=1.54 for alginate, as can be observed in Fig.4 and Table 4). In the case of CMC/k-carr beads, the low maximum potential growth obtained (A=0.66) reflects the inactivity of *L. casei* during the first hours of cultivation.

The lactic acid yield/biomass ($Y_{p/x}$) and the lactose yield/biomass of *L. casei* ($Y_{s/x}$), were greater in the alginate and CMC/k-carr beads than in the alginate/starch beads (Table 4). Thus, lactic acid concentrations and lactose consumption registered in fermentations with alginate and CMC/k-carr beads are high in relation to the amount of biomass quantified inside the beads. These results confirm the low contribution of *L. casei* cells encapsulated in these hydrogels to the production of lactic acid, which can be attributed to the cell leakage phenomenon.

Regarding the performance of *P. taetrolens*, the maximum potential growth (A) was obtained for fermentations with alginate/starch beads (Table 4). However, the maximum specific growth rate was not the highest (μ_m=0.17 h^{-1} compared to 0.74 h^{-1} with *L. casei* encapsulated in alginate beads). This low rate can be explained by the gradual growth of *P. taetrolens* throughout the experiment, without a decrease in the number of cells, whereas in fermentations with *L. casei* encapsulated in alginate the maximum growth (much lower) was reached earlier (Fig. 4).

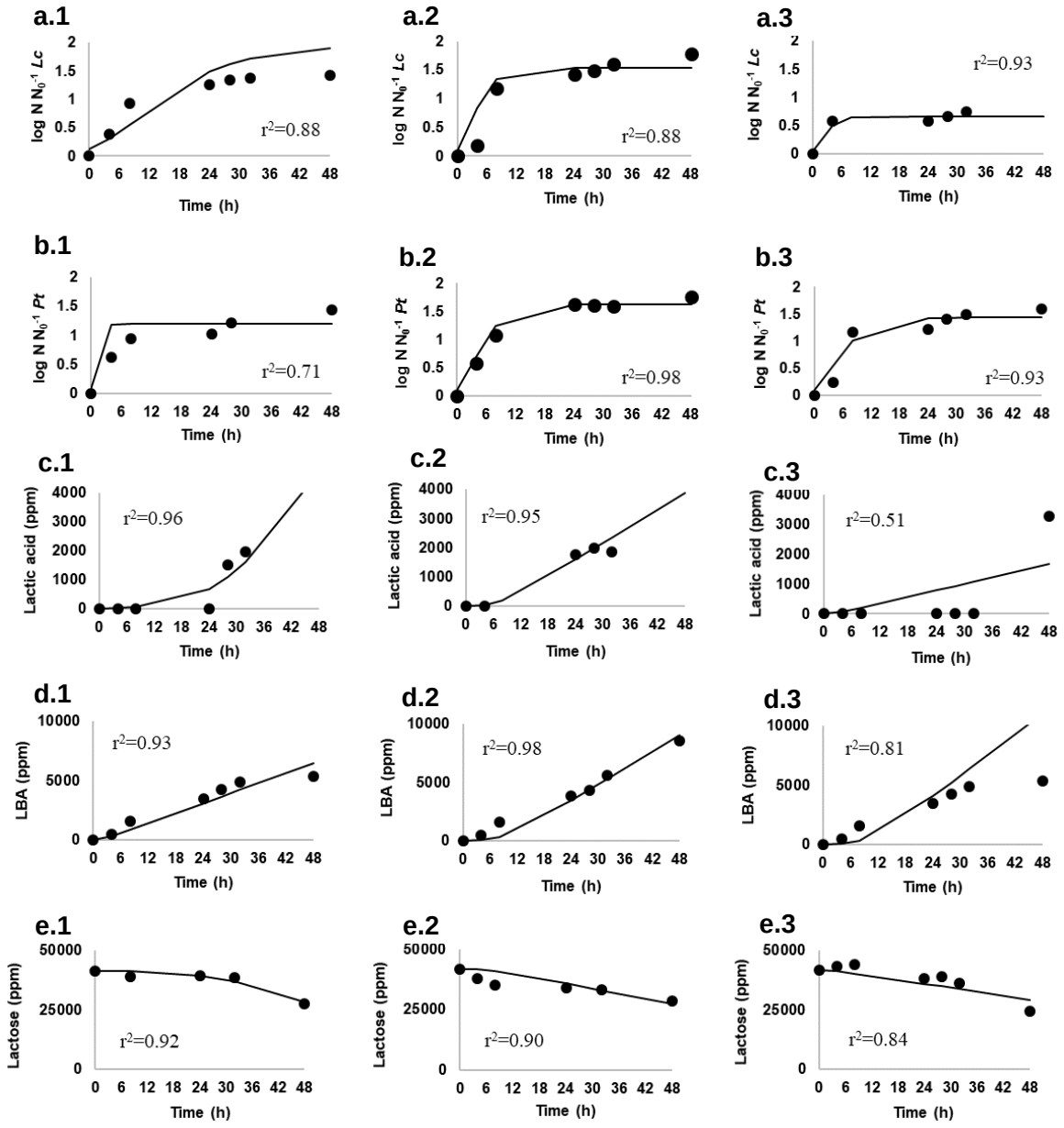

Figure 4 – Fitting of experimental data (●) to the kinetic model (-) corresponding to the growth curves of *L. casei* (a), *P. taetrolens* (b), the lactic acid (c), lactobionic acid (d) and lactose concentrations (e) for mixed cultures with *L. casei* encapsulated in alginate (1), alginate/starch (2) and CMC/k-carr beads (3).

Lactobionic acid yields/biomass were similar for *L. casei* encapsulated in alginate/starch and CMC/k-carr beads (0.19 and 0.18 g lactobionic acid/g biomass, respectively), higher values than that obtained in the case of alginate beads, while no major differences were observed for the $Y_{s/x}$ results (Table 4).

