Universidad de Oviedo
Universidá d'Uviéu
University of Oviedo

Departamento de Ingeniería Química y
Tecnología del Medio Ambiente

Incorporación de probióticos y ácido lactobiónico en biomateriales de aplicación alimentaria

Programa de Doctorado en Ingeniería
Química, Ambiental y Bioalimentaria

TESIS DOCTORAL POR

Sara Sáez Orviz

Julio, 2022

Universidad de Oviedo

RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

1.- Título de la Tesis	
Español/Otro Idioma: Incorporación de probióticos y ácido lactobiónico en biomateriales de aplicación alimentaria	Inglés: Incorporation of probiotics and lactobionic acid in biomaterials for food application

2.- Autor	
Nombre: Sara Sáez Orviz	DNI/Pasaporte/NIE:
Programa de Doctorado: Programa de Doctorado en Ingeniería Química, Ambiental y Bioalimentaria	
Órgano responsable: Comisión del Programa de Doctorado	

RESUMEN (en español)

En las últimas décadas, el mercado del envasado o *packaging* alimentario ha crecido enormemente. Los materiales no biodegradables son los mayoritarios, lo que da lugar a problemas ambientales, tanto por la contaminación durante los procesos de producción como por los residuos sólidos que se generan y se deben tratar. Dentro de las nuevas estrategias que se plantean, destaca la elaboración de materiales de envasado a partir de polímeros naturales y renovables, ya que son una alternativa mucho más sostenible. Los principales biopolímeros que se emplean en el desarrollo de biomateriales alimentarios son las proteínas, los polisacáridos y los lípidos. Una de las características más atractivas de este tipo de materiales es la facilidad con la que se pueden incorporar compuestos bioactivos, como prebióticos y probióticos. Dentro de los prebióticos, un compuesto que ha generado un gran interés en el ámbito alimentario en los últimos años es el ácido lactobiónico. Esta sustancia, además de por su capacidad prebiótica, destaca por sus otras propiedades bioactivas como antimicrobiano, antioxidante, etc.

En este contexto, esta tesis doctoral explora por primera vez la incorporación de ácido lactobiónico como compuesto prebiótico junto con bacterias probióticas del género *Lactobacillus* en biomateriales de uso alimentario. En primer lugar, se analizó la capacidad de dos bacterias probióticas, *Lactobacillus plantarum* CECT 9567 y *Lactobacillus paracasei* CB L74, para emplear el ácido lactobiónico exclusivamente como sustrato. Para la incorporación del ácido lactobiónico y las bacterias probióticas se seleccionaron dos matrices diferentes: proteínas y polisacáridos.

Empleando matrices proteicas se desarrollaron *films* como biomateriales de uso alimentario. Las proteínas seleccionadas fueron gelatina y proteína de yema de huevo delipidada. En el caso de la proteína de yema de huevo delipidada, se usó una fracción poco valorizada en la industria alimentaria que permitió obtener *films* de muy buenas características, ya que presentaron una muy baja solubilidad en agua, lo cual es una característica muy favorable para recubrir alimentos, ya que la gran mayoría tiene un alto grado de humedad. En el caso de los polisacáridos, se abordó el desarrollo de biomateriales diferentes a *films*, como micropartículas, *coatings* y cápsulas. Las micropartículas se elaboraron empleando una mezcla de polisacáridos mientras que los *coatings* y las cápsulas se prepararon empleando alginato de sodio como matriz.

Todos los biomateriales desarrollados fueron debidamente caracterizados atendiendo a sus propiedades fisicoquímicas y mecánicas. Además, se examinó el impacto que tuvo la incorporación del ácido lactobiónico y las bacterias probióticas en la formulación de los mismos. En todos los materiales desarrollados, se estudió el efecto sinérgico del ácido lactobiónico sobre los dos probióticos, y se observó que mejoró su viabilidad durante el periodo de almacenamiento. Además, los biomateriales desarrollados que fueron sometidos a pruebas de digestión *in vitro* simuladas confirieron protección a las bacterias probióticas, manteniendo la viabilidad de estas. Asimismo, se analizó el comportamiento de los biomateriales prebióticos, probióticos y sinbióticos en modelos alimentarios reales, empleando gelatina comercial y queso fresco de cabra.

Por tanto, la presente tesis doctoral aborda el desarrollo, caracterización y aplicación de biomateriales de uso alimentario que tienen integrados en su matriz ácido lactobiónico como compuesto prebiótico y *L. plantarum* CECT 9567 y *L. paracasei* CB L74 como

bacterias probióticas, así como el estudio del efecto sinérgico entre ambos y la influencia del prebiótico en la viabilidad de los microorganismos probióticos durante el periodo de almacenamiento y pruebas de digestión *in vitro* simuladas.

RESUMEN (en inglés)

In recent decades, the food packaging market has grown significantly. Non-biodegradable materials are the majority. This causes environmental problems, both due to contamination during production processes and due to the solid waste, that is generated and must be properly treated. Among the new strategies that are proposed, the development of packaging materials from natural and renewable polymers stands out, since they are a much more sustainable alternative. The main biopolymers used in the development of food biomaterials are proteins, polysaccharides, and lipids. One of the most attractive characteristics of this type of materials is the ease form in which bioactive compounds, such as prebiotics and probiotics, can be incorporated. Within prebiotics, a compound that has generated a great interest in the food field in recent years is lactobionic acid. This compound, in addition to its prebiotic capacity, highlights for its other bioactive properties such as antimicrobial, antioxidant, etc.

In this context, this thesis explores for the first time the incorporation of lactobionic acid as a prebiotic compound together with probiotic bacteria of the genus *Lactobacillus* in biomaterials for food use. Firstly, the ability of two probiotic bacteria, *Lactobacillus plantarum* CECT 9567 and *Lactobacillus paracasei* CB L74, to use lactobionic acid exclusively as a substrate was analyzed. For the incorporation of lactobionic acid and probiotic bacteria, two different matrices were selected: proteins and polysaccharides.

Using protein matrices, films were developed as biomaterials for food use. The selected proteins were gelatine and delipidated egg yolk protein. In the case of the delipidated egg yolk protein, a fraction that is not highly valued in the food industry was used. As a result, the films developed had very good characteristics, since they presented a very low solubility in water, which is a very favourable characteristic for coating food, since the vast majority have a high degree of humidity. In the case of polysaccharides, the development of biomaterials other than films, such as microparticles, coatings and capsules, was addressed. The microparticles were made using a mixture of polysaccharides while the coatings and capsules were prepared using sodium alginate as matrix.

All the developed biomaterials were fully characterized according to their physicochemical and mechanical properties. In addition, the impact of incorporating lactobionic acid and probiotic bacteria in their formulation was studied. In all the materials developed, the synergistic effect of lactobionic acid on the two probiotics was analysed, and it was observed that it improved their viability during the storage period. In addition, the developed biomaterials that were subjected to simulated *in vitro* digestion tests conferred protection to the probiotic bacteria, maintaining their viability. Likewise, the behaviour of prebiotic, probiotic and synbiotic biomaterials in real food models was studied, using commercial gelatine and goat cottage cheese.

Therefore, the present addresses the development, characterization, and application of biomaterials for food use that have lactobionic acid integrated in their matrix as a prebiotic compound and *L. plantarum* CECT 9567 and *L. paracasei* CB L74 as probiotic bacteria, as well as the study of the synergistic effect between both and the influence of the prebiotic on the viability of the probiotic microorganisms during the storage period and simulated *in vitro* digestion tests.

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

ÍNDICE

RESUMEN

En las últimas décadas, el mercado del envasado o *packaging* alimentario ha crecido enormemente. Los materiales no biodegradables son los mayoritarios, lo que da lugar a problemas ambientales, tanto por la contaminación durante los procesos de producción como por los residuos sólidos que se generan y se deben tratar. Dentro de las nuevas estrategias que se plantean, destaca la elaboración de materiales de envasado a partir de polímeros naturales y renovables, ya que son una alternativa mucho más sostenible. Los principales biopolímeros que se emplean en el desarrollo de biomateriales alimentarios son las proteínas, los polisacáridos y los lípidos. Una de las características más atractivas de este tipo de materiales es la facilidad con la que se pueden incorporar compuestos bioactivos, como prebióticos y probióticos. Dentro de los prebióticos, un compuesto que ha generado un gran interés en el ámbito alimentario en los últimos años es el ácido lactobiónico. Esta sustancia, además de por su capacidad prebiótica, destaca por sus otras propiedades bioactivas como antimicrobiano, antioxidante, etc.

En este contexto, esta tesis doctoral explora por primera vez la incorporación de ácido lactobiónico como compuesto prebiótico junto con bacterias probióticas del género *Lactobacillus* en biomateriales de uso alimentario. En primer lugar, se analizó la capacidad de dos bacterias probióticas, *Lactobacillus plantarum* CECT 9567 y *Lactobacillus paracasei* CB L74, para emplear el ácido lactobiónico exclusivamente como sustrato. Para la incorporación del ácido lactobiónico y las bacterias probióticas se seleccionaron dos matrices diferentes: proteínas y polisacáridos.

Empleando matrices proteicas se desarrollaron *films* como biomateriales de uso alimentario. Las proteínas seleccionadas fueron gelatina y proteína de yema de huevo delipidada. En el caso de la proteína de yema de huevo delipidada, se usó una fracción poco valorizada en la industria alimentaria que permitió obtener *films* de muy buenas características, ya que presentaron una muy baja solubilidad en agua, lo cual es una característica muy favorable para recubrir alimentos, ya que la gran mayoría tiene un alto grado de humedad. En el caso de los polisacáridos, se abordó el desarrollo de biomateriales diferentes a *films*, como micropartículas, *coatings* y cápsulas. Las micropartículas se elaboraron empleando una mezcla de polisacáridos mientras que los *coatings* y las cápsulas se prepararon empleando alginato de sodio como matriz.

Todos los biomateriales desarrollados fueron debidamente caracterizados atendiendo a sus propiedades fisicoquímicas y mecánicas. Además, se examinó el impacto que tuvo la incorporación del ácido lactobiónico y las bacterias probióticas en la formulación

de los mismos. En todos los materiales desarrollados, se estudió el efecto sinérgico del ácido lactobiónico sobre los dos probióticos, y se observó que mejoró su viabilidad durante el periodo de almacenamiento. Además, los biomateriales desarrollados que fueron sometidos a pruebas de digestión *in vitro* simuladas confirieron protección a las bacterias probióticas, manteniendo la viabilidad de estas. Asimismo, se analizó el comportamiento de los biomateriales prebióticos, probióticos y sinbióticos en modelos alimentarios reales, empleando gelatina comercial y queso fresco de cabra.

Por tanto, la presente tesis doctoral aborda el desarrollo, caracterización y aplicación de biomateriales de uso alimentario que tienen integrados en su matriz ácido lactobiónico como compuesto prebiótico y *L. plantarum* CECT 9567 y *L. paracasei* CB L74 como bacterias probióticas, así como el estudio del efecto sinérgico entre ambos y la influencia del prebiótico en la viabilidad de los microorganismos probióticos durante el periodo de almacenamiento y pruebas de digestión *in vitro* simuladas.

ABSTRACT

In recent decades, the food packaging market has grown significantly. Non-biodegradable materials are the majority. This causes environmental problems, both due to contamination during production processes and due to the solid waste, that is generated and must be properly treated. Among the new strategies that are proposed, the development of packaging materials from natural and renewable polymers stands out, since they are a much more sustainable alternative. The main biopolymers used in the development of food biomaterials are proteins, polysaccharides, and lipids. One of the most attractive characteristics of this type of materials is the ease form in which bioactive compounds, such as prebiotics and probiotics, can be incorporated. Within prebiotics, a compound that has generated a great interest in the food field in recent years is lactobionic acid. This compound, in addition to its prebiotic capacity, highlights for its other bioactive properties such as antimicrobial, antioxidant, etc.

In this context, this thesis explores for the first time the incorporation of lactobionic acid as a prebiotic compound together with probiotic bacteria of the genus *Lactobacillus* in biomaterials for food use. Firstly, the ability of two probiotic bacteria, *Lactobacillus plantarum* CECT 9567 and *Lactobacillus paracasei* CB L74, to use lactobionic acid exclusively as a substrate was analyzed. For the incorporation of lactobionic acid and probiotic bacteria, two different matrices were selected: proteins and polysaccharides. Using protein matrices, films were developed as biomaterials for food use. The selected proteins were gelatine and delipidated egg yolk protein. In the case of the delipidated egg yolk protein, a fraction that is not highly valued in the food industry was used. As a result, the films developed had very good characteristics, since they presented a very low solubility in water, which is a very favourable characteristic for coating food, since the vast majority have a high degree of humidity. In the case of polysaccharides, the development of biomaterials other than films, such as microparticles, coatings and capsules, was addressed. The microparticles were made using a mixture of polysaccharides while the coatings and capsules were prepared using sodium alginate as matrix.

All the developed biomaterials were fully characterized according to their physicochemical and mechanical properties. In addition, the impact of incorporating lactobionic acid and probiotic bacteria in their formulation was studied. In all the materials developed, the synergistic effect of lactobionic acid on the two probiotics was analysed, and it was observed that it improved their viability during the storage period.

In addition, the developed biomaterials that were subjected to simulated *in vitro* digestion tests conferred protection to the probiotic bacteria, maintaining their viability. Likewise, the behaviour of prebiotic, probiotic and synbiotic biomaterials in real food models was studied, using commercial gelatine and goat cottage cheese.

Therefore, the present addresses the development, characterization, and application of biomaterials for food use that have lactobionic acid integrated in their matrix as a prebiotic compound and *L. plantarum* CECT 9567 and *L. paracasei* CB L74 as probiotic bacteria, as well as the study of the synergistic effect between both and the influence of the prebiotic on the viability of the probiotic microorganisms during the storage period and simulated *in vitro* digestion tests.

Capítulo 1.
Introducción a la tesis

1.1. Introducción

En las últimas décadas, el mercado del envasado o *packaging* alimentario ha crecido enormemente. El material dominante es el plástico, debido a su alta relación precio-rendimiento, su peso ligero y su versatilidad a la hora de recubrir diferentes productos alimentarios (Jeya Jeevahan et al., 2020). Los materiales plásticos, junto con el cartón, el metal y el vidrio, representan casi la mitad de la producción de envases de uso alimentario (Ribeiro, Estevinho, & Rocha, 2021). Sin embargo, estos materiales, a excepción del cartón, están clasificados como no biodegradables, dando lugar a serios problemas ambientales, tanto por la contaminación durante los procesos de producción como por la cantidad de residuos sólidos que se generan y se deben tratar (Amin et al., 2021) (Parreidt, Müller, & Schmid, 2018). Para intentar paliar este problema, actualmente muchas compañías e investigaciones están desarrollando nuevas estrategias para la elaboración de materiales de envasado a partir de polímeros naturales renovables, ya que suponen una alternativa mucho más sostenible.

Los principales biopolímeros que se emplean en el desarrollo de envases, recubrimientos y otros materiales de uso alimentario son las proteínas, los polisacáridos y los lípidos, siendo estos últimos los menos usados. Entre los polisacáridos más empleados destacan la celulosa, el almidón y el alginato (Amin et al., 2021) y entre las proteínas más habituales se encuentran la caseína y la gelatina (Zoghi, Khosravi-Darani, & Mohammadi, 2020). Todos estos compuestos pueden ser obtenidos de diferentes fuentes, tanto animales como vegetales, así como de subproductos de las industrias agroalimentarias, permitiendo su reutilización en términos de economía circular (Ribeiro et al., 2021). Estos biopolímeros se pueden emplear como matrices para el desarrollo de recubrimientos alimentarios, como *films* y *coatings*, y también para elaborar otros materiales de uso alimentario como micropartículas y cápsulas (Espitia, Batista, Azeredo, & Otoni, 2016). Además, una de las principales características del empleo de estos polímeros naturales es la facilidad con la que se les pueden incorporar una gran variedad de compuestos diferentes. Por otro lado, en la actualidad los consumidores están demandando productos alimentarios, que además de cumplir con las demandas nutricionales tengan la capacidad de mejorar la salud o reducir el riesgo de padecer ciertas enfermedades (Ashwell, 2002), conocidos como alimentos funcionales. Estas reclamaciones han dado lugar al desarrollo de recubrimientos y materiales de uso alimentario a los que se les han incorporado compuestos bioactivos, como antimicrobianos o antioxidantes (Batista et al., 2017). Cuando se añaden compuestos

bioactivos, los materiales desarrollados se “funcionalizan”, así como los alimentos a los que se incorporan. Dentro de los compuestos bioactivos que se pueden añadir, destacan los probióticos y los prebióticos.