Table 4 – Values of parameters λ (h), A [$\log(N_\infty/N_0)$], μ_{max} (h^{-1}), $Y_{p/x}$ (g product/g biomass) and $Y_{s/x}$ (g substrate/g biomass) resulting from the fitting of experimental data to the kinetic model.

	P. taetrolens					*L. casei*				
Hydrogel	**λ**	**A**	**μ_{max}**	**$Y_{p/x}$**	**$Y_{s/x}$**	**λ**	**A**	**μ_{max}**	**$Y_{p/x}$**	**$Y_{s/x}$**
Alginate	0	1.19	0.74	0.12	0.01	0	1.54	0.07	2.02	5.35
Alginate/starch	0	1.62	0.17	0.19	0.01	0	1.95	0.21	0.29	1.06
CMC/k-carr	0	1.43	0.13	0.18	0.03	0	0.66	0.13	0.69	4.30

3.5. *Mixed fermentations with immobilized* P. taetrolens *and free* L. casei

Mixed fermentations with entrapped *P. taetrolens* and free *L. casei* in the medium were carried out to evaluate the effect of the encapsulation of the sensitive strain on its competitive exclusion. Because of the low mechanical resistance of the CMC/k-carr beads and the poor fermentative capacity of *L. casei* entrapped in this gelling mixture, experiments were carried out employing only the alginate and the alginate/starch beads, in order to establish the influence of the porosity of the encapsulating material on the behaviour of *P. taetrolens*.

Fig. 5a shows the increase in the CFU number of *P. taetrolens* inside the bead during fermentations, from an initial concentration of 10^8 $CFUmL^{-1}$. During the first 48 hours of incubation, a very low increase in the biomass concentration in the two encapsulating materials can be observed, the highest growth occurring from this moment onwards. Nevertheless, the CFU number only increased 20 times with respect to the inoculation value, compared to the increase of up to 60 times found in the alginate/starch beads when *L. casei* was encapsulated. This low growth is consistent with the low lactobionic acid concentrations registered during cultures. The encapsulation of *P. taetrolens* did not lead to an improvement in lactobionic acid synthesis with respect to that obtained in mixed fermentations with both microorganisms free (Fig. 5b). Lactobionic acid productivities of 0.09 and 0.11 $gL^{-1}h^{-1}$ were achieved with *P. taetrolens* entrapped in alginate and alginate/starch beads, respectively, not significantly

higher than that achieved in free cell cultures (0.10 $gL^{-1}h^{-1}$). Improved lactobionic acid production was obtained with *L. casei* entrapped in alginate/starch beads, but not when *P. taetrolens* was encapsulated. The acidic micro-environment that is created inside the beads seems to be the main cause of the damage to the *P. taetrolens* cells. Entrapped cells of *P. taetrolens* are forced to suffer the acidic stress at earlier stages than free bacteria, becoming non-lactobionic-acid-producing cells and therefore leading to low productivities [6]. On the contrary, LAB such as *L. casei* have an acid tolerance response, preserving the proper physiological functions in the cells and surviving at low pH [35]. This ability makes the immobilized LAB more able to survive within the acidic environment inside the bead than the *P. taetrolens* strain.

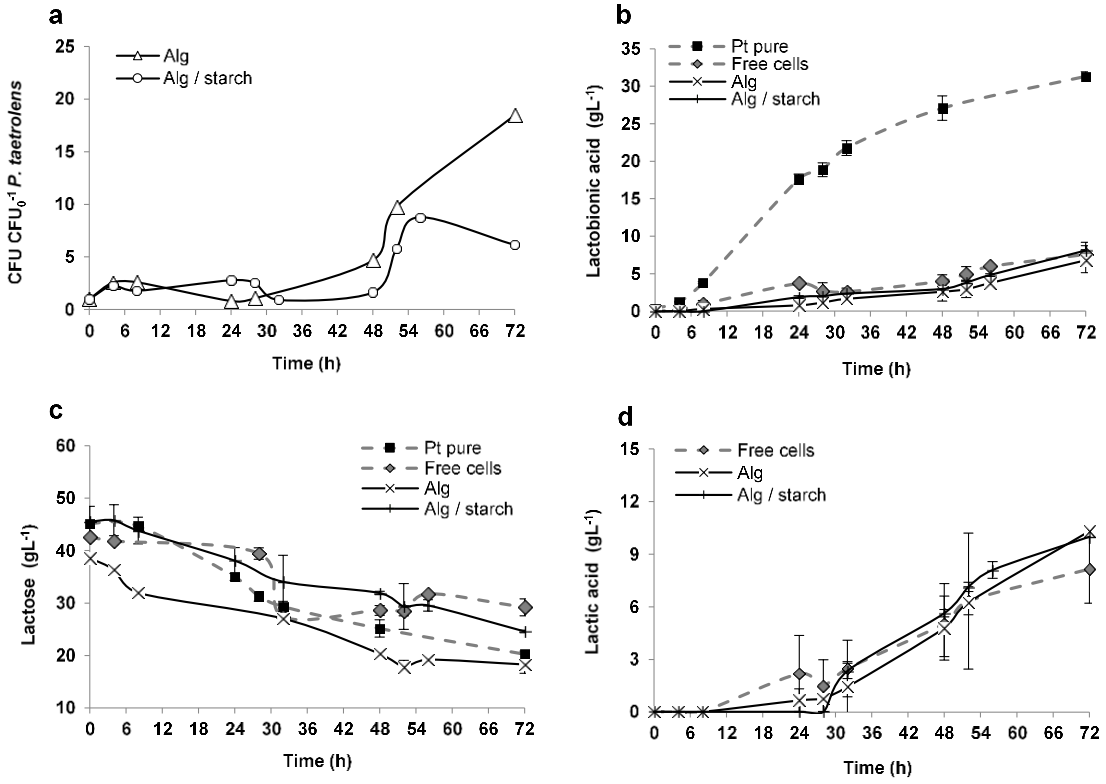

Figure 5 - Evolution of *P. taetrolens* growth inside beads (A), lactobionic acid production (B), lactose consumption (C) and lactic acid production (D) in mixed fermentations with *P. taetrolens* encapsulated in alginate and alginate/starch beads. Pure cultures of *P. taetrolens* and mixed fermentations with both microorganisms free in the medium are used as controls.