Los probióticos se pueden definir como microorganismos viables que ejercen efectos beneficiosos sobre la salud de los consumidores cuando son ingeridos en las cantidades adecuadas (FAO/WHO, 2002). Para que una bacteria sea considerada como probiótica debe de cumplir una serie de requisitos, recogidos en la Figura 1.1. Los probióticos que se usan de manera habitual en la industria alimentaria pertenecen al grupo de las bacterias del ácido láctico (BAL), entre las que destacan los géneros *Lactobacillus* y *Bifidobacterium* (Zendeboodi, Khorshidian, Mortazavian, & da Cruz, 2020). Es importante destacar que los efectos probióticos son cepa-específicos (Espitia et al., 2016). Además, para que puedan ser usados en alimentos, estos microorganismos deben tener la categoría QPS (*Qualified Presumption of Safety*) otorgada por la *European Food Safety Authorities* (EFSA) (Barlow et al., 2007) o la categoría GRAS (*Generally Recognize as Safe*) otorgada por la *Food and Drug Administration* (FDA) (FDA, 2016).

Figura 1.1. Requisitos que debe cumplir una bacteria para que sea considerada como probiótica (FAO/WHO, 2002).

El concepto de prebiótico fue definido por primera vez en 1995 por Gibson y Roberforid como ingredientes no digeribles que afectan beneficiosamente al consumidor, mejorando la salud del huésped a través de la estimulación del crecimiento y/o actividad de un número limitado de microorganismos que se sitúan en el tracto gastrointestinal (Gibson & Roberfroid, 1995). El desarrollo de la metagenómica y otras herramientas "ómicas" ha permitido conocer en mayor profundidad la complejidad de las interacciones que tienen lugar entre la flora gastrointestinal y el huésped (Moreno, Corzo, Montilla, Villamiel, & Olano, 2017), por lo que el concepto de prebiótico ha sido actualizado. Actualmente, los prebióticos se definen como ingredientes que son fermentados por microorganismos de la microflora gastrointestinal de manera selectiva proporcionando beneficios al huésped (Gibson et al., 2017). Al igual que sucede con los probióticos, los prebióticos deben de cumplir una serie de requisitos para que sean considerados como tal, que se describen en la Figura 1.2. Los prebióticos más estudiados son los galactanos (como los galacto-oligosacáridos; (GOS)), fructanos (como los fructo-oligosacáridos (FOS) y la inulina) y la lactulosa. Sin embargo, en la actualidad se están considerando otra multitud de compuestos como sustancias prebióticas como los soja-oligosacáridos (SOS), isomalto-oligosacáridos (IMO) y otras fibras no digeribles (FAO, 2008). Entre estas sustancias se encuentra el ácido lactobiónico.

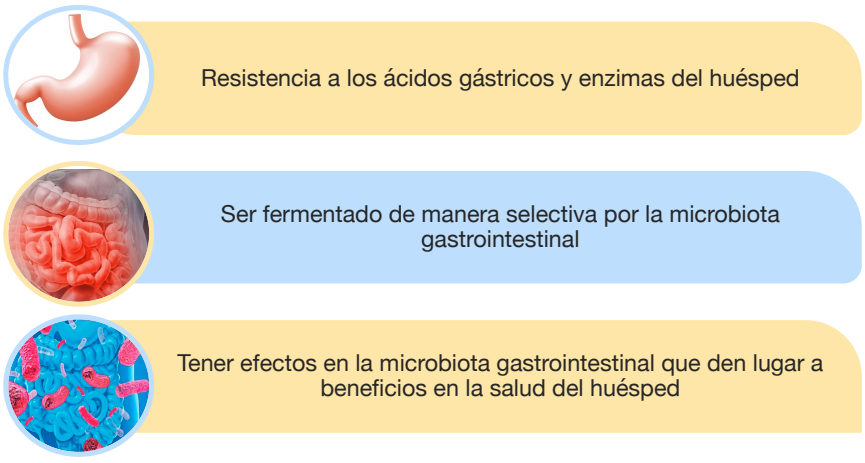


Figura 1.2. Requisitos que debe cumplir un compuesto para ser considerado como prebiótico (Paulo, Baú, Ida, & Shirai, 2021) (Gibson et al., 2017).

El ácido lactobiónico es un polihidroxiácido natural derivado de la oxidación de la lactosa, cuyo nombre sistemático es el 4-O-β-D-galactopiranosil-D-ácido glucónico. Como su nombre indica, está formado por la unión de una molécula de galactosa y ácido glucónico (Figura 1.3) y este compuesto pertenece a la familia de los ácidos

aldobiónicos (Cardoso, Marques, Dagostin, & Masson, 2019) (Alonso, Rendueles, & Díaz, 2013a).

El ácido lactobiónico se puede producir por síntesis química, enzimática o microbiológica. La síntesis microbiológica presenta una serie de ventajas frente a las otras alternativas, ya que no genera subproductos contaminantes ni emplea catalizadores tóxicos (Oh et al., 2020). En la síntesis biológica, se pueden emplear como sustratos residuos o subproductos del ámbito alimentario, como los sueros lácteos de la industria lechera (Alonso et al., 2013a), ya que se estima que un 50% del suero que se genera no se aprovecha, generando pérdidas económicas y problemas de contaminación (Fernandes, Leonardo M. Guimaraes Jonas T., Pimentel Tatiana C., Esmerino Erick A., Freitas Mônika Q., Carvalho Carlos Wanderlei P., 2020). Además, se pueden emplear diferentes especies bacterianas como *Burkholderica cepacia*, *Zymomonas mobilis* o *Acetobacter orientales*. Sin embargo, una de las bacterias más empleadas y estudiadas es *Pseudomonas taetrolens* (Sarenkova, Sáez-Orviz, Ciprovica, Rendueles, & Díaz, 2022) (De Giorgi, Raddadi, Fabbri, Gallina Toschi, & Fava, 2018) (García, Rendueles, & Díaz, 2017) (Alonso, Rendueles, & Díaz, 2011).

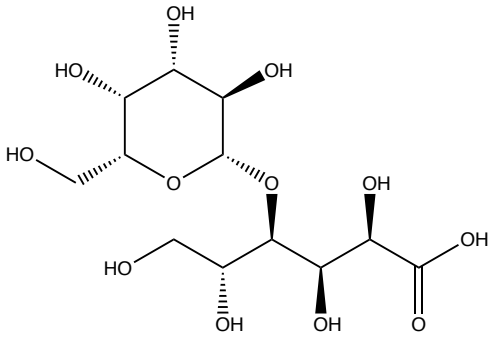


Figura 1.3. Fórmula estructural del ácido lactobiónico.

El ácido lactobiónico ha recibido mucha atención en los últimos años por parte de diferentes industrias como la alimentaria, cosmética y farmacéutica debido a sus numerosas propiedades bioactivas (Cardoso, Marques, Sotiles, Dagostin, & Masson, 2019). Concretamente, en el caso de la industria alimentaria destaca por sus propiedades como agente quelante, acidulante, antioxidante, estabilizante, espesante, inductor de la coagulación, emulsificante y antimicrobiano (Cardoso, Marques, Dagostin, et al., 2019) (Alonso et al., 2013a). Además, una de las propiedades más interesantes es su potencial prebiótico, al ser resistente a los enzimas digestivos, absorbido pobremente en el intestino delgado y pudiendo ser fermentado por los microorganismos probióticos del colon beneficiosos para la salud (Saarela, Hallamaa, Mattila-Sandholm, & Mättö, 2003) (Schaafsma, 2008) (Alonso, Rendueles, & Díaz,

2013b). A pesar de que hay mucha investigación de su aplicación directa en alimentos, tanto por sus propiedades como conservante, prebiótico y antimicrobiano, la regulación de este compuesto es escasa. Sin embargo, en países como Japón, hay productos comercializados desde hace años con este prebiótico. Es el caso del *Caspian Sea yogurt* (Kiryu et al., 2009) (Kiryu et al., 2012). Este yogur contiene ácido lactobiónico en forma de su sal más común (lactobionato de calcio) en una concentración de 0,45 mg/ g de yogurt y está considerado en este país como un producto FOSHU (*Food for specified health use*) (Ohama, Ikeda, & Moriyama, 2014). En países occidentales como en EEUU, la FDA aprobó en el año 2011 su uso como lactobionato de calcio (E-399) (FDA, 2017), mientras que las autoridades europeas (EFSA) aún están estudiando su regulación, aunque se espera que se apruebe próximamente. Uno de los problemas que se presentan es la falta de ensayos *in vivo* en diferentes poblaciones con este compuesto y la necesidad de establecer una concentración adecuada para su consumo (Cardoso, Marques, Dagostin, et al., 2019). A pesar de que hay muy pocos estudios sobre esta temática, algunos autores han concluido que se pueden ingerir hasta 24 g/día de ácido lactobiónico sin la aparición de efectos adversos, teniendo en cuenta que los efectos secundarios serían similares a los causados por intolerancia a la lactosa (Dokkum, Wezendonk, Aken-Schneijder, & Kistemaker, 1994).

La combinación de microorganismos probióticos y compuestos prebióticos se conoce como “sinbióticos”. Los probióticos y los prebióticos conjuntamente en un mismo producto tienen un comportamiento sinérgico (Figueroa-González, Quijano, Ramírez, & Cruz-Guerrero, 2011). Varios autores han observado una mejora en el mantenimiento de la viabilidad de los probióticos durante los procesos de producción y almacenamiento (Orozco-Parra, Mejía, & Villa, 2020) (Oliveira-Alcântara et al., 2020) (Pereira et al., 2019).

En consecuencia, el desarrollo de materiales de uso alimentario a partir de biopolímeros naturales y biodegradables es una de las alternativas sostenibles y necesarias para la disminución del uso de materiales plásticos y derivados del petróleo en el ámbito alimentario. La inclusión de compuestos bioactivos como probióticos y prebióticos en dichos materiales permite obtener alimentos funcionales, que cada vez van a ser más demandados en el mercado por los consumidores. El estudio del comportamiento de estos materiales en alimentos reales es uno de los factores clave para su inclusión activa en la industria alimentaria.

1.2. Objetivos

La incorporación de ácido lactobiónico junto con bacterias probióticas del género *Lactobacillus* en biomateriales de aplicación alimentaria es el principal objetivo de la presente tesis doctoral.

En particular, se han planteado los siguientes objetivos específicos:

- Evaluar la capacidad de dos cepas probióticas (*Lactobacillus plantarum* CECT 9567 y *Lactobacillus paracasei* CB L74) para usar de manera exclusiva el ácido lactobiónico como sustrato.
- Desarrollar y caracterizar *films* de uso alimentario empleando como matriz dos proteínas diferentes, gelatina y proteína delipidada de yema de huevo.
- Elaborar y caracterizar otro tipo de biomateriales, como micropartículas, *coatings* y cápsulas de uso alimentario empleando distintos polisacáridos como matriz.
- Convertir en funcionales los biomateriales desarrollados añadiendo de forma novedosa ácido lactobiónico como prebiótico y bacterias del género *Lactobacillus* como probiótico.
- Analizar la influencia del prebiótico y el probiótico en las propiedades de los biomateriales elaborados.
- Investigar el posible efecto sinérgico en la viabilidad del probiótico durante el almacenamiento y en pruebas de digestión *in vitro* simulada en los biomateriales sinbióticos.
- Estudiar el comportamiento de dichos biomateriales en modelos alimentarios reales, como en queso fresco de cabra y en gelatina comercial.
- Examinar el posible efecto protector del ácido lactobiónico sobre los microrganismos probióticos durante las pruebas de digestión *in vitro* simulada.

1.3. Estructura de la memoria

Esta memoria de tesis doctoral se presenta como un compendio de publicaciones, que se enmarcan en el ámbito de los materiales alimentarios bioactivos. Todas las publicaciones siguen el esquema tradicional, distinguiéndose en cada una de ellas los apartados de introducción, materiales y métodos, resultados y discusión, y conclusiones. Estas publicaciones han sido aceptadas o están siendo evaluadas por revistas incluidas en el *Science Citation Index*. La presente memoria consta de 9 capítulos, subdivididos en sus correspondientes apartados y subapartados.

El capítulo 1 se corresponde con la introducción (*apartado 1.2*), dónde se hace una breve exposición de los motivos que han llevado al desarrollo de esta tesis doctoral. Asimismo, en este capítulo se indican los objetivos que se han pretendido alcanzar (*apartado 1.3*).

El capítulo 2 se corresponde con un estudio preliminar, dividido en dos apartados. En el primero se presenta una revisión bibliográfica sobre la repercusión del ácido lactobiónico como compuesto bioactivo, destacando sus propiedades como antimicrobiano y prebiótico (*apartado 2.1*). También se detallan las últimas investigaciones en las que se ha añadido en diferentes productos alimentarios. En el segundo (*apartado 2.2*), se muestra una revisión bibliográfica sobre la influencia que tiene la adición de microrganismos probióticos y compuestos prebióticos en la formulación de *films* y *coatings*, tanto a nivel de procesos de producción como de las interacciones moleculares que tienen lugar dentro de estas matrices.

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 detalla en la sección de “materiales y métodos” de cada una de las publicaciones. En este capítulo se muestran, de manera general, las técnicas más comunes que han sido empleadas en la elaboración de las diferentes partes experimentales que componen la presente memoria.

En el capítulo 4 se presentan los resultados obtenidos empleando proteínas como matriz en el desarrollo de materiales alimentarios bioactivos. Este capítulo se divide en dos apartados, el primero (*apartado 4.1*) dedicado a la gelatina y el segundo (*apartado 4.2*) dedicado a la proteína de yema de huevo.

En el capítulo 5 se presentan los resultados obtenidos empleando polisacáridos como matriz en el desarrollo de biomateriales alimentarios. Este capítulo se divide a su vez en dos apartados, el primero dedicado a la investigación desarrollada con una mezcla

de varios carbohidratos (*apartado 5.1*) y el segundo dedicado al alginato de sodio (*apartado 5.2*).

El capítulo 6 muestra una discusión general de los resultados obtenidos y en el capítulo 7 se recogen las conclusiones más importantes que se derivan del trabajo realizado.

El capítulo 8 recoge una bibliografía común a la presente memoria. En este capítulo se omiten las referencias específicas asociadas a los capítulos 2, 3, 4 y 5, que pueden ser consultadas en la sección de "referencias" dentro del capítulo, apartado o publicación correspondiente.

Por último, el capítulo 9 está destinado a los "Anexos". En este capítulo se recogen las listas de figuras (*apartado A1*), tablas (*apartado A2*), ecuaciones (*apartado A3*) abreviaturas (*apartado A4*) y símbolos (*apartado A5*) Además, se muestra la difusión de la tesis doctoral en forma de artículos científicos (*apartado A6.1*) y comunicaciones a congresos de ámbito nacional e internacional (*apartado A6.2*) así como otras publicaciones elaboradas no derivadas de la presente tesis doctoral (*A6.3*).

Capítulo 2.

Estudio preliminar

2.1. El ácido lactobiónico como compuesto bioactivo

En este capítulo se expone una revisión bibliográfica sobre las propiedades del ácido lactobiónico como compuesto bioactivo. El ácido lactobiónico está clasificado químicamente como un ácido aldobiónico o biónico y está formado por la unión de una galactosa con ácido glucónico (Alonso, Rendueles, & Díaz, 2013). En los últimos años, el ácido lactobiónico ha generado un interés creciente en la industria alimentaria debido a sus numerosas propiedades. En algunos países, como en Japón, está aprobado su uso directo (Cardoso, Marques, Dagostin, & Masson, 2019), mientras que en otros como EEUU sólo se puede emplear bajo la forma de lactobionato de calcio (CaLb) (E-399) (FDA., 2017), su sal más frecuente y las autoridades europeas (EFSA) aún están estudiando su regulación.

El propósito de esta revisión bibliográfica es detallar las diferentes propiedades bioactivas del ácido lactobiónico y conocer, evaluar y analizar su inclusión y comportamiento en diferentes tipos de matrices alimentarias o materiales de uso alimentario. En cuanto a las propiedades antimicrobianas, se muestra en detalle el mecanismo de acción de este compuesto en las bacterias Gram-positivas y Gram-negativas y se recoge el espectro de acción del ácido lactobiónico frente a bacterias patógenas y responsables del deterioro de alimentos. También se describen otras propiedades bioactivas como su capacidad como conservante, antioxidante y agente gelificante. Entre sus características, destaca su papel como compuesto prebiótico, ya que es resistente a los enzimas digestivos humanos y puede ser fermentado por la microflora intestinal. Además, se recogen los resultados de las investigaciones más recientes en las que se ha usado este compuesto en productos alimentarios de diferente naturaleza teniendo en cuenta las propiedades bioactivas comentadas.

- **Publicación:** Sáez-Orviz, S., Marcet, I., Rendueles, M., Díaz, M. 2022. The antimicrobial and bioactive properties of lactobionic acid. *Journal of the Science of Food and Agriculture*, (https://doi.org/10.1002/jsfa.11823).
- **Estado**: publicada
- **Índice de impacto:** 3,639 (2020)

The antimicrobial and bioactive properties of lactobionic acid

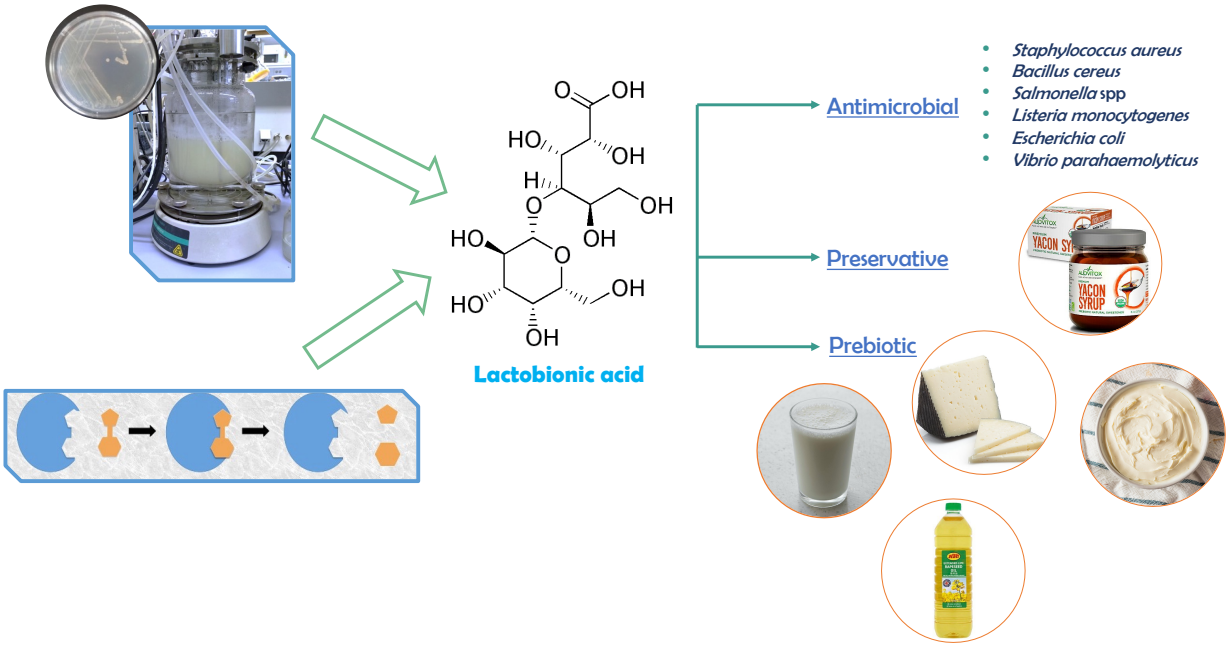
S. Sáez-Orviz[a], I. Marcet[a], M. Rendueles[a*], M. Díaz[a]

[a]Department of Chemical and Environmental Engineering, University of Oviedo, C/Julián Clavería 8, 33006, Oviedo, Spain.

[*]Corresponding author

E-mail address: mrenduel@uniovi.es (Manuel Rendueles)

Graphical abstract



Abstract

Lactobionic acid (LBA) is a bioactive molecule that has generated keen interest in different industries. However, its future application in the food area is one of the most promising. Chemically, it is a polyhydroxy acid formed by the union of two molecules (galactose and gluconic acid) linked by an ether-bond, showing many interesting and unusual properties due to its structure and composition, although it is traditionally known in the food industry for its chelating, moisturizing, gelling and antioxidant properties. As a result, there has been much research into the production of LBA, either by microbial fermentation or biocatalytic approaches such as enzymatic synthesis, but its use in foodstuffs, to produce new functional products and to evaluate its antimicrobial activity against food-borne pathogens, is a relatively new topic that has attracted the interest of the international research community recently. Furthermore, in

spite of the potential of LBA, it has been approved only by the US Food and Drug Administration (FDA), and for its use as the salt form, but the publication of new comprehensive studies, able to agglutinate all the new food-related LBA research results, could disseminate knowledge about this compound and have an influence on its current regulation status. Thus, the aim of the present review is to describe the most recent advances and research on its antimicrobial potential, as well as summarizing the significant aspects that make LBA a promising bioactive compound for the food sector.

Keywords: organic acid, bioactive compound, antimicrobial, preservative, prebiotic.

1. Introduction

Lactobionic acid (LBA) is a natural polyhydroxy acid (Figure 2.1) whose systematic name is 4-O-β-D-galactopyranosyl-D-gluconic acid and is produced by the oxidation of the glucose component of lactose to gluconic acid. This compound has attracted the attention of the international research community owing to its particular chemical composition and properties. According to several authors, LBA exerts chelating, antioxidant, emulsifying and antimicrobial properties that have been exploited by different industries (Alonso, Rendueles, & Díaz, 2013) (Alonso, 2018) (Coroli et al., 2021). In addition, owing to its high biocompatibility it has been widely used as an organ preservative, preventing cell swelling, and as an antioxidant, preventing the oxidation of lipid-based cosmetic products (Alonso et al., 2013).

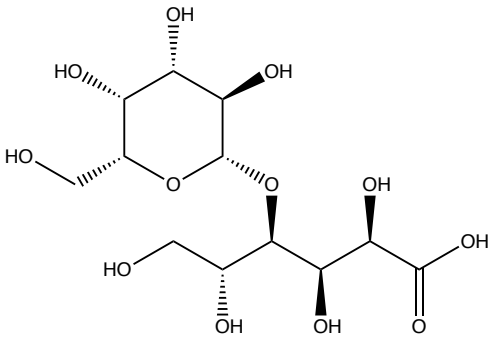


Figure 2.1. Structural formula of LBA.

LBA is produced mainly by chemical, enzymatic or microbial synthesis. Although, at present, chemical synthesis is the predominant method employed to produce this organic acid (Pleissner et al., 2019), the method requires expensive, toxic metal catalysts together with the production of undesirable by-products (Murzina et al., 2008). One alternative to chemical synthesis for the production of LBA is the enzymatic oxidation of lactose, producing a high lactose conversion rate in an eco-friendly process. However, this method suffers from enzyme deactivation problems and the use

of additional enzymes and high-cost cofactors (Oh et al., 2020). Finally, a third option is the microbial fermentation of whey to oxidize lactose into LBA, which has been developed using different *Pseudomonas* species, in particular *P. taetrolens*, although other bacterial species such as *Burkholderia cepacia*, *Zymomonas mobilis* or *Acetobacter orientalis* have been reported as LBA producers (Alonso et al., 2013) (Sarenkova & Ciprovica, 2018). In this case, it is an eco-friendly process that does not generate problematic by-products. However, the LBA produced by microbial fermentation subsequently requires expensive purification processes (Minal, Bharwade, Balakrishnan, Chaudhary, & Jain, 2017). With respect to large scale commercial production, bearing in mind that LBA is a growing market, it is no surprise that several international companies have this organic acid in their portfolio, among them Solvay (Germany), Frieslandcampina Domo (New Zealand), Sadoz (Germany), Reliable Biopharmaceutical Corp. (United States) and U.S. Dairy Ingredient Comp. (USA), although there are also many other chemical-related companies from China involved in LBA production.

At the present time there is only one commercial food product marketed with LBA as a prebiotic compound, namely “Caspian Sea yoghurt”. This product is sold in Japan and is considered as a FOSHU (“Food for specified health use”) (Kiryu et al., 2009) (Kiryu et al., 2012) and it contains LBA in its salt form (calcium lactobionate, CaLb) at a concentration of 0.45 mg CaLb/g yoghurt. The US Food and Drug Administration (FDA) has approved LBA for use as additive, antioxidant, stabilizer and gelling agent in dessert products. There are also patents in existence referring to its use as an aging inhibitor for bread, as a water retainer for processed meats and a flavour enhancer for foods or beverages, among others (Sarenkova & Ciprovica, 2018).

One of the applications that has attracted attention in recent research is its potential as an antimicrobial. The use of natural bioproducts to control food-borne spoilage and pathogenic bacteria is considered a novel antimicrobial strategy (Xu et al., 2017) (Goñi, Tomadoni, Moreira, & Roura, 2013) and LBA could play a key role. Knowing the mechanism of action of this bionic acid is one of the main points to improving and extending their use in the food field.

Within this context, the objective of this comprehensive study is to provide an overview of the antimicrobial potential of LBA. Recent discoveries about the antimicrobial properties of this organic acid and its incorporation in food matrices for antimicrobial purposes will be analysed. Thus, the mechanism of action will be summarised and discussed, in order to clarify the potential use of this organic acid as a protective agent

against foodborne pathogens. In addition, recent research of food products containing LBA as bioactive compound will also be highlight.

2. Antimicrobial properties in food

Foodborne pathogens are an important public health problem (Rivera et al., 2018). In fact, the World Health Organisation estimated that in 2015 one out of ten people suffered from a food-borne disease (World Health Organization, 2015). In this context, the use of natural preservatives with antimicrobial capacity is an important topic in food science since these preservatives avoid the use of potentially harmful chemicals and can help to reduce food spoilage and contamination. One of these antimicrobial preservatives is LBA, which has recently been studied in depth in order to understand its mechanism of action. In addition, its antimicrobial capacities have been tested in various food matrices in order to estimate its potential range of application.

2.1. Mechanism of action of LBA

One of the key requirements for the promotion of the use of LBA in the food field as a natural preservative with antimicrobial capacity is to understand its mechanism of action and to know what its targets are inside the bacterial cells. The mechanism of action of LBA against a Gram-positive bacteria, *Staphylococcus aureus*, has been investigated extensively, as staphylococcal food poisoning is the most prevalent foodborne intoxication worldwide (Fetsch & Johler, 2018). The mechanism of action is summarized in Figure 2.2-A.

Cao et al. (2019) treated a suspension of *S. aureus* with a microbial load of 6 $\log_{10}$ CFU/mL with 7.5 and 15 mg/mL LBA and analysed the growth of the bacteria. As a result, they found that LBA negatively affected the growth of *S. aureus* in a dose-dependent manner, damaging the integrity of the cell membrane. In this case, the possible disruptive effect of the LBA on the cell wall membrane was suggested by the presence of alkaline phosphatase and nucleotides in the culture medium and by the transmission electron microscopy (TEM) observations. In a further study, Cao and Zheng (2021) provided a deeper insight into this subject by carrying out an iTRAQ-based quantitative proteomic analysis, which suggested that the LBA was affecting the expression of ABC transporters, altering the cellular metabolism, and decreasing the levels of proteins involved in the survival of the bacteria under stress conditions. Likewise, LBA could attenuate the virulence of *S. aureus* and reduce its capacity of infection. These authors observed the morphological changes in *S. aureus* caused by LBA and visualized by TEM and SEM.

Kang et al. (2019) thoroughly studied the mechanism of action of LBA on methicillin-resistant *S. aureus* variants (MRSA). These variants are resistant to a wide range of antibiotics such as penicillin, oxacillin and tetracycline and are highly resistant to environmental factors. Therefore, they can contaminate a variety of foodstuffs such as meat and dairy products and so imply a risk to the health of food handlers and consumers (Fetsch & Johler, 2018). In this case, Kang et al. (2019) studied the antimicrobial effect of LBA on MRSA N315 using quantitative proteomics, experiments of reactive oxygen species (ROS), virulence-associated gene expression and quantitative real-time PCR. As a result, they found that LBA's mode of action against MRSA was similar to that against non- methicillin-resistant *S. aureu*s, causing cell wall damage and loss of membrane integrity. Furthermore, there was inhibition of DNA repair and protein synthesis, induction of oxidative stress and inhibition of metabolic pathways. Regarding cell surface proteins and virulence factors, after a 2-hour treatment with LBA, the virulence, biofilm production and adhesion to the MRSA host were decreased.

Although the mechanism of action has been studied in depth in a Gram-positive bacteria (*S. aureus* species), research about the mechanism of action has also been done on Gram-negative bacteria (Figure 2.2-B). The antimicrobial effect of LBA has been studied in *Pseudomonas fluorescens* (Kang et al., 2021). This bacterium is responsible for the spoilage of foods of animal origin, such as meat, poultry, milk, and fish. It is also the most common psychrotroph bacterium found in milk and dairy products (Gutiérrez-Larraínzar et al., 2012). In this study, *P. taetrolens* ATCC 13525 cells at an exponential growth phase were incubated with an amount of LBA of 6.25 mg/mL and a proteomic analysis was performed. As a result, it was found that LBA causes cell damage at different levels. SEM and TEM results showed that LBA may cause cell membrane dysfunction. Besides, folate biosynthesis may be inhibited, blocking DNA and protein synthesis, causing cell death. Regarding the outer membrane, LBA could increase rapidly its permeability in a dose-dependent manner. Therefore, this increase of the permeability of the outer membrane could cause an hypoosmotic shock. In addition, in another study with the same strain, it was also observed that LBA was able to intercalate into bacterial DNA, affecting normal cellular functions (Kang et al., 2020). Another Gram-negative microorganism on which the mechanism of action has been recently studied is *Vibrio parahaemolyticus* (Fan et al., 2022). This bacterium is the main pathogenic bacteria associated with seafood-borne illnesses. The antimicrobial mechanism was studied in two different strains: *V. parahaemolyticus* ATCC 17802 and

ATCC 33847 and with a concentration of 4 and 8 mg/mL of LBA. The antimicrobial effect of LBA was studied by measuring intracellular ATP concentrations, leakage of proteins, changes in bacterial morphology and membrane integrity. As a result, it was observed that LBA treatment was able to reduce intracellular ATP concentration and increase protein loss. As for changes in morphology, it was observed that LBA caused damage to cell morphology and membrane integrity.

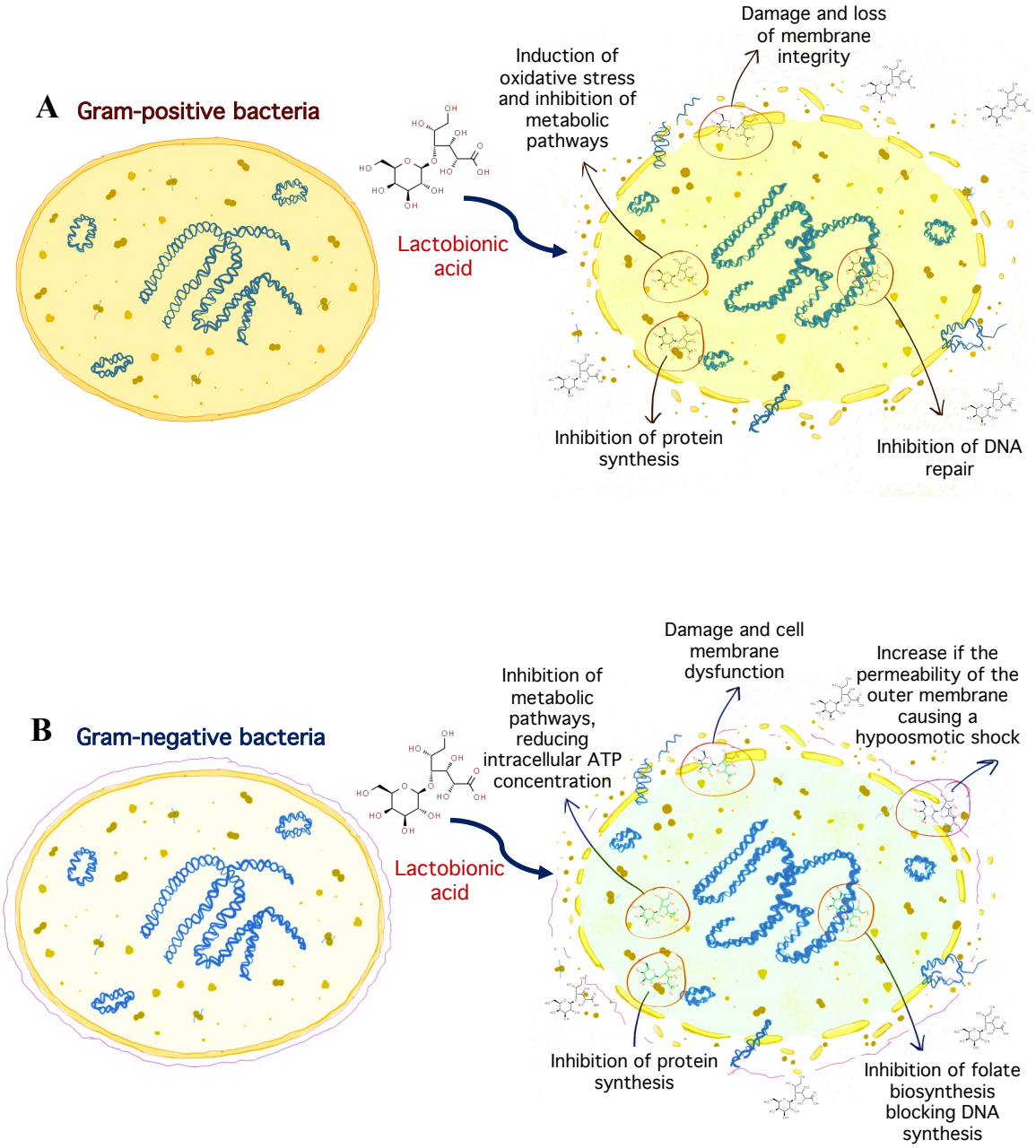


Figure 2.2. Summary of the mechanism of action of LBA. (A) On *S. aureus* as Gram-positive bacteria and (B) *P. fluorescens* as Gram-negative bacteria. Observed effects include induction of oxidative stress, loss of membrane integrity, inhibition of metabolic pathways, protein synthesis and DNA repair. Besides, on Gram-negative bacteria it was observed an increase of the permeability of the outer membrane that causes hypoosmotic shocks.