With respect to lactic acid, an improvement in its production was registered, particularly in the case of the *P. taetrolens* encapsulated in alginate, corresponding to a greater degradation of lactose (Fig. 5c and 5d).

4. Conclusions

This study has revealed the potential of microbial encapsulation to act as a barrier that minimizes the inhibitory effect in mixed fermentations in which antagonistic strains coexist in the same niche. The entrapment of *L. casei* in alginate/starch beads not only maintains the healthy status of the LAB, but also allows an improvement in the bioconversion performance of free *P. taetrolens*. Therefore, it can be proposed as a feasible strategy to achieve the co-production of lactic and lactobionic acids, in the context of its possible application to the production of fermented dairy products enriched in lactobionic acid. The protection of *P. taetrolens* when *L. casei* was encapsulated is especially significant, because it implies that encapsulation, beyond simply creating a protective environment for entrapped cells, can protect a sensitive strain in suspension against an entrapped dominant strain. The results also highlight the need to evaluate the behaviour of immobilized microorganisms, since those strains which are not able to have a tolerance response to acid stress may not be suitable for encapsulation.

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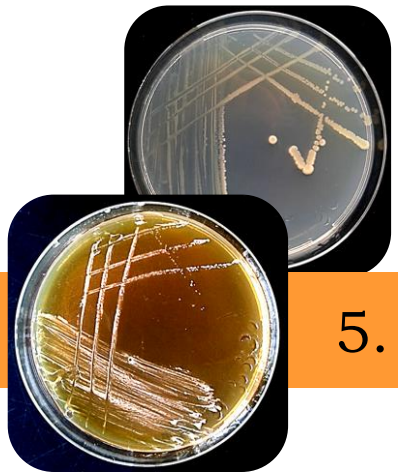

5. DISCUSIÓN GENERAL

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La presente tesis doctoral aborda la interacción entre las bacterias *P. taetrolens* y *L. casei* para su aplicación como *starter* mixto en fermentaciones alimentarias. Los resultados obtenidos, que se discutirán a continuación, pueden resultar de interés para la industria de los alimentos fermentados, en el contexto del diseño de cultivos iniciadores y estrategias de cultivo para la obtención de productos con propiedades mejoradas.

El proceso biotecnológico de desarrollo de un alimento implica estudiar el crecimiento de los microorganismos, su papel en la fermentación y definir la combinación de fermentos. Por ello, como primer paso en esta tesis se estudió el comportamiento de *P. taetrolens* y *L. casei* al ser inoculados en el medio de fermentación de forma simultánea. Inicialmente estas fermentaciones se llevaron a cabo con biomasa en suspensión sobre un sustrato de suero de queso, buscando al mismo tiempo alternativas de valorización para este subproducto alimentario (**subcapítulo 4.1**). Para la evaluación de las interacciones en fermentaciones mixtas alimentarias otros autores han llevado a cabo estudios semi-cuantitativos basados en el método clásico de difusión en agar. Esta técnica permite evaluar el crecimiento bacteriano, pero no la actividad fermentativa, pudiendo resultar difícil extrapolar los resultados a la fermentación en medio líquido (Nehme et al., 2008). En el presente trabajo, durante el desarrollo de las fermentaciones mixtas se monitorizó la viabilidad bacteriana y se cuantificaron el consumo de lactosa y la síntesis de ácidos orgánicos. Se detectó entonces una reducción de la producción de ácido lactobiónico del 87 % (Tabla 5.1) frente al cultivo puro de *P. taetrolens*, no atribuible a las condiciones de operación. Estos resultados corroboraron el efecto negativo observado sobre el crecimiento de la *Pseudomona*, con el desarrollo de colonias de morfología atípica desde el inicio de la fermentación y la incapacidad total para crecer en medio sólido después de 8 horas de incubación.

El empleo de la técnica de citometría de flujo permitió además detectar la existencia de una población de células viables, pero no cultivables (VPNC) de *P. taetrolens* a partir de ese momento, culminando en muerte celular a las 48 horas. Durante el estado fisiológico VPNC los microorganismos son capaces de mantener su integridad de membrana e incorporar oxígeno a una tasa reducida, pero no de formar colonias en medio sólido (Oliver, 2005). La aparición de estas poblaciones ha sido observada en otras especies de *Pseudomonas* sometidas a un ambiente desfavorable (Mock et al., 2015; Trevors, 2011). Esta población es imposible de detectar mediante el empleo de las técnicas tradicionales de recuento en placa, por lo que la combinación

de estas técnicas con la citometría de flujo resulta especialmente útil para la monitorización del estado fisiológico de los microorganismos, particularmente en el caso de los cultivos mixtos. Así, se ha comprobado que, mientras que en cultivos puros la siembra en placa puede aportar resultados de recuento similares a los obtenidos mediante citometría de flujo, en cultivos mixtos las discrepancias aumentan por la aparición de las poblaciones VPNC de forma más frecuente (Bunthof y Abee, 2002; Schellenberg et al., 2006).

El daño y muerte de las células de *P. taetrolens*, junto con la acusada disminución en la síntesis de ácido lactobiónico, se atribuyó en este trabajo a la producción por *L. casei* de metabolitos antimicrobianos, como bacteriocinas o ácido láctico, típicamente sintetizados por las BAL. La monitorización de la actividad fermentativa de *L. casei*, sin embargo, demostró su capacidad para crecer y sintetizar ácido láctico de forma comparable a su comportamiento en cultivo puro. La entrada de *L. casei* en el estado VPNC solo se detectó cuando se alteraron sus condiciones ambientales óptimas (agitación y temperatura). Aunque en esa situación se vio mermada su capacidad fermentativa, no la perdió totalmente gracias a la habilidad de las BAL, y específicamente de la especie *L. casei*, para desarrollar una respuesta de tolerancia frente a diferentes fuentes de estrés (Wu et al., 2012; Zotta et al., 2014).