2.2. LBA-spectrum of action against food-borne pathogenic and spoilage bacteria

The antimicrobial capacity of LBA has been studied in several bacterial species (Table 2.1), including the most important foodborne pathogenic bacteria such as *Salmonella* spp., *Escherichia coli* and *Listeria* (Rivera et al., 2018). Antimicrobial activity can be measured as MIC or the minimum bactericidal concentration (MBC). The MIC is the lowest concentration which prevents visible growth of bacteria while MBC is the lowest concentration of an antibacterial agent require to kill a specific bacterium. In the case of LBA, different concentrations have been tested with various spoilage and pathogenic foodborne bacteria in order to determine their MIC or MBC.

Table 2.1. Summary of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of lactobionic acid (LBA) for some spoilage and pathogenic food-borne bacteria.

Microorganism strain	MIC/MBC (mg/mL)	Reference
Staphylococcus aureus ATCC 25923	15/50	(Cao et al., 2019)
Methicillin-resistant *Staphylococcus aureus* (MRSA) N315	18.75/NA	(Kang et al., 2019)
Bacillus cereus ATCC 11778	300-400/NA	(Cardoso, Marques, Sotiles, et al., 2019)
Salmonella spp. ATCC 13076	300-400/NA	(Cardoso, Marques, Sotiles, et al., 2019)
Pseudomonas fluorescens ATCC 13525	12.5/NA	(Kang et al., 2020)
Listeria monocytogenes Scott A	10.0/20.0	(H. Chen & Zhong, 2017)
Escherichia coli O157:H7 ATCC 43895	10.0/20.0	(H. Chen & Zhong, 2017)
Escherichia coli ATCC 25922	300-400/NA	(Cardoso, Marques, Sotiles, et al., 2019)
Enterococcus faecalis 29212	35.8/NA	(Wojciechowska et al., 2020)
Vibrio parahaemolyticus ATCC 17802	4.0/4.0	(Fan et al., 2022)
Vibrio parahaemolyticus ATCC 33847	4.0/4.0	(Fan et al., 2022)

NA: not available

In addition to studying the mechanism of action, Cao et al. (2019) calculated the MIC and MBC for *S. aureus* ATCC 25923. To determine the MIC, the agar well diffusion

method was employed, and the inhibition zones were measured. The MBC was calculated on a 96-well plate where the final LBA concentrations tested ranged from 10 to 100 mg/mL. Kang et al. (2020) also determined the MIC of MRSA N315 and *P. fluorescens* ATCC 13525 by broth microdilution. Besides that, Chen and Zhong (2017) determined the MIC and MBC for *L. monocytogenes* and *E. coli* O157:H7 ATCC 43895. In this case, the MIC and MBC were determined by the serial microbroth dilution method using a microtiter plate reader to measure optical density (OD^{630nm}). Fan et al. (2022) calculated the MIC and MBC of *V. parahaemolyticus* ATCC 17802 and ATCC 33847, using the modified broth dilution method with increasing concentrations of LBA (from 1 to 32 mg/mL). As a result, the MIC and MBC for these strains was observed to be 4 mg/mL. Cardoso et al. (2019) calculated the MIC for *B. cereus* ATCC 11778, *Salmonella* spp. ATCC 13076 and *E. coli* ATCC 25922 by the diffusion disk test. The inhibition of microbial growth was determined by measuring in mm the inhibition halos. The same method was used by Wojciechowska et al. (2020) to determine LBA MIC for *Enterococcus faecalis* 29212.

As a result, it can be seen that LBA has a wide range of activity, affecting the growth of both Gram-positive (such as *S. aureus*, or *Listeria*) and Gram-negative bacteria (*E. coli* or *Salmonella* spp., among others) (Table 2.1). Regarding the concentration of LBA needed to inhibit microbial growth, as expected, it was highly dependent on the bacterial species. Due to the broad spectrum of action of LBA, it should be considered as one of the promising natural acids in the food area for the prevention of food spoilage and control of food-borne diseases.

2.3. Incorporation of LBA in food matrices for antimicrobial purposes

Although LBA has many properties of interest to the food industry, in particular from an antimicrobial point of view, there are very few studies in which these properties have been tested in a real food model (Table 2.2).

Kang et al. (2020) analysed LBA antibacterial activity against *P. fluorescens* ATCC 13525 and MRSA N315 in whole milk for 12 days at 4 ºC. For this purpose, a concentration of 3 $\log_{10}$ CFU/mL of each of the microorganisms was added to the whole milk together with LBA concentrations of 12.5 and 18.75 mg/mL (MIC concentration) for *P. fluorescens* and MRSA, respectively. As a result, there was a reduction in the growth of *P. fluorescens* and MRSA with regard to control of 2.29 and 2.13 $\log_{10}$ CFU/mL respectively. Chen and Zhong (2017) studied the combined effect of LBA with other antimicrobial agents against *L. monocytogenes* Scott A and *E. coli* O157:H7 ATCC 43895 in 2% reduced fat and whole milk for 120 h at 21 ºC. The best results were

obtained when a ternary combination of 500 IU/mL of nisin, 2 mg/mL thymol and 10 mg/mL of LBA was employed. In 2% reduced fat milk, this combination caused a reduction in both the bacterial populations to a level below the detection limit (1 $\log_{10}$ CFU/mL). In the case of whole milk, there was a large reduction in the concentration of both bacteria, which was maintained throughout the 120 h of testing. In both cases, the presence of LBA increased the synergistic effect of nisin and thymol against the two microorganisms tested. Furthermore, the importance of the study matrix was also reflected, as in whole milk the antimicrobial effect of the ternary combination was weaker compared to that for the reduced fat milk. Cardoso et al. (2021) used LBA in the production of *requeijão cremoso* with the aim of studying the effects of LBA as a food additive. They used different concentrations of LBA (0.25 0.50; 0.75 and 1.00 g LBA/100 g of food product) and monitored it for 22 days, to investigate the effect on different spoilage and pathogenic bacteria. The best results were obtained for a concentration of 0.75 mg LBA/100 g product. For all concentrations tested, the inhibition halos measured in order from highest to lowest were obtained for *L. monocytogenes* ATCC 7644, *S. aureus* ATCC 25923, *S. enteritidis* ATCC 13076 and *Bacillus cereus* ATCC 11778. Despite this variation, after 22 days all the samples showed a loss of antimicrobial activity.

In recent years there has been little investigation into the antimicrobial properties of LBA in real food products and the few studies into this topic were carried out using dairy products. However, as was seen in Section 2.2, LBA possesses notable *in vitro* antimicrobial properties against some of the most worrying food borne pathogens, but it is important to note that this characteristic has not been studied using processed meat, fish or vegetable foodstuffs. In this sense, more research is necessary to assess interactions between LBA and other food compounds that may tend to suppress or enhance its antimicrobial properties, and thus evaluate the real potential of LBA as an antimicrobial agent of practical use the food industry.

Table 2.2. Summary of recent studies on food products to which LBA has been added as an antimicrobial compound.

Food product	Concentration of LBA used	Microorganisms tested	Highlighted results	References
Whole milk	12.5 mg/mL 18.75 mg/mL	*Pseudomonas fluorescens* ATCC 13525 MRSA N315	There was a reduction in the growth of *P. fluorescens* and MRSA with respect to control of approximately 2.29 and 2.13 $\log_{10}$ CFU/mL, respectively.	(Kang et al., 2020)
2% reduced fat milk and whole milk	10 mg LBA/mL in combination with 500 IU of nisin and 2 mg/mL thymol	*Listeria monocytogenes* Scott A *Escherichia coli* O157:H7 ATCC 43895	In 2% reduced fat milk, this combination reduced the bacterial populations to a level below the limit of detection (1 $\log_{10}$ CFU/mL). In the case of whole milk, a large reduction was maintained in both bacterial populations throughout 120 h of testing.	(H. Chen & Zhong, 2017)
Cream cheese (*requeijão cremoso*)	0.75 mg LBA/100 g of food product	*Listeria monocytogenes* ATCC 7644 *Staphylococcus aureus* ATCC 25923 *Salmonella enteritidis* ATCC 13076 *Bacillus cereus* ATCC 11778	The highest inhibition halos (in order from highest to lowest) were obtained for *Listeria*, *S. aureus*, *Salmonella* and *Bacillus*. Nonetheless, after 22 days all samples showed a loss of antimicrobial activity.	(Cardoso et al., 2021)

3. LBA as bioactive compound in innovative food products

In the last years, several new food products have been developed in which LBA plays a fundamental role, and which have been designed to benefit from other of its properties such as its preservative and prebiotic capacity. Like other bioactive compounds, such as galactosyl derivatives (Wojciechowska et al., 2020) and glucooligosaccharides (Grimoud et al., 2010), LBA can show a prebiotic or antimicrobial effect depending on the bacterial type and dose. A recent study has shown that the utilisation of prebiotics by lactic acid bacteria have species and strains specificity (Cui & Qu, 2021). In the case of bifidobacteria and other bacteria of the gastrointestinal tract (GIT) microflora, LBA acts as a prebiotic, which implies that it is an ingredient that is selectively fermented by this type of bacteria (Delgado-Fernández, Corzo, Olano, Hernández-Hernández, & Moreno, 2019) while shows antimicrobial activity against several food pathogens. The most recent research work about the incorporation of LBA in food-related products in order to take advantage of its bioactive properties are summarized in Table 2.3.

Regarding its properties as a preservative, Marques et al. (2020) prepared yacon juice and tested the effect of several heat treatments and acids, including LBA, on some of its properties. In this case, an LBA concentration of 1% combined with steam blanching was found to be the best option for preserving the pH, antioxidants, colour and total polyphenols content of this juice. When the same yacon juice was tested for a long-term storage (120 days), the appearance of moulds and yeasts was delayed from day 60 to the control to day 120 for the juice with LBA added (Marques et al., 2021). Goderska (2019) added LBA dissolved in glycerol to rapeseed oil to check the preservative properties of this organic acid on a lipid-based product. In this research and as the author expected, the larger the LBA concentration, the higher antioxidant effect observed. The best results were obtained at the highest LBA concentration tested (1%, w/v), with a decrease in the peroxide value of 19.9%. There have also been recent studies on dairy food products. Cardoso et al. (2021) prepared a kind of acid-set crema cheese (*requeijão cremoso*) using LBA and/or lactic acid, testing their effect of these acids on several cheese properties under refrigerated storage up to 22 days. LBA showed no effect on the water activity, pH and colour of the cheese produced and the antioxidant properties of the cheese were higher only during the first day of storage. Kang et al. (2020) added LBA to whole milk at a concentration of 12.5 mg/mL, testing the sensory properties of the milk during 12 days of storage at 4ºC. In this case, the milk with LBA exhibited less thickness and sourness, had less precipitate after boiling and its natural milk colour was preserved for longer when compared to the control milk.

All in, according to all these recent research papers, it is noticeable that the preservative effect of the LBA was most significant when it was incorporated in liquid food products, but when it was added to a more complex, concentrated food matrix, such as the *requeijão cremoso*, these preservative properties were almost unnoticeable (Cardoso et al., 2021). These results suggest some kind of interaction in cheese between the LBA and the milk compounds, resulting to some extent in the suppression of the preservative properties of LBA. In any case, no other study was found in the literature about the preservative effect of LBA in cheese that might have been able to provide a deeper insight into this question.

Regarding its prebiotic properties, several new food products have been developed. García et al. (2018) prepared a novel dairy product by fermenting milk sequentially with *P. taetrolens* LMG 2336 and then with *Lactobacillus casei* CECT 475 in order to obtain a synbiotic product enriched in LBA. This double fermented milk contained, in its best formulation, 30 g/L of LBA and an active *L. casei* population of 9 $\log_{10}$ CFU/mL. In addition, the textural properties of this dairy product were improved by the gelling capacity of the LBA. Part of the newly research has focused on the preparation of biomaterials. In this sense, some authors have prepared microparticles loaded with LBA using casein, gelatine, maltodextrin and gum arabic (Sáez-Orviz, Camilleri, Marcet, Rendueles, & Díaz, 2019). Several studies have shown that LBA is consumed by different probiotic bacteria belonging to the genera *Bifidobacterium* and *Lactobacillus* (Goderska, 2019) (Sáez-Orviz, Passannanti, et al., 2021), which are generally present in dairy products. Therefore, LBA was introduced in the microparticles in order to prevent it from being consumed by the lactic acid bacteria that are naturally present in this kind of foodstuff with the aim to reach the low GIT intact. Results showed that the concentration of LBA contained in the microparticles remained almost invariable for 12 days in the cheese matrix. Furthermore, LBA was released abundantly in stomach and intestine, which suggests that the LBA is able to reach the low GIT in a free form, exerting its prebiotic function. If LBA is combined with probiotic bacteria, synbiotic products and bioactive packaging are developed. In this respect, Sáez-Orviz et al. (2020) prepared an alginate-based synbiotic coating that contained LBA as prebiotic and *Lactobacillus plantarum* CET 9567 as probiotic in order to coat cottage cheese. The presence of LBA in the coating increased the survival of the probiotic during the analysis (15 days). Thus, a simulated *in vitro* digestion of the bioactive coating was made, an only those coating with both, probiotic and prebiotic in their composition met the minimum legal requirements to be considered as probiotic (6.0 $\log_{10}$ CFU/g cheese).

Also, synbiotic films and coatings using delipidated egg yolk protein as the matrix, *L. plantarum* CECT 9567 as a probiotic microorganism and LBA (10 g/L) as a prebiotic compound were developed (Sáez-Orviz, Marcet, Rendueles, & Díaz, 2021).The resulting bioactive packaging possessed suitable properties for coating foodstuffs for 15 days at 4 ºC. Furthermore, LBA exerted a protective effect on the probiotic bacteria, and when pieces of gelatine were coated by dipping in the film-forming solution containing LBA and probiotic, the LBA also exerted a protective effect on the probiotic population subjected to *in vitro* oral gastrointestinal digestion. It is of interest that LBA can exerted a protective effect on the bacterium when encapsulated or trapped in a film or coating, enabling a greater degree of survival in the simulated *in vitro* digestion studies mentioned above (Sáez-Orviz, Puertas, Marcet, Rendueles, & Díaz, 2020a) (Sáez-Orviz, Marcet, et al., 2021), which is an issue that deserves more attention. LBA, like other prebiotics, would show a protective effect when it is encapsulated or trapped with probiotic bacteria, since it would provide an extra nutritional contribution that improves their survival in the acidic conditions of the digestion process (Song, Mao, Siu, Tai, & Wu, 2019) (Iyer & Kailasapathy, 2005) (Huan Liu et al., 2019). However, the industrial use of these new products is limited since these laboratory concepts have not been scaled-up. In addition, despite the fact that the prebiotic capacity of LBA has been studied by several authors (Saarela, Hallamaa, Mattila-Sandholm, & Mättö, 2003) (Schaafsma, 2008) and LBA is known to be resistant to digestive enzymes and able to reach the lower GIT to be fermented by the GIT microflora, more *in vivo* research is necessary.

Current food applications for LBA are scarce and it may be due to a lack of regulation in Europe discouraging research initiatives in the food area. At the moment, LBA has been approved by the Food and Drug Administration (FDA) for its use as a food preservative only in its salt form (CaLb, E-399) (*FDA.*, 2011), although it is expected that its human consumption will be approved by other food authorities, such the European Food Safety Agency (EFSA), in the near future. On the other hand, Japan has approved its consumption In different types of foodstuff, such as “Caspian Sea yoghurt” and other dairy products (Takaaki Kiryu et al., 2016). One of the challenges facing the approval of LBA for use in the food sector is the lack of *in vivo* test (Cardoso, Marques, Dagostin, & Masson, 2019) and the establishment of concentrations suitable for its consumption. Although very few studies have been conducted, some authors have found that up to 24 g per day of LBA can be ingested without adverse effects, effects which would be similar to those caused by lactose intolerance (Van Dokkum W, Wezendonk LJW, van Aken-Schneider P, 1994).

Table 2.3. Summary of the most recent research works about the incorporation of LBA in food-related products.

Food product	LBA activity	Final product parameters tested	Highlighted results	Reference
Yacon Juice	Preservative (up to 48 h).	Juice antioxidant and phenolic content, pH, colour and enzymatic browning protector capacity.	The best preservative tested was a combination of LBA (1%) and steam blanching. All the parameters	(Marques et al., 2020)
Yacon Juice	Preservative (up to 120 days). Prebiotic.	Juice colour, antioxidant content, polyphenols bioavailability, Fe^{2+} and Ca^{2+} chelating capacity, gastrointestinal digestion, prebiotic assessment.	The best preservative tested was LBA at a concentration of 1%.	(Marques et al., 2021)
Cream cheese (*requeijão cremoso*)	Preservative (up to 22 days). Acidulant.	Cheese pH, moisture, water activity, colour, rheology, antioxidant content, iron chelating capacity and antimicrobial properties.	When the cheese was produced using LBA instead of lactic acid, better antioxidant and antimicrobial results against *Listeria monocytogenes* were obtained after one day of storage.	(Cardoso et al., 2021)
Whole milk	Preservative (up to 12 days).	Sensory properties.	Milk with added LBA showed a better score on the sensory parameters tested over the whole time of storage assessed than the whole milk control.	(Kang et al., 2020)
Synbiotic coatings to cover cheese pieces	Prebiotic.	*L. plantarum* survival under storage conditions and after being subjected to simulated gastrointestinal tract conditions. Textural properties of the pieces of cheese.	The coexistence of LBA and *Lactobacillus plantarum* in the coatings noticeably increased the survival of the bacteria.	(Sáez-Orviz et al., 2020a)

Microparticles loaded with LBA in order to protect it from its consumption by lactic acid bacteria	NA	LBA encapsulation efficiency, microparticle solubility, microstructure and enriched cottage cheese digestibility and textural analysis.	Microparticles exert a protective effect against consumption of lactobionate by lactic acid bacteria found in the cheese matrix.	(Sáez-Orviz et al., 2019)
Rapeseed oil	Antioxidant.	Lipid oxidation level.	At the highest concentration of LBA tested (1%, w/v) a 19.9% antioxidant effect was observed.	(Goderska, 2019)
Synbiotic dairy product	Prebiotic and gelling agent	Concentration of LBA in the final product and textural properties	Final product can be marketed as synbiotic due to an adequate amount of probiotic and prebiotic. The final product had higher viscosity due to the gelling effect of LBA.	(García et al., 2018)
Bioactive packaging based on delipidated edible egg yolk protein	Prebiotic	*L. plantarum* survival under storage conditions and after being subjected to simulated gastrointestinal tract conditions.	The coexistence of LBA and *Lactobacillus plantarum* in the films and coatings increased noticeably the viability and survival of the bacteria after the digestion test, reaching final values above 6 $\log_{10}$ CFU/mL	(Sáez-Orviz, Marcet, et al., 2021)

NA: not available.

4. Conclusions

In this review, the great potential of LBA as a natural food additive that is of interest for its antimicrobial capacity has been highlighted. The antimicrobial mechanism of action involves loss of cell membrane integrity, inhibition of DNA and protein synthesis and the induction of oxidative stress. LBA could also intercalate into bacterial DNA and is able to decrease virulence factors in some cases. Regarding Gram-negative bacteria, there is an increase in the permeability of the outer membrane causing hypoosmotic shock. LBA has a spectrum of action against a variety of foodborne bacteria (Gram-positive and Gram-negative) and some studies have already demonstrated this antimicrobial activity in dairy products, but more research is needed in order to assess antimicrobial capacity when LBA is included in other food matrices. Something similar must be said about its preservative properties. There is a general lack of studies in the literature into the possible interactions of LBA with biopolymers such as lipids or proteins from a variety of food sources. The study of these interactions could clarify its antimicrobial and preservative potential as a valuable additive for the food industry. As to the innovative products containing LBA and developed in the last five years, it is clear that most of them have been prepared only on laboratory scale and they have not been subjected to any sensory testing. All in all, from the perspective of the food industry, LBA may be considered to remain a promising but understudied compound. Therefore, further in-depth studies into this subject would be valuable for the food research community.

Acknowledgements

This work was financially supported by the Spanish Ministry of Science and Innovation, through the project MCIU-19-RTI-2018-094218-B-100 and by the grant *Programa Severo Ochoa de Ayudas Predoctorales para la investigación y docencia* (grant number BP19-127 to Sáez-Orviz, Sara.

5. References

Alonso, S. (2018). Exploiting the bioengineering versatility of lactobionic acid in targeted nanosystems and biomaterials. *Journal of Controlled Release*, *287*(August), 216–234. https://doi.org/10.1016/j.jconrel.2018.08.030

Alonso, S., Rendueles, M., & Díaz, M. (2013). Bio-production of lactobionic acid: Current status, applications and future prospects. *Biotechnology Advances*, *31*(8), 1275–1291. https://doi.org/10.1016/j.biotechadv.2013.04.010

Cao, J., Fu, H., Gao, L., & Zheng, Y. (2019). Antibacterial activity and mechanism of lactobionic acid against Staphylococcus aureus. *Folia Microbiologica*, *64*(6), 899–906. https://doi.org/10.1007/s12223-019-00705-3

Cao, J., & Zheng, Y. (2021). iTRAQ-based quantitative proteomic analysis of the antimicrobial mechanism of lactobionic acid against Staphylococcus aureus . *Food & Function*, *1*(120), 1349–1360. https://doi.org/10.1039/d0fo02491k

Cardoso, T., Dias, M. C. G. C., Dagostin, J. L. A., & Masson, M. L. (2021). Direct acidification of requeijão cremoso model by lactobionic acid: physical, chemical and antimicrobial effects. *Journal of Food Science and Technology*, *58*(2), 660–671. https://doi.org/10.1007/s13197-020-04580-6

Cardoso, T., Marques, C., Dagostin, J. L. A., & Masson, M. L. (2019). Lactobionic Acid as a Potential Food Ingredient: Recent Studies and Applications. *Journal of Food Science*, *84*(7), 1672–1681. https://doi.org/10.1111/1750-3841.14686

Cardoso, T., Marques, C., Sotiles, A. R., Dagostin, J. L. A., & Masson, M. L. (2019). Characterization of lactobionic acid evidencing its potential for food industry application. *Journal of Food Process Engineering*, *42*(7), 1–11. https://doi.org/10.1111/jfpe.13277

Chen, H., & Zhong, Q. (2017). Lactobionic acid enhances the synergistic effect of nisin and thymol against Listeria monocytogenes Scott A in tryptic soy broth and milk. *International Journal of Food Microbiology*, *260*(August), 36–41. https://doi.org/10.1016/j.ijfoodmicro.2017.08.013

Coroli, A., Romano, R., Saccani, A., Raddadi, N., Mele, E., & Mascia, L. (2021). An in-vitro evaluation of the characteristics of zein-based films for the release of lactobionic acid and the effects of oleic acid. *Polymers*, *13*(11). https://doi.org/10.3390/polym13111826

Cui, Y., & Qu, X. (2021). Genetic mechanisms of prebiotic carbohydrate metabolism in lactic acid bacteria: Emphasis on Lacticaseibacillus casei and Lacticaseibacillus paracasei as flexible, diverse and outstanding prebiotic carbohydrate starters. *Trends in Food Science and Technology*, *115*(June), 486–499. https://doi.org/10.1016/j.tifs.2021.06.058

Delgado-Fernández, P., Corzo, N., Olano, A., Hernández-Hernández, O., & Moreno, F. J. (2019). Effect of selected prebiotics on the growth of lactic acid bacteria and physicochemical properties of yoghurts. *International Dairy Journal*, *89*, 77–85. https://doi.org/10.1016/j.idairyj.2018.09.003

Fan, Q., Yuan, Y., Zhang, T., Song, W., Sheng, Q., & Yue, T. (2022). Inhibitory effects of lactobionic acid on Vibrio parahaemolyticus planktonic cells and biofilms. *Food Microbiology*, *103*(December 2021), 103963. https://doi.org/10.1016/j.fm.2021.103963

FDA. Code of Federal Regulations, Title 21, 21 CFR 172.720.US Food and Drug Administration; (2011). Retrieved from http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfCFR/CFRSearch.cfm?fr=172.720.

Fetsch, A., & Johler, S. (2018). Staphylococcus aureus as a Foodborne Pathogen. *Current Clinical Microbiology Reports*, *5*(2), 88–96. https://doi.org/10.1007/s40588-018-0094-x

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. https://doi.org/10.1111/1471-0307.12558

Goderska, K. (2019). The antioxidant and prebiotic properties of lactobionic acid. *Applied Microbiology and Biotechnology*, 3737–3751. https://doi.org/10.1007/s00253-019-09754-7

Goñi, M. G., Tomadoni, B., Moreira, M. R., & Roura, S. I. (2013). Application of tea tree and clove essential oil on late development stages of Butterhead lettuce: Impact on microbiological quality. *LWT - Food Science and Technology*, *54*(1), 107–113. https://doi.org/10.1016/j.lwt.2013.04.021

Grimoud, J., Durand, H., Courtin, C., Monsan, P., Ouarné, F., Theodorou, V., & Roques, C. (2010). In vitro screening of probiotic lactic acid bacteria and prebiotic glucooligosaccharides to select effective synbiotics. *Anaerobe*, *16*(5), 493–500. https://doi.org/10.1016/j.anaerobe.2010.07.005

Gutiérrez-Larraínzar, M., Rúa, J., Caro, I., de Castro, C., de Arriaga, D., García-Armesto, M. R., & del Valle, P. (2012). Evaluation of antimicrobial and antioxidant activities of natural phenolic compounds against foodborne pathogens and spoilage bacteria. *Food Control*, *26*(2), 555–563. https://doi.org/10.1016/j.foodcont.2012.02.025

Iyer, C., & Kailasapathy, K. (2005). Effect of co-encapsulation of probiotics with prebiotics on increasing the viability of encapsulated bacteria under in vitro acidic and bile salt conditions and in yogurt. *Journal of Food Science*, *70*(1), 1–6. https://doi.org/10.1111/j.1365-2621.2005.tb09041.x

Kang, S., Kong, F., Liang, X., Li, M., Yang, N., Cao, X., Yang, M., Tao, D., Yue, X. & Zheng, Y. (2019). Label-Free Quantitative Proteomics Reveals the Multitargeted Antibacterial Mechanisms of Lactobionic Acid against Methicillin-Resistant Staphylococcus aureus (MRSA) using SWATH-MS Technology. *Journal of Agricultural and Food Chemistry*, *67*(44), 12322–12332. https://doi.org/10.1021/acs.jafc.9b06364

Kang, S., Kong, F., Shi, X., Han, H., Li, M., Guan, B., Yang, M., Cao, X., Tao, D., Zheng, Y. & Yue, X. (2020). Antibacterial activity and mechanism of lactobionic acid against Pseudomonas fluorescens and Methicillin-resistant Staphylococcus aureus and its application on whole milk. *Food Control*, *108*(September 2019), 106876. https://doi.org/10.1016/j.foodcont.2019.106876

Kang, S., Shi, C., Chang, J., Kong, F., Li, M., Guan, B., Zhang, Z., Shi, X., Zhao, H., Peng, Y., Zheng, Y. & Yue, X. (2021). Label free-based proteomic analysis of the food spoiler Pseudomonas fluorescens response to lactobionic acid by SWATH-MS. *Food Control*, *123*(July 2020), 107834. https://doi.org/10.1016/j.foodcont.2020.107834

Kiryu, T., Kiso, T., Nakano, H., Ooe, K., Kimura, T., & Murakami, H. (2009). Involvement of Acetobacter orientalis in the production of lactobionic acid in Caucasian yogurt ("Caspian Sea yogurt") in Japan. *Journal of Dairy Science*, *92*(1), 25–34. https://doi.org/10.3168/jds.2008-1081

Kiryu, T., Yamauchi, K., Masuyama, A., Ooe, K., Kimura, T., Kiso, T., Nakano, H. & Murakami, H.(2012). Optimization of Lactobionic Acid Production by *Acetobacter orientalis* Isolated from Caucasian Fermented Milk, "Caspian Sea Yogurt." *Bioscience, Biotechnology, and Biochemistry*, *76*(2), 361–363. https://doi.org/10.1271/bbb.110608

Kiryu, Takaaki, Kiso, T., Koma, D., Tanaka, S., Nakano, H., & Murakami, H. (2016). *Biological Production of Lactobionic Acid for Food*. 137–141.

Liu, H., Cui, S. W., Chen, M., li, Y., Liang, R., Xu, F., & Zhong, F. (2019). Protective approaches and mechanisms of microencapsulation to the survival of probiotic bacteria during processing, storage and gastrointestinal digestion: A review. *Critical Reviews in Food Science and Nutrition*, *59*(17), 2863–2878. https://doi.org/10.1080/10408398.2017.1377684

Marques, C., Bortolan Toazza, C. E., Sari, R., Mitterer-Daltoé, M. L., Amaral, W. do, & Masson, M. L. (2021). Long-term storage of yacon (Smallanthus sonchifolius) juice: Phytochemical profile, in vitro prebiotic potential and discriminant bioactive properties. *Food Bioscience*, *41*(February). https://doi.org/10.1016/j.fbio.2021.100970

Marques, C., Wojeicchowski, J. P., Cardoso, T., Mafra, M. R., Mitterer-Daltoé, M. L., & Masson, M. L. (2020). Lactobionic acid as a suitable food preservative for yacon juice. *Innovative Food Science and Emerging Technologies*, *64*(January), 102400. https://doi.org/10.1016/j.ifset.2020.102400

Minal, N., Bharwade, Balakrishnan, S., Chaudhary, N. N., & Jain, A. K. (2017). Lactobionic Acid: Significance and Application in Food and Pharmaceutical. *International Journal of Fermented Foods*, *6*(1), 25. https://doi.org/10.5958/2321-712x.2017.00003.5

Murzina, E. V., Tokarev, A. V., Kordás, K., Karhu, H., Mikkola, J. P., & Murzin, D. Y. (2008). d-Lactose oxidation over gold catalysts. *Catalysis Today*, *131*(1–4), 385–392. https://doi.org/10.1016/j.cattod.2007.10.080

Oh, Y. R., Jang, Y. A., Lee, S. S., Kim, J. H., Hong, S. H., Han, J. J., & Eom, G. T. (2020). Enhancement of Lactobionic Acid Productivity by Homologous Expression of Quinoprotein Glucose Dehydrogenase in Pseudomonas taetrolens. *Journal of Agricultural and Food Chemistry*, *68*(44), 12336–12344. https://doi.org/10.1021/acs.jafc.0c04246

Pleissner, D., Dietz, D., van Duuren, J. B. J. H., Wittmann, C., Yang, X., Lin, C. S. K., & Venus, J. (2019). Biotechnological production of organic acids from renewable resources. *Advances in Biochemical Engineering/Biotechnology*, *166*, 373–410. https://doi.org/10.1007/10_2016_73

Rivera, D., Toledo, V., Reyes-Jara, A., Navarrete, P., Tamplin, M., Kimura, B., Wiedmann, M., Silva, P. & Moreno Switt, A. I. (2018). Approaches to empower the implementation of new tools to detect and prevent foodborne pathogens in food processing. *Food Microbiology*, *75*, 126–132. https://doi.org/10.1016/j.fm.2017.07.009

Saarela, M., Hallamaa, K., Mattila-Sandholm, T., & Mättö, J. (2003). The effect of lactose derivatives lactulose, lactitol and lactobionic acid on the functional and technological properties of potentially probiotic Lactobacillus strains. *International Dairy Journal*, *13*(4), 291–302. https://doi.org/10.1016/S0958-6946(02)00158-9

Sáez-Orviz, S., Camilleri, P., Marcet, I., Rendueles, M., & Díaz, M. (2019). Microencapsulation of Calcium Lactobionate for Protection From Microorganisms in a Solid Phase Food. *Biochemical Engineering Journal*, *150*(June), 107281. https://doi.org/10.1016/j.bej.2019.107281

Sáez-Orviz, S., Marcet, I., Rendueles, M., & Díaz, M. (2021). Bioactive packaging based on delipidated egg yolk protein edible films with lactobionic acid and Lactobacillus plantarum CECT 9567: Characterization and use as coating in a food model. *Food Hydrocolloids*, *119*(January). https://doi.org/10.1016/j.foodhyd.2021.106849

Sáez-Orviz, S., Passannanti, F., Gallo, M., Cante, R. C., Nigro, F., Budelli, A. L., Rendueles, M., Nigro, R. & Díaz, M. (2021). Lactic Acid Bacteria Co-Encapsulated with Lactobionic Acid : Probiotic Viability during In Vitro Digestion. *Appl. Sci.*, *11*(11404). https://doi.org/https://doi.org/ 10.3390/app112311404

Sáez-Orviz, S., Puertas, C., Marcet, I., Rendueles, M., & Díaz, M. (2020). Bioactive synbiotic coatings with lactobionic acid and Lactobacillus plantarum CECT 9567 in the production and characterization of a new functional dairy product. *Journal of Functional Foods*, *75*(July). https://doi.org/10.1016/j.jff.2020.104263

Sarenkova, I., & Ciprovica, I. (2018). The current status and future perspectives of lactobionic acid production: A review. *Research for Rural Development*, *1*, 233–239. https://doi.org/10.22616/rrd.24.2018.037

Schaafsma, G. (2008). Lactose and lactose derivatives as bioactive ingredients in human nutrition. *International Dairy Journal*, *18*(5), 458–465. https://doi.org/10.1016/j.idairyj.2007.11.013

Song, A. X., Mao, Y. H., Siu, K. C., Tai, W. C. S., & Wu, J. Y. (2019). Protective effects of exopolysaccharide of a medicinal fungus on probiotic bacteria during cold storage and simulated gastrointestinal conditions. *International Journal of Biological Macromolecules*, *133*, 957–963. https://doi.org/10.1016/j.ijbiomac.2019.04.108

Van Dokkum W, Wezendonk LJW, van Aken-Schneider P, K. C. (1994). Tolerance of lactobionic acid in man. TNO Report. *The Netherlands: TNO Nutri- Tion and Food Research*.