El cultivo mixto de *P. taetrolens* y *L. casei*, por tanto, reveló la existencia de una relación antagónica entre ambos microorganismos, en la que *P. taetrolens* se ve inhibida por la coexistencia mientras que *L. casei* permanece inalterada. Se trata de una interacción de tipo amensalista, en la que uno de los participantes se ve perjudicado por la coexistencia, mientras que el otro no sufre ningún efecto. Este tipo de interacción amensalista ha sido detectada en otras fermentaciones mixtas alimentarias, en las que las BAL se establecen como las cepas dominantes gracias a su liberación de múltiples sustancias antimicrobianas, como las bacteriocinas, que producen efectos bactericidas o bacteriostáticos sobre microorganismos sensibles (Jamuna y Jeevaratnam, 2004; Smid y Lacroix, 2013). Por otra parte, la habilidad para volverse dominantes en fermentaciones mixtas es mayor en BAL que desarrollan la vía homofermentativa, como es el caso de *L. casei* CECT 475, debido a la rápida acidificación del medio por la acumulación de grandes cantidades de ácido láctico (Moon et al., 2018).

En fermentaciones alimentarias se han encontrado interacciones de tipo amensalista no solo entre BAL y otras cepas bacterianas, sino también en la combinación de BAL y levaduras que participan por ejemplo en el proceso de elaboración del vino y la sidra (Alexandre et al., 2004; Herrero et al., 1999). Con el objetivo de controlar y estandarizar estos procesos tradicionales, la fermentación espontánea ha dejado paso a la selección de inóculos y al estudio del momento

óptimo para la inoculación de cada cepa, optándose frecuentemente en este caso por un sistema secuencial en el que *Saccharomyces cerevisiae* es introducida en primer lugar, y las BAL en segundo lugar, una vez que la fermentación alcohólica ha terminado. La revisión bibliográfica llevada a cabo en el capítulo 2 muestra que este sistema secuencial es una estrategia utilizada en otras fermentaciones mixtas alimentarias (Devanthi et al., 2018; Lu et al., 2018). Se trata de un mecanismo que permite que cada cepa ejerza su metabolismo sin la influencia del resto de la microbiota, minimizando los efectos indeseados de los antagonismos sobre el producto final, y, por otra parte, permitiendo implementar en cada etapa las condiciones operacionales más adecuadas para cada cepa.

En vista de la interacción amensalista detectada entre *P. taetrolens* y *L. casei*, haciendo imposible la coexistencia de ambos microorganismos con fines productivos, se exploraron a continuación otras estrategias fermentativas, comenzando por este sistema secuencial. Se diseñó entonces un sistema de fermentación de dos etapas, teniendo en cuenta el carácter sensible de *P. taetrolens* (**subcapítulo 4.2**). Por ello se estableció su inoculación en primer lugar, evitando así el efecto negativo de los metabolitos antimicrobianos sintetizados por la BAL. En los sistemas secuenciales, el intervalo entre la primera y la segunda inoculación constituye un parámetro clave, ya que determina la duración de la actividad metabólica del primer microorganismo introducido (Ciani et al., 2014). En este caso, el momento de la inoculación de *L. casei* en la segunda etapa se estableció en función de la acidificación del sustrato debido a la acumulación del ácido lactobiónico durante la etapa oxidativa de *P. taetrolens*. Para determinar el valor de pH inicial limitante, se llevaron a cabo previamente fermentaciones de *L. casei* sobre un sustrato sintético conteniendo diferentes concentraciones de ácido lactobiónico. Este experimento demostró su incapacidad para crecer en sustratos con concentraciones de ácido lactobiónico superiores al 1% (p/v), que daban lugar a un pH inicial del medio <5. Estos resultados mostraron concordancia con los obtenidos por otros autores para diferentes cepas de *Lactobacillus* (Adebola et al., 2014). La explicación se encuentra en los requerimientos ambientales de las BAL: se trata de microorganismos neutrófilos con un rango óptimo de pH entre 5 y 9. Como se mencionó anteriormente, son capaces de desarrollar una respuesta de tolerancia al estrés, pudiendo crecer en el ambiente ácido generado por sus propios productos de fermentación hasta valores de pH inferiores a 4 (Shah et al., 2000). Sin embargo, esta respuesta de tolerancia se ha detectado durante la fase de crecimiento exponencial y después de su entrada en la fase estacionaria, pero no al comienzo de la fase *lag* (Saarela et al.,

2004; van de Guchte et al., 2002). Un valor inicial de pH menor que 5 impide el desarrollo de su ciclo normal de crecimiento.

El pH del medio, por tanto, fue el criterio utilizado para el diseño del sistema de fermentación secuencial, de manera que la fase oxidativa de *P. taetrolens* se detuvo transcurridas las 32 h, al alcanzarse un pH de 5.2 por la acumulación del ácido lactobiónico. Se procedió entonces a iniciar la segunda etapa introduciendo *L. casei*. En esta segunda etapa la BAL se mostró capaz de fermentar el medio oxidado, aunque alcanzando una baja productividad. Esta baja productividad del ácido láctico se atribuyó al largo tiempo de fermentación requerido por *L. casei*, comparado con los *starters* tradicionales del yogur (Ma et al., 2015) y a sus necesidades nutricionales, que pueden no verse satisfechas por el permeado de suero de queso empleado como sustrato. Así, otros autores han confirmado la necesidad de suplementar el permeado de suero de queso con minerales, aminoácidos y bases nitrogenadas para alcanzar elevadas productividades de ácido láctico empleando *L. casei* como microorganismo fermentador (Pauli y Fitzpatrick, 2002). Por otra parte, aunque se ha comprobado la habilidad de esta bacteria para fermentar los carbohidratos contenidos en residuos lácteos (Alonso et al., 2010; Panesar et al., 2007), se ha visto que muestra un patrón jerárquico de consumo de los azúcares, metabolizando de forma más eficiente la glucosa y la sacarosa que la lactosa (Alonso et al., 2010).