Wojciechowska, A., Klewicki, R., & Klewicka, E. (2020). The potential of new bionic acids as prebiotics and antimicrobials. *Lwt*, *125*(November 2019). https://doi.org/10.1016/j.lwt.2020.109246

World Health Organization. (2015). *WHO Estimates of the Global Burden of Food- borne Diseases*. *46*, 1–15.

Xu, C., Li, J., Yang, L., Shi, F., Yang, L., & Ye, M. (2017). Antibacterial activity and a membrane damage mechanism of Lachnum YM30 melanin against Vibrio parahaemolyticus and Staphylococcus aureus. *Food Control*, *73*, 1445–1451. https://doi.org/10.1016/j.foodcont.2016.10.048

2.2. Impacto de la incorporación de prebióticos y probióticos en las características de *films* y *coatings* de uso alimentario

En este apartado se hace una revisión bibliográfica sobre la influencia en diferentes parámetros fisicoquímicos y mecánicos que tiene la adición de probióticos y prebióticos en la formulación de films y coatings de uso alimentario. Ambos recubrimientos se diferencian en que los films deben ser secados previamente antes de su uso en alimentos, mientras que los coatings se aplican como materiales fluidos o geles sobre los productos alimentarios (Hellebois, Tsevdou, & Soukoulis, 2020).

En esta revisión, se describen los diferentes tipos de biopolímeros que se pueden emplear en la elaboración de estos dos recubrimientos de uso alimentario (proteínas, polisacáridos, lípidos o una mezcla de ellos). Asimismo, se describen los requisitos que deben cumplir los microorganismos probióticos y compuestos prebióticos para definirse como tal. También se recogen las principales especies de microorganismos probióticos que se usan de manera habitual en el ámbito alimentario y ejemplos de materiales bioactivos con probióticos desarrollados en los últimos cinco años. Por otro lado, también se describen los principales compuestos prebióticos (galactanos, fructanos y lactulosa). La revisión detalla además el mecanismo de formación de films y coatings y recoge los diferentes métodos de producción para ambos tipos de recubrimiento (solving casting y extrusión para el caso de los films e inmersión y pulverización para el caso de los coatings). Además, se hace hincapié en los diferentes factores que hay que tener en cuenta durante el proceso de producción al añadir prebióticos y probióticos, siendo especialmente importante en el caso de los probióticos ya que se puede ver comprometida su viabilidad. Por último, se explican la diferentes interacciones covalentes y no covalentes que tienen lugar dentro de las matrices una vez formados los films y coatings. Además, se detalla la influencia que tiene la presencia de probióticos y prebióticos en esas interacciones, ya que las alteraciones que se producen a nivel molecular ocasionan cambios en las diferentes propiedades físicas y mecánicas de los materiales desarrollados. Finalmente, se resumen los aspectos relacionados con la regulación y comercialización de los materiales alimentarios bioactivos.

- **Publicación:** Sáez-Orviz, S., Rendueles, M., Díaz, M. Impact of adding prebiotics and probiotics on the characteristics of edible films and coatings- a review.
- **Estado:** enviada para su evaluación en la revista *Trends in Food Science and Technology*.

Molecular interactions and production methods of edible films and coatings: impact of adding probiotics and prebiotics- a review

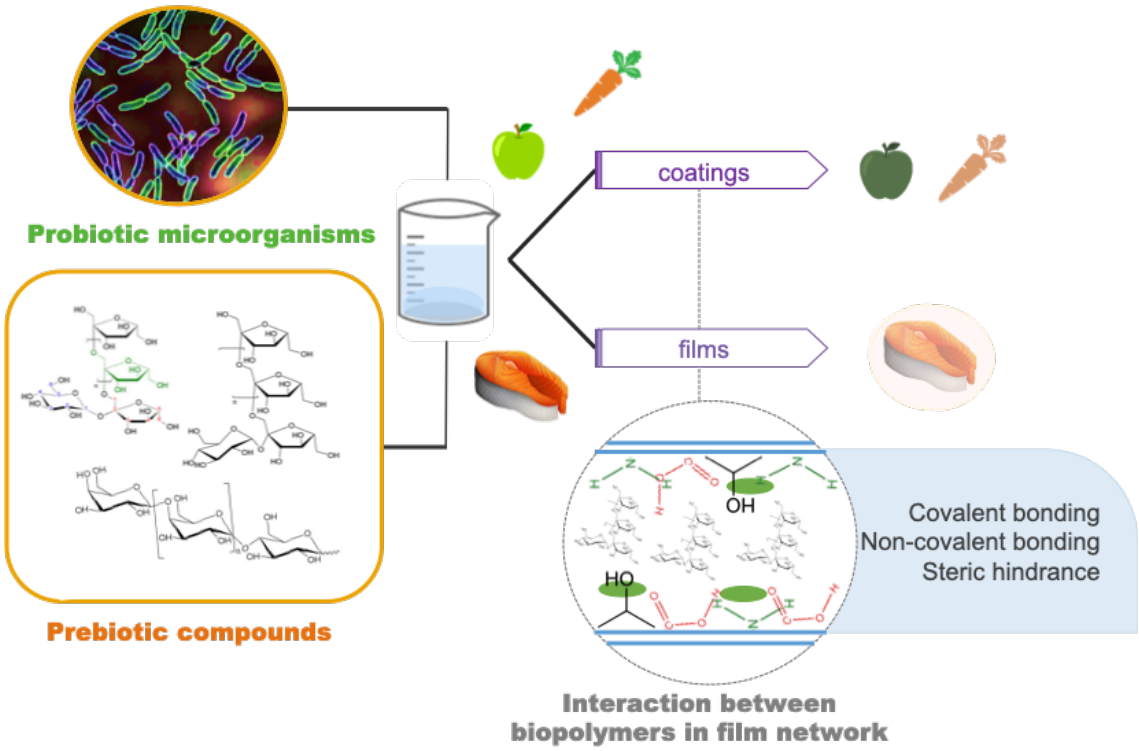
S. Sáez-Orviz [a], M. Rendueles [a*], M. Díaz [a]

[a]Department of Chemical and Environmental Engineering, University of Oviedo, C/Julián Clavería 8, 33006, Oviedo, Spain.

[*]Corresponding author

E-mail address: mrenduel@uniovi.es (M. Rendueles)

Graphical abstract



Abstract

Nowadays, conventional packaging materials made using non-renewable sources are being replaced by other more sustainable alternatives such as natural biopolymers (proteins, polysaccharides, and lipids). Within edible packaging, one can differentiate between edible films (as dry covering materials) or edible coatings (as fluid liquids or gels covering the surface of foodstuff). This edible packaging can be additivated with bioactive compounds to develop edible and functional food packaging, which allow to

improve the state of health and/or the risk of disease. Among the active compounds that can be added to edible packaging are probiotics and prebiotics. The most used probiotics in edible packaging belong to the *Lactobacillus* and *Bifidobacterium* genera. The most common prebiotics belong to the galactans, fructans and lactulose groups. There are different production methods for films (wet and dry processes) and for coatings (dipping and spraying). When bioactive compounds such as probiotics and prebiotics are added, different factors must be considered to select the most suitable production processes. Specially as probiotics are living microorganisms, they are more sensitive to physical and chemical factors. The interactions that take place inside the matrix (covalent and non-covalent bindings) change depending on the biopolymers used and due to the presence of probiotic bacteria and prebiotics. Having a better knowledge of the influence of these compounds on the interactions that occur within the films and coatings will allow to a better control on their physical and mechanical properties and to a better understanding of the behaviour of edible packaging additivated with probiotics and prebiotics.

Keywords: packaging, edible films, edible coatings, probiotic, prebiotic, preparation method, biopolymers interactions

Abbreviations			
EFSA	European Food Safety Authority	IMO	Isomalto-oligosaccharides
EU	European Union	LAB	Lactic acid bacteria
FDA	Food and drug administration	QPS	Qualified presumption of safety
FOS	Fructo-oligosaccharides	SEM	Scanning electron microscopy
FTIR	Fourier transform infrared	SOS	Soya-oligosaccharides
GIT	Gastrointestinal tract	WVP	Water vapour permeability
GOS	Galacto-oligosaccharides	WVTR	Water vapour transmission rate
GRAS	Generally recognized as safe	XOS	Xylo-oligosaccharides

1. Introduction

Packaging is an ancient technology, that has been used since past times to prevent or delay the deterioration of food products (Ribeiro, Estevinho, & Rocha, 2021). Packaging is a key point to consider during the storage and transport of food products as there are many physical, chemical and microbiological factors that can affect the stability of foodstuff and compromise their quality (Jeya Jeevahan et al., 2020) (Ribeiro et al., 2021).

The most used materials in food packaging are paper or paperboard, metal, or glass, but plastic is the preferred type of packaging. Plastic is a cheap, lightweight and very versatile material (Jeya Jeevahan et al., 2020). Nevertheless, this material has serious environmental drawbacks, as it is non-biodegradable and comes from a non-renewable source (Parreidt, Müller, & Schmid, 2018). Thus, during the development of this type of packaging various emissions (such as carbon monoxide, hydrochloric acid, amines, benzenes, etc. (Amin et al., 2021)) are caused. These emissions, besides to environmental issues, also causes health problems (Mangaraj, Yadav, Bal, Dash, & Mahanti, 2019). It is for these reasons that nowadays conventional packaging materials derived from petroleum and other non-renewable sources are being replace by more sustainable alternatives.

Nowadays consumers are demanding minimally processed food. The use of edible packaging can help to extend their shelf life and improve the quality of food products. In this context, the use of biopolymers of natural and renewable origin is drawing attention (Parreidt et al., 2018). In this sense, proteins, polysaccharides, and lipids have been used as primary material for the development of edible biodegradable packaging. All these biopolymers can be obtained from animal or vegetable sources. Edible biodegradable packaging have been categorized as films and coatings (Amin et al., 2021), being its main difference the form of application in the food products (Parreidt et al., 2018). Edible films and coatings are very versatile and have the potential to include bioactive compounds in their matrix. This is one way in which value can be added to the packaging market since food products can be transformed into “functional” foodstuff. Functional food can be defined as “the one that beneficially affects one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease” (Ashwell, 2002). Among the active compounds that can be added are probiotics (Espitia, Batista, Azeredo, & Otoni, 2016) and prebiotics. (Paulo, Baú, Ida, & Shirai, 2021).

Regarding probiotics, they are living microorganisms with beneficial effects on humans and animals (Food and Agriculture Organization (FAO) of the United Nations, 2002) (Espitia et al., 2016). The most common probiotics employed in food products belong to the genera *Lactobacillus* and *Bifidobacterium* (Espitia et al., 2016). Probiotic bacteria have been used since ancient times to fermented food products, such as yogurt, cheese, dry cured meat, fermented vegetables, etc. (Hellebois, Tsevdou, & Soukoulis, 2020). Traditionally, probiotics are mostly employed in dairy food matrices, but their

inclusion in other food matrix such as fruits, meat, cereals or bakery products, have been currently studied (Fernandes, Leonardo M. Guimaraes Jonas T., Pimentel Tatiana C., Esmerino Erick A., Freitas Mônika Q., Carvalho Carlos Wanderlei P., 2020). For probiotics to be effective, they must reach alive and in an adequate amount to the gastrointestinal tract (GIT) (Zendeboodi, Khorshidian, Mortazavian, & da Cruz, 2020). Their inclusion in edible films and coatings is a way to increase their viability and survival during food production processes, since probiotics are living microorganisms and there are several physical and chemical factors that can minimize their survival (Mbye et al., 2020).

As for prebiotics, they comprise a wide range of compounds of both animal and vegetal origin. These compounds have been commonly used directly in products such as beverages, bakery products or dairy products (Paulo et al., 2021). However, during production processes, its functionality can be compromised due to the presence of very high or very low temperatures, changes in pH and Maillard reactions (Neri-Numa & Pastore, 2020). Thus, edible films and coatings have the capacity to protect them. In addition, several studies have noticed that when probiotic bacteria are combined with prebiotic compounds, their viability is improved, both during the food production process and during storage and even in *in vitro* digestion tests (Espitia et al., 2016). The combination of probiotics and prebiotics in the same food product allows obtaining synbiotic foodstuff (Hellebois et al., 2020).

When selecting the production methods for edible films and coatings, it is necessary to bear in mind what parameters can affect the viability of probiotics (Mbye et al., 2020). Besides, the addition of prebiotics and probiotics leads to changes in the molecular interactions between the components of the biopolymeric matrix that will produce structural changes, affecting the physical and mechanical properties of the edible films and coatings developed.

Therefore, this review aims to summarize the natural biopolymers that can be used to develop edible films and coatings, as well as the probiotics and prebiotics commonly used in this type of packaging. In addition, the advantages and disadvantages of the different production methods and the key factors to consider when adding probiotics and prebiotics will be analysed. This review also highlights the molecular interactions that take place inside the packaging matrix, how they are affected by the presence of probiotics and prebiotics and the changes in physical and mechanical properties that occur in edible films and coatings. Finally, the aspects in the regulation and commercialization of this type of packaging will be discussed.

2. Use of biopolymers as matrix in edible packaging

Nowadays, the use of conventional petroleum-based and synthetic plastics is being replaced, at least partially, by environmentally friendly alternatives (Rojas-Lema et al., 2021) (Liminana, Garcia-Sanoguera, Quiles-Carrillo, Balart, & Montanes, 2018). In this context, edible packaging using biodegradable polymers as matrix has emerged. The main biopolymers used in the development of edible packaging are proteins, polysaccharides, and lipids. In addition, these compounds can be obtained from different sources, both animal and vegetable. Thus, these biopolymers can also be obtained from by-products of other industries, such as agro-industrial and marine wastes, which would make it possible to reuse and take advantage of these by-products in terms of circular economy (Ribeiro et al., 2021) (Chiralt, Menzel, Hernandez-García, Collazo, & Gonzalez-Martinez, 2020).

2.1. Proteins

Proteins come from different sources of animal and vegetable origin. In addition, proteins can be obtained from by-products of different agro-industries. Some of the most common proteins used in the preparation of edible packaging are whey protein, casein, corn zein, gelatine, soy protein and wheat gluten (Amin et al., 2021) (Zoghi, Khosravi-Darani, & Mohammadi, 2020). Proteins have hydrophobic and hydrophilic parts, which allows them to be used in combination with different bioactive compounds (Calva-Estrada, Jiménez-Fernández, & Lugo-Cervantes, 2019) (Hassan, Chatha, Hussain, Zia, & Akhtar, 2018). To form the films or coatings, the proteins must undergo a denaturation process so that the interaction between molecules can take place to form a cohesive film or coating (Calva-Estrada et al., 2019). This denaturation can be carried out through changes in pH (using acids and/or bases), the use of solvents or by applying heat. Other processes that can be employed include irradiation, chemical crosslinking or the use of enzymes such as transglutaminase (W. Chen et al., 2021). The final characteristics of the films and coatings developed depend to a large extent on the type of protein they are made of, since properties such as molecular weight, conformation, charge, flexibility, and thermal stability will be different (Koshy, Mary, Thomas, & Pothan, 2015). Packaging made with protein as a matrix usually has very good mechanical and barrier properties, blocking the release of aromas and volatile compounds (Calva-Estrada et al., 2019) (Ribeiro et al., 2021). However, these films present a major problem, since they are usually very soluble in water, which makes their use in the food industry very difficult, since most foods contain a high percentage of water (Gopalakrishnan, Xu, Zhong, & Rotello, 2021).

2.2. Polysaccharides

As in the case of proteins, polysaccharides can be obtained from animal and vegetable sources. The most studied polysaccharides to develop films and coatings are cellulose (and its derivatives), chitosan, starch, pectin, alginate, carrageenan, pullulan and kefiran (Cazón, Velazquez, Ramírez, & Vázquez, 2017) (Amin et al., 2021) (Hassan et al., 2018) (W. Chen et al., 2021). Packaging made from these polymers is colourless, tough, transparent, and elastic, with good mechanical properties (Ribeiro et al., 2021) (Amin et al., 2021). In general, packaging made from polysaccharides has good barrier properties against the transfer of gases such as O_2 and CO_2, and also of volatile compounds such as aromas or organic vapours (Cazón et al., 2017) (Vieira, Da Silva, Dos Santos, & Beppu, 2011). This is due to the tightly-packed hydrophilic polymer network in its matrix (Ribeiro et al., 2021) (Corbo, Campaniello, Speranza, Bevilacqua, & Sinigaglia, 2015). However, these materials are quite hydrophilic, which makes them have poor water vapor barrier properties (Cazón et al., 2017) (Hellebois et al., 2020). Some of them, such as alginate and carrageenan, are very hygroscopic, so they can protect the moisture loss of certain foods before the films or coatings dehydrate (Cazón et al., 2017) (Hassan et al., 2018).