A pesar de la baja productividad de ácido láctico, *L. casei* alcanzó un rendimiento en sustrato de 0.95 g/g. La mayor parte de este consumo de sustrato correspondió a la metabolización de lactosa, mostrando una preferencia clara por la lactosa como fuente de carbono, frente al ácido lactobiónico que apenas fue consumido. Sin embargo, el experimento realizado sobre el medio sintético reveló la capacidad de la cepa utilizada de *L. casei* para metabolizar el ácido lactobiónico en ausencia de otra fuente de carbono, sintetizando ácido láctico con elevados rendimientos. Este hallazgo resultó vital para el objetivo global de obtener un producto con propiedades simbióticas. Así, la baja preferencia del probiótico *L. casei* CECT 475 por el ácido lactobiónico, en presencia de lactosa, permite que el producto fermentado siga conteniendo ácido lactobiónico al final del proceso de manufactura. Este ácido lactobiónico podría ejercer entonces su papel prebiótico en el tracto intestinal, siendo consumido por el probiótico en el colon, donde otras fuentes de carbono son escasas. Un efecto realmente sinérgico entre probiótico y prebiótico es una característica fundamental para poder calificar un producto como simbiótico, y la especificidad de la interacción se da a nivel de cepa (Adebola et al., 2014; Duncan y Flint, 2013). Actualmente se comercializan como simbióticas diferentes

preparaciones en las que no hay evidencias reales de que el compuesto prebiótico ayude al crecimiento de la cepa probiótica introducida.

El sistema de fermentación secuencial, por tanto, se configuró como una estrategia factible para el cultivo mixto de *P. taetrolens* y *L. casei* CECT 475, dando lugar a un producto potencialmente simbiótico. El proceso fue entonces ensayado sobre un nuevo sustrato de leche desnatada vaca (**subcapítulo 4.3**), con el objetivo de hacer una aproximación a la obtención de un producto interesante para la industria. El cambio de sustrato permitió incrementar la síntesis de ácido lactobiónico en más del 250% en fermentaciones secuenciales sin control de pH. Este aumento puede explicarse por la mayor concentración proteica de la leche frente al permeado de suero, donde la fuente de nitrógeno era prácticamente inexistente. La especie *P. taetrolens* fue aislada originalmente a partir de alimentos en proceso de putrefacción (como carne y huevos), siendo por tanto capaz de beneficiase de la presencia de proteínas (Alonso et al., 2011; West 2004). En este estudio sobre leche de vaca, además, el proceso secuencial se escaló a nivel de biorreactor con control de pH. Así, durante la etapa de oxidación de *P. taetrolens* se implementó una estrategia de control de pH diseñada previamente por Alonso et al. (2011) para optimizar la producción de ácido lactobiónico. Al contrario que *L. casei*, *P. taetrolens* no es capaz de desarrollar una respuesta de tolerancia al estrés ácido y el incremento en la acidez del medio produce una disminución de su viabilidad y por tanto una pobre bioconversión de la lactosa (Alonso et al., 2015). En la etapa fermentativa de *L.casei* no se aplicó control de pH, dejándolo variar libremente hasta el cese del crecimiento de la bacteria.

Gracias a la estrategia de control de pH aplicada en la primera etapa y al nuevo sustrato rico en proteína, se alcanzó una concentración de 34 g/L de ácido lactobiónico en el producto final, muy superior a la obtenida sobre suero de queso y sin control del pH (Tabla 5.1). En relación a su papel como prebiótico, esta cantidad resultaría suficiente para estimular el crecimiento de *L. casei* en ausencia de otra fuente de carbono, teniendo en cuenta su capacidad para crecer a partir de concentraciones de solo 5-10 g/L de ácido lactobiónico (subcapítulo 4.2). Por otra parte, 30 g/L suponen 3 g de prebiótico en una ración típica de producto fermentado de 100 mL. Para otros prebióticos como la inulina, 2.5-5 g se considera la dosis mínima diaria para producir efectos bifidogénicos (Kelly, 2008). En el caso de no darse un efecto realmente sinérgico entre probiótico y prebiótico se requieren dosis de prebiótico más elevadas (> 6g/día) para ejercer un efecto beneficioso sobre la microbiota intestinal (Kolida y Gibson, 2011). Se ha comprobado mediante el desarrollo de estudios *in vivo* que cantidades de hasta 24 g/día de ácido lactobiónico son bien toleradas por el organismo (Schaafsma, 2008).

La producción de ácido láctico también se vio incrementada en el sistema secuencial, pero lo más relevante fue la elevada población viable de *L. casei* cuantificada en el producto final: 10^9 UFC/mL. En productos procesados se recomienda una población de 10^7-10^8 UFC/mL de probiótico en el momento de la venta del producto, manteniéndose en el rango de 10^6-10^7 UFC/mL al alcanzar la fecha de caducidad. Se considera que esta cantidad puede ejercer efectos beneficiosos con un aporte diario de 100 mL de producto (Cruz et al., 2009). A las dosis de prebiótico y probiótico encontradas en el producto final se añade su bajo contenido en lactosa: 0.73 g en 100 mL de producto, gracias a la acción sinérgica de *P. taetrolens* y *L. casei* metabolizando la lactosa. Este supone un contenido mucho más bajo que el 3% típicamente encontrado en un yogur tradicional (Cutrim et al., 2016), y que según la legislación vigente permitiría clasificar el producto como "bajo en lactosa" (Morlock et al., 2014).

Además del análisis químico y microbiológico se llevó también a cabo la caracterización textural del producto final. Todas las fermentaciones desarrolladas en el biorreactor con control de pH dieron lugar a un producto de textura líquida, similar a la de los yogures batidos, debido a la desestabilización de la red de caseína por la agitación mecánica. Sin embargo, en las fermentaciones secuenciales llevadas a cabo a nivel de incubador orbital, se observó un incremento de la viscosidad con respecto a la obtenida en productos fermentados únicamente por *L. casei* (11840 y 4571 cP, respectivamente). Este incremento significativo en la viscosidad puede atribuirse, además de a la mayor concentración de sólidos, al efecto espesante conferido por el ácido lactobiónico (Alonso et al., 2013; Gutiérrez et al., 2012). Debido al elevado número de grupos hidroxilo presentes en su molécula (Figura 1.2), el ácido lactobiónico es muy higroscópico, con la capacidad de formar geles en una atmósfera de humedad (Gutiérrez et al., 2012). De forma adicional, en esta investigación se exploró el efecto que tendría la suplementación con otros agentes texturizantes, como el k-carragenano, siendo necesarios más estudios para determinar una concentración que pudiese complementar al ácido lactobiónico sin generar un efecto de estabilización excesiva.

El sistema de fermentación secuencial desarrollado en esta parte del trabajo, por tanto, se plantea como un bioproceso de interés para la industria láctea, no solo por el potencial funcional del producto (siendo necesario el desarrollo de futuros ensayos *in vivo* para confirmar el efecto de su consumo), sino también por los beneficios tecnológicos que aporta el ácido lactobiónico al integrarse en el propio proceso de manufactura. Se satisfaría así la demanda cada vez mayor de los consumidores de productos con aditivos "naturales", frente a la incorporación de otros prebióticos o de ácido lactobiónico obtenido mediante los procesos de síntesis química y

enzimática implementados por otros autores, que resultan caros, laboriosos, poco estables y que generan subproductos indeseados (Gutiérrez et al., 2012a; Kuusisto et al., 2007; Van Hecke et al., 2009).

Además de la fermentación secuencial, en esta tesis doctoral se ha explorado otra estrategia para el control de cultivos mixtos antagónicos: la encapsulación bacteriana (**subcapítulo 4.4**). El objetivo de este último estudio fue determinar la capacidad de la encapsulación para impedir o minimizar la exclusión competitiva ejercida por *L. casei* sobre *P. taetrolens*. Para ello se llevaron a cabo fermentaciones mixtas sin control de pH encapsulando una u otra bacteria de forma alternativa, y evaluando la efectividad de agentes encapsulantes de diferente naturaleza, pero todos ellos de aplicación alimentaria (alginato, alginato/almidón y CMC/k-carragenano). El bioproceso se ensayó también a nivel de biorreactor con control de pH, pero no supuso una mejoría debido a la ruptura de las cápsulas a causa de la agitación interna.

La mezcla polimérica de 2% alginato/2% almidón mostró las mejores propiedades, con la mayor eficiencia de encapsulación y resistencia mecánica, y la menor pérdida de células encapsuladas a lo largo de la incubación. La encapsulación de *L. casei* en esta mezcla de alginato y almidón en las fermentaciones sin control de pH permitió mejorar la bioconversión de la lactosa por la BAL, incrementando su producción de ácido láctico en un 31% frente a los cultivos mixtos con células libres. Esta mejora puede atribuirse al ambiente protector creado en el interior de la cápsula frente a las condiciones ambientales aplicadas (temperatura y agitación), más favorables a *P. taetrolens* y muy alejadas del óptimo de la BAL. Pero la encapsulación de *L. casei* en alginato/almidón se tradujo además en un incremento de la producción de ácido lactobiónico del 182% con respecto a los cultivos mixtos con ambos microorganismos en suspensión (Tabla 5.1). La inmovilización de *P. taetrolens*, sin embargo, no implicó ninguna ventaja, ni en términos de crecimiento bacteriano ni de productividad.

Este resultado supuso un hallazgo sorprendente, ya que la encapsulación se ha estudiado de forma tradicional como una técnica para proteger del estrés ambiental a los microorganismos encapsulados, especialmente probióticos (Champagne y Fustier, 2007; Sandoval-Castilla et al., 2010). Sin embargo, en este estudio la inmovilización de la cepa dominante permitió no solo mejorar su propio comportamiento fermentativo, sino también proteger a la cepa sensible libre en el medio. Otros autores han observado la aparición de cambios fisiológicos en los microorganismos encapsulados, con la expresión de genes de tolerancia al estrés que les permiten ser más resistentes a compuestos inhibidores (Doleyres et al., 2004; Westman et al., 2012). Sin embargo, la mejora de *P. taetrolens* en este caso solo puede explicarse por la eficacia

del biopolímero encapsulante frenando la difusión de los compuestos antimicrobianos, de dentro hacia fuera de la cápsula. Esta función de la encapsulación, actuando como una barrera física, puede verse potenciada por la incorporación de almidón a la mezcla polimérica, actuando este como un "relleno" que sella los poros y contribuye a la estabilización de la matriz de alginato (Chan et al., 2011; Martin et al., 2013). En la retención del ácido en el interior de la cápsula, sin embargo, puede encontrarse la causa para el efecto negativo observado sobre *P. taetrolens* encapsulada. En esta situación las células de la *Pseudomona*, incapaces de adaptarse al microambiente ácido, se ven forzadas a sufrir estrés en una etapa más temprana que en estado libre, convirtiéndose en células no productoras de ácido lactobiónico (Alonso et al., 2015).

Por otra parte, se ha visto que las cápsulas de mayor tamaño, como las obtenidas mediante la técnica de extrusión, liberan los materiales encapsulados con menor rapidez, debido al menor ratio superficie/volumen (Heidebach et al., 2012; Zhang et al., 2016). Esta propiedad entra en conflicto con el tamaño deseado de las cápsulas de cara a su posible aplicación alimentaria, ya que cápsulas de gran tamaño tendrían un impacto negativo sobre las propiedades sensoriales del producto. Considerando el diámetro de las cápsulas obtenidas en este trabajo (en torno a los 3 mm), resultaría de interés el ensayo futuro de otras técnicas encapsulantes.