2.3. Lipids

Lipids come from different sources, such as animal and vegetable native oils and fats (peanut, coconut, cocoa, milk butters, jojoba bees, etc.) (Barbosa, Andrade, Vilarinho, Fernando, & Silva, 2021) (Debeaufort & Voilley, 2009). They are generally small hydrophobic molecules. The lipids that can be incorporated include triglycerides, acetylated monoglycerides, fatty acids, waxes and oils (Aguirre-Joya, J.A.; Álvarez, B.; Ventura, J.M.; García-Galindo, J.O.; De León-Zapata, M.A.; Rojas, R.; Sauceso, S.; Aguilar, 2016) (Amin et al., 2021). However, the most common lipids are fatty acids between 14 and 18 carbons, fatty alcohols and hydrogenated and non-hydrogenated vegetable oils (Milovmovic, 2001). The main characteristic of packaging developed using lipids as a matrix is that they are very effective reducing water vapour permeability due to their apolar nature (Debeaufort & Voilley, 2009) and can provide an attractive and shiny character on the surface of certain foods, such as fruits (W. Chen et al., 2021). In the specific case of fatty acids, it was observed that the barrier properties increased when the carbon chains had a length between 12 to 18, but from 18 onwards these properties decreased (Milovmovic, 2001). Films and coatings developed exclusively with lipids show low mechanical strength, organoleptic quality reduction (rancidity) and low transparency (Barbosa et al., 2021) (Amin et al., 2021). Thus, the adhesion to foods

that have hydrophilic surfaces is weak (Falguera, Quintero, Jiménez, Muñoz, & Ibarz, 2011) (Yousuf, Sun, & Wu, 2021). Lipids are usually added as an extra layer or in the form of an emulsion to packaging made with other matrices, such as proteins and polysaccharides (Aguirre-Joya, J.A.; Álvarez, B.; Ventura, J.M.; García-Galindo, J.O.; De León-Zapata, M.A.; Rojas, R.; Sauceso, S.; Aguilar, 2016), since films and coatings made exclusively with lipids have very limited applications (Yousuf et al., 2021). These combinations are known as composites.

2.4. Composites

Composites consist of the combination of different biopolymers with the objective of taking advantage of the different functional characteristics of each compound while minimizing the disadvantages of each one of them (Ribeiro et al., 2021). In general, a combination of layers of hydrophilic biopolymers (proteins and polysaccharides) with hydrophobic biopolymers (lipids) is produced (Vargas, Pastor, Chiralt, McClements, & González-Martínez, 2008). The addition of a lipid layer improve the water vapour resistance and proteins/polysaccharides layers provide integrity, structural cohesion and selective permeability to O_2 and CO_2 (Vargas et al., 2008). In this type of packaging, the method of preparation and the compatibility between the different types of biopolymers is also important. Composites have been successfully used in different types of foodstuff such as fruits (N. Kumar et al., 2021) (Pinzon et al., 2020).

2.5. Additives

In addition to the biopolymers necessary to form the matrix of the edible films and coatings, other food grade additives can also be added, such as plasticizers, antioxidants, antimicrobials, antifungals, surfactants, natural pigments, etc. (Parreidt et al., 2018) (Hellebois et al., 2020).

Plasticizers are commonly needed to develop edible packaging. They are low molecular weight and non-volatile compounds that are miscible with the polymer matrix. Thus, the majority are hydrophilic and hygroscopic (Suhag, Kumar, Petkoska, & Upadhyay, 2020). Plasticizers are used to improve the characteristics of edible packaging, such as flexibility, or thermoplasticity (Hellebois et al., 2020). Besides, they can reduce brittleness and improve its strength. The most common and effective plasticizer in hydrophilic matrix is water (Parreidt et al., 2018), due to its ability to interact with the biopolymers of the matrix (Hellebois et al., 2020). Other plasticizers are sorbitol, glycerine, glycol, polyethylene glycol, glycerol, and sucrose, among others (Suhag et al., 2020) (S. Kumar et al. 2014). The addition of hydrophobic plasticizers generally

makes it possible to improve the water vapor permeability (WVP) (Jost, Kobsik, Schmid, & Noller, 2014). Therefore, the addition of plasticizers allows the characteristics of edible packaging to be modified. Among the factors that must be considered are their nature and the proportion that is added.

Other common additives can be added with the aim to develop bioactive edible packaging. This type of packaging can be defined as the one that protect foodstuff not only in a conventional way but minimizing undesirable interactions with the food product with the aim to extend their shelf-life (Espitia et al., 2016). Among these additives there is a wide variety of antimicrobial (nisin (Xiong, Chen, Warner, & Fang, 2020) (Venkatachalam & Lekjing, 2020), essential oils (Çakmak, Özselek, Turan, Fıratlıgil, & Karbancioğlu-Güler, 2020) (Socaciu et al., 2020), etc.), antifungal (Aguilar-Sánchez et al., 2019) (Robledo et al., 2018) and antioxidant compounds (Rodríguez, Sibaja, Espitia, & Otoni, 2020) (Dou, Li, Zhang, Chu, & Hou, 2018) (Moghadam, Salami, Mohammadian, Khodadadi, & Emam-Djomeh, 2020).

3. Probiotics and prebiotics in edible films and coatings

3.1. Probiotics

In 2002, the WHO and FAO defined probiotics as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (Food and Agriculture Organization (FAO) of the United Nations, 2002). This definition is accepted by the majority of the research community. In recent years certain nuances have been added depending on their functionality, so they can be re-defined as true probiotics (when microorganisms are viable and active), pseudoprobiotics (if they are viable but inactive) and ghost probiotics (when they are not viable). (Zendeboodi et al., 2020). Among the characteristics that microorganisms must have to be considered probiotics, taking into account their relationship with the host, are (i) being resistant to gastric acids and bile acids, (ii) being able to adhere to mucus and/or human epithelial cells, (iii) having antimicrobial activity against potentially pathogenic bacteria, (iv) having the ability to reduce the adhesion of these pathogens to surfaces and (v) having bile salt hydrolase activity (Food and Agriculture Organization (FAO) of the United Nations, 2002). Besides, probiotic effects are strain specific (Espitia et al., 2016). Some of the common beneficial effects on the host are an improvement in the response of the immune system of the GIT, prevention against infections and strengthening of the intestinal barrier, colonization resistance, regulation of the intestinal transit, among others (Zendeboodi et al., 2020) (Espitia et al., 2016) (Hellebois et al., 2020). The probiotics commonly used in the food industry belong to the group of lactic acid

bacteria (LAB) (Zendeboodi et al., 2020) (Guimarães, Abrunhosa, Pastrana, & Cerqueira, 2018). LAB have been used since ancient times in the production of fermented dairy products, such as cheeses and yoghurts. Besides, many probiotic microorganisms are native to the oral cavities and the GIT (Zendeboodi et al., 2020). LAB have the category of "qualified presumption of safety" (QPS) supplied by the EFSA (European Food Safety Authority, European Union (EU) (Barlow et al., 2007)) or "generally recognized as safe" (GRAS) given by the FDA (Food and Drug Administration, USA (*FDA.*, 2016)), allowing its use in food products. The most common probiotics used in edible films and coatings and the latest research in which they have been employed are shown in Table 2.4.

When probiotics are added to food matrices or edible films or coatings, they must be able to survive after passing through the digestive tract, where they will proliferate. Probiotics are only effective if the dosage is sufficiently high. Although there is no scientific consensus on the concentration required to obtain beneficial health effects, some researchers have suggested the amount between 10^6 and 10^9 CFU per day, but these bacteria have to reach the low GIT (Espitia et al., 2016) (Saad, Delattre, Urdaci, Schmitter, & Bressollier, 2013). One of the advantages of adding probiotics to edible films and coatings is that it can help them survive the stomach acids and bile salts, increasing their survival. This phenomenon has been observed in numerous investigations in which *in vitro* digestion tests have been carried out (Alvarez et al., 2021) (Sáez-Orviz, Marcet, Rendueles & Díaz, 2021) (Sáez-Orviz et al., 2020b) (Soukoulis et al., 2014) (Valerio et al., 2020). In addition to the packaging in which the probiotics are embedded, the composition of the food matrix they cover is also important. Although probiotics are used especially and mainly in dairy products such as yoghurts, fermented milks, ice creams and cheeses (Gao et al., 2021), when they are added to the formulation of edible films and coatings they are used in numerous other food products. Among these products stand out fruits (Bambace et al., 2019) (Monteiro et al., 2022) (Wong et al., 2021), vegetables (Shigematsu et al., 2018) (Dianin et al., 2019), dairy (El-Sayed et al., 2021) (Sáez-Orviz et al., 2020b) (Angiolillo, Conte, Faccia, Zambrini, & Nobile, 2014), bakery (Soukoulis et al., 2014) (Gregirchak et al., 2020), meat (Pavli et al., 2017) (Odila Pereira et al., 2018) and fish products (Mozaffarzogh et al., 2020) (López de Lacey, López-Caballero, & Montero, 2014). The interactions between the matrices, the packaging and the probiotics are also decisive in their viability and must be considered. Another factor that influences the viability of probiotics is the presence of other compounds such as prebiotics.

Table 2.4. Common probiotics used in edible films and coatings and the 5 last year's research the latest research in which they have been employed.

Probiotic genus	Probiotic species	Type of edible packaging to which they have been added	Concentration used	Reference
Lactobacillus	*L. casei*	Edible biofilms based on whey protein isolate	0.5% (*w/v*)	(Dianin, Oliveira, Pimentel, Hernandes, & Costa, 2019)
		Edible coating based on chitosan, alginate and carboxymethyl cellulose	> 10^7 CFU/mL	(El-Sayed, El-Sayed, Mabrouk, Nawwar, & Youssef, 2021)
		Edible coating based on whey protein isolates	10^9 CFU/mL	(Odila Pereira, Soares, J.P. Monteiro, Gomes, & Pintado, 2018)
		Edible films based on carboxymethyl cellulose-sodium caseinate	10^9 CFU/mL	(Mozaffarzogh, Misaghi, Shahbazi, & Kamkar, 2020)
		Edible films based on suck feet gelatine	~ 10^{10} CFU/g film-forming solution-dry basis	(Abedinia et al., 2021)
		Edible films based on citrus pectin	10^9-10^{10} CFU/mL	(Nisar et al., 2022)
	L. rhamnosus	Alginate-based coatings	10^9 CFU/mL	(Bambace, Alvarez, & Moreira, 2019)
		Edible films based on carboxymethyl cellulose-sodium caseinate	10^9 CFU/mL	(Mozaffarzogh et al., 2020)
		Edible films based on citrus pectin	10^9-10^{10} CFU/mL	(Nisar et al., 2022)
	L. salivarius	Edible coating based on gelatine additivated with inulin	10^{10} CFU/mL	(Monteiro et al., 2022)
		Spray-coating based on milk powder and sucrose	10^8 CFU/mL	(Wang, Lin, & Zhong, 2021)
	L. plantarum	Bilayer edible coating containing carboxymethyl cellulose in the primary coating and zein in the secondary	8.78 ± 0.10 log CFU/ mL	(Wong, Mak, & Li, 2021)
		Edible film based on delipidated egg yolk protein	10^8 CFU/mL	(Sáez-Orviz, S., Marcet, I., Rendueles, M., Díaz, 2021)
		Edible coatings based on sodium alginate and prebiotics	10^9 CFU/mL	(Sáez-Orviz, Puertas, Marcet, Rendueles, & Díaz, 2020b)
		Edible films based on alginate	10^9 CFU/mL	(Pavli et al., 2017)
		Nanocomposite film based on whey protein isolate and polydextrose	10^9 CFU/mL	(Karimi, Alizadeh, Almasi, & Hanifian, 2020)

		Nanocomposite film based on carboxymethyl cellulose and inulin	~ 10^9 CFU/mL	(Zabihollahi, Alizadeh, Almasi, Hanifian, & Hamishekar, 2020)
	L. acidophilus	Edible coating based on chitosan, alginate and carboxymethyl cellulose	> 10^7 CFU/mL	(El-Sayed et al., 2021)
		Edible films based on carboxymethyl cellulose-sodium caseinate	10^9 CFU/mL	(Mozaffarzogh et al., 2020)
		Edible films based on citrus pectin	10^9-10^{10} CFU/mL	(Nisar et al., 2022)
		Edible coating based on sodium alginate	7.36 log CFU/g	(Shigematsu et al., 2018)
		Edible coatings based on sodium alginate, whey, and glycerol	90 mg of dry bacteria/ 10mL of film-forming solution	(Gregirchak, Stabnikova, & Stabnikov, 2020)
	L. pentosus	Edible films based on alginate	10^9 CFU/mL	(Pavli et al., 2017)
		Emulsion film based on gelatine/polydextrose/camellia oil	10^9 CFU/mL	(Zong et al., 2021)
	L. reuteri	Edible films based on carboxymethyl cellulose-sodium caseinate	10^9 CFU/mL	(Mozaffarzogh et al., 2020)
	L. paracasei	Hydrogels of soy protein isolate and sugar beet pectin	10^{10} CFU/mL	(Yan et al., 2021)
		Edible films based on chitosan and *Aloe vera*	2.8 x 10^9 CFU/mL	(Barragán-Menéndez et al., 2020)
		Edible coating based on pectin	1.5 x 10^9 CFU/g of coated apple	(Valerio et al., 2020)
Bifidobacterium	*B. lactis*	Edible coating based on chitosan, alginate and carboxymethyl cellulose	> 10^7 CFU/mL	(El-Sayed et al., 2021)
		Edible coatings based on alginate, glycerol, inulin and oligofructose	5 x 10^{11} CFU/mL	(Alvarez, Bambace, Quintana, Gomez-Zavaglia, & Moreira, 2021)
	B. animalis	Edible coating based on whey protein isolates	10^9 CFU/mL	(Odila Pereira et al., 2018)
		Edible films based on alginate or whey protein and prebiotics	10^9 CFU/mL	(Pereira et al., 2019)
	B. bifidum	Edible films based on carboxymethyl cellulose-sodium caseinate	10^9 CFU/mL	(Mozaffarzogh et al., 2020)
		Edible films based on citrus pectin	10^9-10^{10} CFU/mL	(Nisar et al., 2022)

3.2. Prebiotics

The concept of a prebiotic was firstly introduced by Gibson and Roberfroid in 1995. They defined a prebiotic as a "non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health" (Gibson & Roberfroid, 1995). The most current definition of a prebiotic is very similar, as it is defined as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (Gibson et al., 2017). Some of the benefits conferred by prebiotics are the stimulation of the immune system of the GIT, the inhibition of the growth of pathogens in the GIT and the reduction of blood lipids, among others (Gibson et al., 2017).

For a compound to be considered a prebiotic, it must meet various requirements. These include (i) the resistance to gastric acids and host enzymes, (ii) to be selectively fermented by large intestinal microbiota, and (iii) to have selective effect on the microbiota resulting on health-promoting effects on the host (Gibson et al., 2017) (Paulo et al., 2021). The dietary prebiotics most extensively documented to have health benefits in humans and are considered as prebiotics are galactans (such as galacto-oligosaccharides (GOS)), fructans (such as fructo-oligosaccharides (FOS) and inulin) and lactulose (Gibson et al., 2017) (Paulo et al., 2021). Other compounds have also been considered prebiotics, such as xylo-oligosaccharides (XOS), isomalto-oligosaccharides (IMO), soya-oligosaccharides (SOS), pyrodextrins, dietary fibers, resistant starches, and other non-digestible oligosaccharides (FAO, 2008), although there is still no scientific consensus to add them to the prebiotic category (Bindels, Delzenne, Cani, & Walter, 2015). Prebiotics can be manufactured from different sources. They can be obtained through plant materials, such as soybeans, fruits, cereals, asparagus, garlic, onions, etc. (Hurtado-Romero, Del Toro-Barbosa, Garcia-Amezquita, & García-Cayuela, 2020) and they can also be obtained by chemical or enzymatic synthesis and through the enzymatic hydrolysis of polysaccharides (Figueroa-González, Quijano, Ramírez, & Cruz-Guerrero, 2011) (Paulo et al., 2021).