Tabla 5.1. Concentración final media de ácido lactobiónico en los productos obtenidos mediante diferentes sistemas experimentales.

Sistema experimental			**Concentración de LBA (g/L)**
Sustrato de permeado de ultrafiltración de suero de queso	Sin control de pH	Cultivo puro Pt	11.10 ± 0.05
		Cultivo simultáneo Pt y Lc	1.40 ± 1.22
		Cultivo secuencial Pt y Lc	5.16 ± 0.14
Sustrato de leche desnatada	Sin control de pH	Cultivo puro Pt	31.32 ± 1.22
		Cultivo simultáneo Pt y Lc	5.99 ± 0.01
		Cultivo simultáneo de Pt y Lc con biomasa encapsulada	16.93 ± 1.58
		Cultivo secuencial Pt y Lc	18.77 ± 1.70
	Con control de pH	Cultivo puro Pt	41.15 ± 2.22
		Cultivo secuencial Pt y Lc	34.08 ± 1.42

6. CONCLUSIONES

6. CONCLUSIONES

Del trabajo realizado en la presente tesis doctoral pueden extraerse las siguientes conclusiones:

- La interacción de *L. casei* CECT 475 y *P. taetrolens* LMG 2336 en fermentaciones mixtas, estudiada por primera vez, da lugar a una relación de tipo amensalista caracterizada por la exclusión competitiva de *P. taetrolens* debido a la producción de metabolitos antimicrobianos por *L. casei*.

- La inhibición de *P. taetrolens* por *L. casei* se traduce en la reducción drástica de su viabilidad y capacidad fermentativa, aunque se mantengan sus condiciones ambientales óptimas. A partir de las 8 horas de incubación la bacteria pierde su capacidad para desarrollar colonias en medio sólido, entrando en un estado VPNC que culmina en daño y muerte celular. *L. casei*, por el contrario, no se ve afectada por la coexistencia, manteniendo su capacidad productiva en niveles comparables al cultivo puro.

- La inoculación secuencial de *L. casei* y *P. taetrolens* en un sustrato de suero de queso, introduciendo a *P. taetrolens* en primer lugar, permite obtener un producto fermentado conteniendo ácido láctico y ácido lactobiónico. Al ser introducida en primer lugar, *P. taetrolens* puede desarrollar libremente su metabolismo sin la inhibición ejercida por *L. casei*.

- *L. casei* es capaz de crecer en el medio previamente oxidado por *P. taetrolens*, produciendo ácido láctico con un rendimiento en lactosa del 95%. El parámetro determinante para la introducción de *L. casei* en la segunda etapa del proceso secuencial es el pH que haya alcanzado el medio como consecuencia de la acumulación de ácido lactobiónico. Un valor de pH<5 impide a la BAL iniciar su ciclo normal de crecimiento.

- En un medio conteniendo lactosa y ácido lactobiónico, la lactosa es metabolizada por *L. casei* de forma preferente. Sin embargo, en ausencia de otra fuente de carbono, la cepa *L. casei* CECT 475 es capaz de fermentar el ácido lactobiónico produciendo ácido láctico con elevados rendimientos. Esta característica permite atribuir propiedades realmente sinérgicas a la combinación de *L. casei* CECT 475 y ácido lactobiónico.

- La leche desnatada de vaca resulta un sustrato idóneo para el crecimiento de *P. taetrolens*. El desarrollo del sistema secuencial sobre este sustrato permite incrementar la producción del ácido lactobiónico en más del 250% con respecto al bioproceso sobre suero de queso.

- La introducción de una estrategia de control de pH durante la etapa oxidativa de *P. taetrolens*, ajustando el pH a 6.5 durante la fase de estacionaria de crecimiento, permite incrementar el rendimiento y la productividad de ácido lactobiónico hasta valores de 0.92 g/g y 0.57 g/L/h, respectivamente. El mantenimiento del pH en 6.5, además, elimina la acidez del medio como factor limitante para el inicio de la fermentación de *L. casei* en la segunda etapa.

- El desarrollo del sistema fermentativo secuencial sobre un sustrato de leche de vaca, e introduciendo una estrategia de control de pH, permite obtener un producto fermentado con propiedades funcionales. Las concentraciones de 34 g/L de ácido lactobiónico, $1.67x10^9$ UFC/mL de *L. casei* y 7.31 g/L de lactosa alcanzadas en el producto final se consideran suficientes para conferirle propiedades prebióticas y probióticas y calificarlo como bajo en lactosa.

- La bioproducción del ácido lactobiónico dentro del propio proceso de manufactura permite incrementar la viscosidad de los productos fermentados (4571 a 11840 cP en productos fermentados por *L. casei* en cultivo puro y productos resultantes del sistema secuencial, respectivamente), sin necesidad de añadir texturizantes adicionales.

- Además de la fermentación secuencial, la encapsulación bacteriana se revela como otra estrategia para eliminar el efecto antagónico en fermentaciones mixtas de *L. casei* y *P. taetrolens*, permitiendo su coexistencia y la co-producción de ácido láctico y ácido lactobiónico. La encapsulación de *P. taetrolens* no resulta viable debido a su incapacidad para tolerar el microambiente ácido creado en el interior de la cápsula. Sin embargo, la encapsulación de *L. casei* manteniendo a *P. taetrolens* libre en el medio permite mejorar significativamente el comportamiento fermentativo de ambos microorganismos.

- La mezcla polimérica de alginato/almidón presenta las mejores propiedades como agente encapsulante, frente al empleo único de alginato y la combinación de CMC/k-carragenano. Así, la encapsulación de *L. casei* en 2% alginato/2% almidón permite incrementar la

producción de ácido láctico en un 31%, y la de ácido lactobiónico en un 182% con respecto a los cultivos mixtos con ambas cepas en suspensión. Cuando *L. casei* es encapsulada en alginato y en CMC/k-carragenano la síntesis de ácido lactobiónico por *P. taetrolens* se incrementa en un 117% y 138%, respectivamente.

7. BIBLIOGRAFÍA

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8. NOMENCLATURA

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8.1. Abreviaturas

AAB	Acetic acid bacteria
BAL	Bacterias ácido lácticas
BU	Bacteriocin units
CECT	Colección Española de Cultivos Tipo
CFU	Colony forming units
CMC	Carboximetilcelulosa
CV6	ChemCrome V6
DGGE	Denaturing gradient gel electrophoresis
DO	Densidad óptica
FCM	Multiparametric flow cytometry
FDA	Food and Drug Administration
GRAS	Generally recognized as safe
HPLC	High performance liquid chromatography
HTS	High throughput sequencing
ILAS S.A.	Industrias Lácteas Asturianas S.A.
IP	Ioduro de propidio
k-carr	k-carragenano
LAB	Lactic acid bacteria
LBA	Lactobionic acid
L. casei	*Lactobacillus casei*
Lc	*Lactobacillus casei*
LMG	Laboratorium voor Microbiologie Universiteit Gent
MALDI-TOF	Matrix assisted laser desorption/ionization time-of-flight mass spectrometry
MBR	Membrane bioreactor
MRS	Caldo de cultivo DeMan, Rogosa y Sharpe
NB	Caldo de cultivo Nutrient Broth

OD	Optical density
P. taetrolens	*Pseudomonas taetrolens*
Pt	*Pseudomonas taetrolens*
PAB	Propionic acid bacteria
PBS	Phospahte-buffered saline
PI	Propidium iodide
PVDF	Fluoruro de polivinilideno
SF	Sphericity factor
TGGE	Temperature gradient gel electrophoresis
UFC	Unidades formadoras de colonias
UHT	Ultra high temperatura processing
VBNC	Viable but non-culturable
VPNC	Viable pero no cultivable

8.2. Símbolos

A	UFC/mL de agente encapsulante después de la encapsulación
B	UFC/mL de agente encapsulante antes de la encapsulación
$\mathbf{d_{max}}$	Diámetro mayor de la cápsula (mm)
$\mathbf{d_{min}}$	Diámetro menor de la cápsula (mm)
N	Tamaño de la población bacteriana (número de células)
Y	Logaritmo del tamaño relativo de población [$\log(N/N_0)$]
A	Máximo crecimiento potencial [$\log(N_\infty/N_0)$]
μ	Velocidad específica de crecimiento (1/h)
$\mathbf{\mu_m}$	Máxima velocidad de crecimiento (1/h)
λ	Duración de la fase lag (h)
$\mathbf{r_s}$	Tasa de consumo de sustrato (g/Lh)
$\mathbf{r_p}$	Tasa de formación de producto (g/Lh)
X	Concentración de biomasa (g/L)
$\boldsymbol{Y_{p/x}}$	Rendimiento producto/biomasa (g de producto/g de biomasa)

$\boldsymbol{Y_{s/x}}$	Rendimiento lactosa/biomasa (g de lactosa/g de biomasa)

9. ANEXOS

9. ANEXOS

9.1. Difusión de la tesis doctoral

9.1.1. Artículos científicos

- **García, C.**, Rendueles, M., Díaz, M., 2017. Microbial amensalism in *Lactobacillus casei* and *Pseudomonas taetrolens* mixed culture. *Bioprocess and Biosystems Engineering* 40, 1111-1122.

- **García, C.**, Rendueles, M., Díaz, M., 2017. Synbiotic fermentation for the co-production of lactic and lactobionic acids from residual dairy whey. *Biotechnology Progress* 33, 1250-1256.

- **García, C.**, Bautista, L., Rendueles, M., Díaz, M., 2018. A new synbiotic dairy food containing lactobionic acid and *Lactobacillus casei*. *International Journal of Dairy Technology* 70, 1-10.

- **García, C.**, Ranieri, G., Rendueles, M., Díaz, M. Exploring encapsulation strategies as a protective mechanism to avoid amensalism in mixed populations of *Pseudomonas taetrolens* and *Lactobacillus casei*. Enviada para su evaluación al *Journal of Industrial Microbiology and Biotechnology*.

- **García, C.**, Rendueles, M., Díaz, M. Liquid-phase food fermentations with microbial consortia involving lactic acid bacteria. Enviada para su evaluación al *Food Research International.*

9.1.2. Comunicaciones a congresos

- **García, C.**, Alonso, S., Rendueles, M., Díaz, M. Simultaneous production of lactic and lactobionic acids from whey in competitive cultures (comunicación oral). XXXV Reunión Bienal de la Real Sociedad Española de Química. A Coruña. Julio 19-23, 2015.

- **García, C.**, Rendueles, M., Díaz, M. Dairy functional foods enriched with lactobionic acid as bioactive compound (comunicación oral). Congreso Nacional de Biotecnología. Murcia. Junio 18-21, 2017.

- Bautista, L., **García, C.**, Rendueles, M., Díaz, M. Preparación de leche fermentada enriquecida en ácido lactobiónico como prebiótico. II Congreso Nacional de Jóvenes Investigadores en Ciencia, Ingeniería y Tecnología de los Alimentos. León. Octubre 19-20, 2017.

- Rendueles, M., **García, C.**, Ranieri, G., Díaz, M. La encapsulación como mecanismo protector para evitar el amensalismo en cultivos bacterianos mixtos. XXXV Jornadas Nacionales de Ingeniería Química. Salamanca. Julio 4-6, 2018.

9.2. Informe sobre el índice de impacto de los artículos de la tesis

Los artículos que conforman el presente trabajo han sido publicados en revistas incluidas en el *Science Citation Index* (Thomson Reuters), cuyos índices de impacto son los siguientes:

- *Bioprocess and Biosystems Engineering* (2017): 2.139
- *Biotechnology Progress* (2017): 1.947
- *International Journal of Dairy Technology* (2018): 1.225