The combination of prebiotics and probiotics is known as "synbiotics". Both compounds together have a synergistic behavior (Figueroa-González et al., 2011). The presence of prebiotics in edible films and coatings allows the viability of probiotics to be improved. Several authors have observed its effectiveness in maintaining viability during storage. Bambace et al. (2019)) observed an improvement in the viability of *L. rhamnosus* CECT 8361 when inulin and FOS (80 g of each prebiotic/ kg of solution) was added to the sodium alginate coating, with counts above 6.2 log CFU/g during 21 days

of analysis. Zabihollahi et al. (2020) developed a carboxymethyl cellulose (CMC) film additivated with *L. platarum* ATCC® 14917™ and inulin (10 and 20 g/100 g CMC). The presence of the prebiotic meaningfully increased the viable cell numbers of the probiotic with no difference between the concentration of inulin employed. Similar results were obtained by Pereira et al. 2019. These authors developed edible films based on alginate and whey protein with *B. lactis* BB-12 and different prebiotics (inulin and FOS). The viability improved with the presence of both prebiotics, but the best result was obtained with inulin (2%, *w/v*), which maintained the viability of the probiotic above 7 log CFU/g during 60 days of storage at 23 ºC. Besides, the presence of prebiotics in edible films and coating also improves the viability during *in vitro* digestion tests. Recently, Orozco-Parra *et al.* (2020) developed an edible film based on cassava starch additivated with inulin (0.5%, *w/v*) and *L. casei*. They observed that inulin was capable of reduce the loss of viability of the probiotic in an *in vitro* digestion test. Similar results were obtained by Sáez-Orviz *et al.* (2020b). These authors developed sodium alginate coatings additivated with *L. plantarum* CECT 9567 and lactobionic acid (20 and 40 g/L) as prebiotic. Results showed that the presence of prebiotic increased about 11% the survival of probiotic after the simulated *in vitro* digestion test. Therefore, synbiotics are a very promising area for the development of new packaging and functional foods.

4. Formation mechanisms of edible films and coatings

There are three different mechanisms of edible film formation. In the case of simple coacervation, the phase change or the precipitation of the hydrocolloid can occur through three different phenomena: (i) the solvent evaporation process, (ii) the incorporation of a non-electrolyte compound (in which the hydrocolloid is not soluble) or (iii) the incorporation of an electrolyte compound (Guilbert, Gontard, & Gorris, 1996) (Parreidt et al., 2018). In the latter case, the electrolyte can be obtained from the pH adjustment of the film-forming solution, which promotes cross-linking (Ribeiro et al., 2021). These three phenomena lead to an increase in the concentration of the biopolymer, resulting in molecular aggregation and the formation of a three-dimensional network (Khwaldia, Ferez, Banon, Desobry, & Hardy, 2004). In the complex coacervation, the interaction or precipitation occurs when two hydrocolloid solutions with opposite electron charges are mixed (Guilbert et al., 1996). Although there are different parameters that can be modified on in the process, such as pH, temperature or concentration of the biopolymers (Warnakulasuriya & Nickerson, 2018), the most important is the charge density (Schmitt, Sanchez, Desobry-Banon, & Hardy, 1998). The last mechanism is the gelation or thermal coagulation. In this case, interaction or precipitation of the

hydrocolloid is achieved by heating the forming-solution causing the denaturation, gelification and or precipitation of the compound (as occurs when denaturing certain proteins such as ovalbumin (Guilbert et al., 1996) or soy protein (Khwaldia et al., 2004)) or by rapid cooling the forming-solution, resulting in a gelling phenomenon (as occurs with agar or gelatine) (Parreidt et al., 2018) (Umaraw & Verma, 2017).

5. Edible films and coatings production methods

Edible films and edible coatings represent different packaging concepts. Edible films are materials that, once they are dried, can be applied directly to foods as wrapping or covering materials. However, edible coatings are applied to food products as fluid liquids or gels (Hellebois et al., 2020) and form a thin layer on the surface of foodstuff (Maringgal, Hashim, Mohamed Amin Tawakkal, & Muda Mohamed, 2020). In both cases, one of the aims of these edible packaging is to extend the shelf-life of food creating a barrier between the foodstuff and the environment and to be consumed together as part of the whole product (Maringgal et al., 2020) (Pop et al., 2020).

5.1. Edible film production methods

The two different methods of producing edible films are shown in Figure 2.3. The solving casting method requires the solubilisation or dispersion of the biopolymers in a solvent followed by the drying of the film-forming solution, whereas the extrusion method uses the biopolymers in powder form, without the use of solvents, and the films are formed by hot press moulding or melt extrusion processes (Suhag et al., 2020).

5.1.1. Wet process or solvent casting method

The solving casting method has three steps (Figure 2.3-A). The first step is the solubilisation phase, in which the biomaterials, plasticisers, bioactive compounds and other additives that may be added are dissolved in a suitable solvent. This solvent can be hydrophilic, such as water, or hydrophobic, such as organic acids, depending on the characteristics of the compounds mentioned above. To avoid the formation of bubbles, the film-forming solution may be subjected to a sonication, centrifugation and/or vacuum processing before the second step. The second stage consists of casting, in which a fixed amount of film-forming solution is poured onto a flat and level surface. The last step is the drying phase, in which the evaporation of the solvent occurs. In general, this drying process takes place in a hot air chamber or oven. After this last stage and once all the solvent has evaporated, the film should be peeled easily from the surface without breaking (Rhim, Mohanty, Singh, & Ng, 2006) (Parreidt et al., 2018) (Ribeiro et al., 2021) (W. Chen et al., 2021).

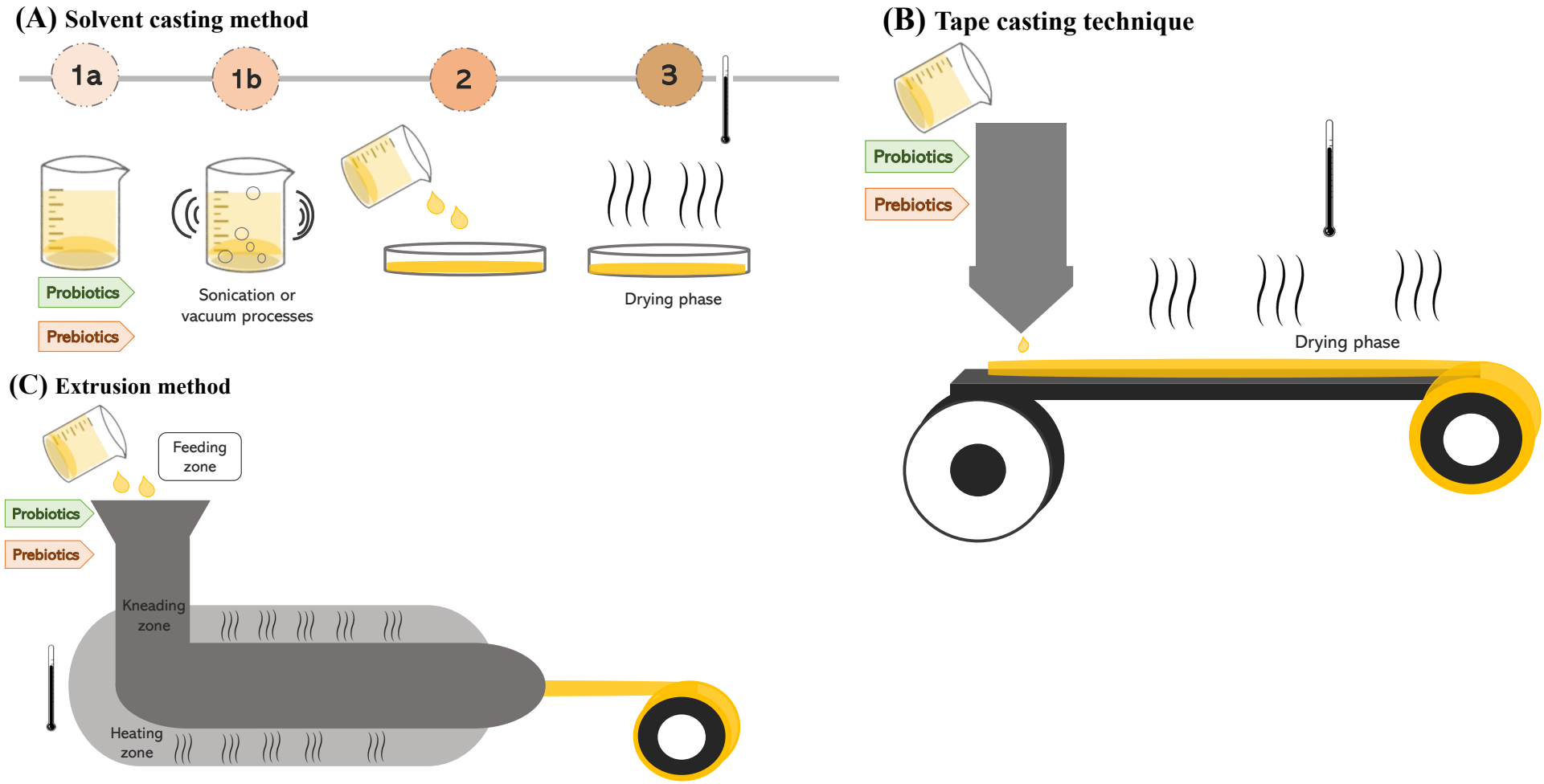


Figure 2.3. *(A)* Schematic diagram of the solvent casting method. 1a- Preparation of the film-forming solution. 1b- Sonication or vacuum processes may be necessary to remove the presence of bubbles. 2- Casting on a plate. 3- Drying phase in a hot air chamber or an oven. *(B)* Schematic diagram of the tape casting technique, as a continuous process similar as the solvent casting method. *(C)* Schematic diagram of the extrusion method. In all cases, prebiotics and probiotics are added to the film forming solution.

Due to its simplicity, as no specialized equipment is need and the cost is low, this is the most frequently film-forming technique at laboratory and pilot scales (Suhag et al., 2020) (Parreidt et al., 2018). However, this method has two disadvantages for scaling up to an industrial level. The first issue is that it is no adequate to develop films much larger than 25-30 cm and there could be variations in different parameters such as the thickness of the films. The second problem is that a large amount of water needs to be evaporated, so the drying times are long, which makes the process more energy-consuming and expensive (De Moraes, Scheibe, Sereno, & Laurindo, 2013) (Jeya Jeevahan et al., 2020). In an attempt to alleviate some of these problems, the tape casting technique (Figure 2.3-B), which is common for other production processes such as paper, plastics, ceramics, and industrial paints (Boch, P.; Chartier, 1998), has been adapted for edible film production. In this technique it is necessary to prepare the film-forming solution, which will be spread on a support with a blade that will allow it to maintain a constant thickness. For the film-forming solution to behave properly under the blade, it is necessary to study the rheology of this suspension previously. Tape casting process can be a continuous process, due to the movement of the tape or a batch process, due to the movement of the blade. The suspension is finally subjected to a drying process (heat conduction, heat convection or infrared radiation) (Ortiz, de Moraes, Vicente, Laurindo, & Mauri, 2017) (Oliveira de Moraes, Scheibe, Augusto, Carciofi, & Laurindo, 2015) (De Moraes et al., 2013). The resulting films have a thickness of between 20 and 100 μm. Thus, with tape casting technique, films can be obtained continuously with shorter drying times than with the casting solvent technique (De Moraes et al., 2013).

In the last three years, a lot of research has been carried out on the production of edible films using the tape casting method. For example, Cai et al. (2020) developed a zein/gelatine edible film loaded with oregano essential oil prepared by continuous casting method. After studying various parameters, they concluded that the long drying times and the increased casting cycle did not affect the thermal stability and mechanical properties of the films. Oldoni et al. (2021) develop continuous edible films using mangoes with an internal breakdown. They found out that the tape casting method allowed for good retention of mango pulp colouring and increased film productivity. Thus, the method they developed can be used to produce edible films from fruits that are discarded due to aesthetical imperfections. De Paola et al. (2021) developed potato starch-based films by the tape-casting method successfully. Firstly, they studied the rheology of the film-forming solutions, optimising the concentrations of the components

to select the one that showed the most suitable behaviour. In this case, the best formulation had a 10% (w/w) of starch, with a viscosity higher than 1 Pa s.

Even so, more research is still needed to investigate and optimise the different parameters, such as feed rate, the type of drying process and number of drying stages, temperature gradient and conveyor speed, among others (Otoni et al., 2018), to achieve an industrial scale-up of edible film production with the tape casting method.

5.1.2. Dry processes or extrusion methods

The dry methods are based on the thermoplastic properties of the polymers. In this case, the film-forming solution which has a low amount of water or solvent and includes the plasticizer compound, such as glycol or sorbitol, is heated above its glass transition temperature (Parreidt et al., 2018) (Ribeiro et al., 2021) (W. Chen et al., 2021) (Suhag et al., 2020). This leads to the conversion of the film-forming solution into an elastic state, due to the thermoplastic behaviour of proteins at low moisture levels (Gómez-Guillén et al., 2009). After compression and/or cooling process, a thin layer of film is formed. The most common methods in dry processes are extrusion (Figure 2.3-C) and compression methods.

Extrusion processes have been widely used to produce conventional plastics (García, Gómez-Guillén, López-Caballero & Barbosa-Cánovas, 2016). Extrusion processes can be divided into three stages. The first one is the preparation of the film-forming solution and its introduction in the feeding zone. This is followed by kneading, where the mixture is compressed and the pressure, temperature and density of the film-forming solution are increased. The last stage is the heating stage, where the film-forming solution is brought to the adequate final characteristics. The film-forming solution is extruded through a screw at a defined speed and, finally, the films are dried (García et al. 2016) (Suhag et al., 2020) (Chen et al., 2021). The two most common extrusion methods are blowing and injection moulding (García et al. 2016). Extrusion processes are very interesting and promising processes with easy scale up possibility to produce edible films through a continuous process. On the other hand, the compression moulding method operates discontinuously. In this case, the film-forming solution is placed in a previously heated mould. After the mould is closed, it is pressed to give the material the desired shape. By changing parameters such as temperature, pressure or operation times, the films are formed (García et al. 2016).

Compared to wet methods, the dry methods have several advantages such as the low energy consumption (fewer evaporation steps are needed) and short times of processing (Suhag et al., 2020) (Parreidt et al., 2018). Moreover, these techniques use

hardly any solvents (Hongsheng Liu, Xie, Yu, Chen, & Li, 2009). In addition, several authors have observed an improvement in certain mechanical and optical properties compared to the casting solvent method (Rhim et al., 2006) (Andreuccetti et al., 2012) (Ochoa-Yepes, Di Giogio, Goyanes, Mauri, & Famá, 2019). Finally, dry techniques make it possible to obtain more diverse shapes than wet methods (Suhag et al., 2020) and extrusion methods can be implemented as a continuous unit process (Gómez-Guillén et al., 2009). Vedove et al. (2021) developed sheets based on cassava starch and anthocyanin using extrusion methods as a large-scale process. This type of studies would facilitate the transfer of this type of technology to an industrial level. Therefore, these methods could be more commonly used in the food field industry (Anukiruthika et al., 2020).

5.2. Coating production methods

Regarding the use of coatings, the two most important factors to consider are the method of application and the ability of the coating to adhere to the surface of the food product (Parreidt et al., 2018). The two usual methods are spraying and dipping methods (Figure 2.4).

5.2.1. Dipping

This process is the most common method (Suhag et al., 2020) and consists of three steps (Figure 2.4-A).

First, the food piece is immersed in the coating-forming solution during a certain period of time. In some cases, it is necessary to repeat this step with another solution that allows a better cross-linking of the coating (Ribeiro et al., 2021), i.e., first a sodium alginate coating solution followed by an immersion in a $CaCl_2$ solution (Parreidt et al., 2018). Then, the excess solution is removed by deposition (Suhag et al., 2020). Finally, the final product is submitted to a drying process (Parreidt et al., 2018) (Ju et al., 2019), and it could be at room temperature or by using heating (Suhag et al., 2020). After the drying period, a layer of coating is formed on the surface of the product. However, the thickness of the layer formed may not be homogeneous and thick coatings may form. This will depend on the density, viscosity and surface tension of the coating forming solution (Ju et al., 2019). Another problem with this method is the low adherence to with surfaces incompatible with the nature of the coating (hydrophilic-hydrophobic and vice versa). This can be resolved by adding successive layers (layer-by-layer technique (Parreidt et al., 2018)) of coating solutions with different characteristics. Thus, the suspension can dilute the outer layer of the food surface, minimizing its functionality.

For example, the natural wax layer of fruits and vegetables can be removed after the dipping process (Silva-Vera et al., 2018). On the other hand, this method has several advantages such as the process is generally simple, short and has a low cost. For these reasons, it is the most common method used in at laboratory scale (Atieno, Owino, Ateka, & Ambuko, 2019). Besides, it allows the food piece to be completely covered, regardless of its shape or structure (Parreidt et al., 2018). That is why the dipping method has been used in different types of food products such as fruits (Strano et al., 2021) (Saleem et al., 2020) (Saleem et al., 2021) (Wong et al., 2021), vegetables (Divya, Smitha, & Jisha, 2018) (Aisyah, Murlida, & Maulizar, 2022), meat (Abdel-Naeem, Zayed, & Mansour, 2021) (Lashkari, Halabinejad, Rafati, & Namdar, 2020) (Gedikoğlu, 2022), bakery (Eom, Chang, Lee, Choi, & Han, 2018) (Nayanakanthi, Senanayake, & Siranjiv, 2021) and dairy products (Sáez-Orviz et al., 2020b) (Jotarkar, Panjagari, Singh, & Arora, 2018) (Siriwardana & Wijesekara, 2021).

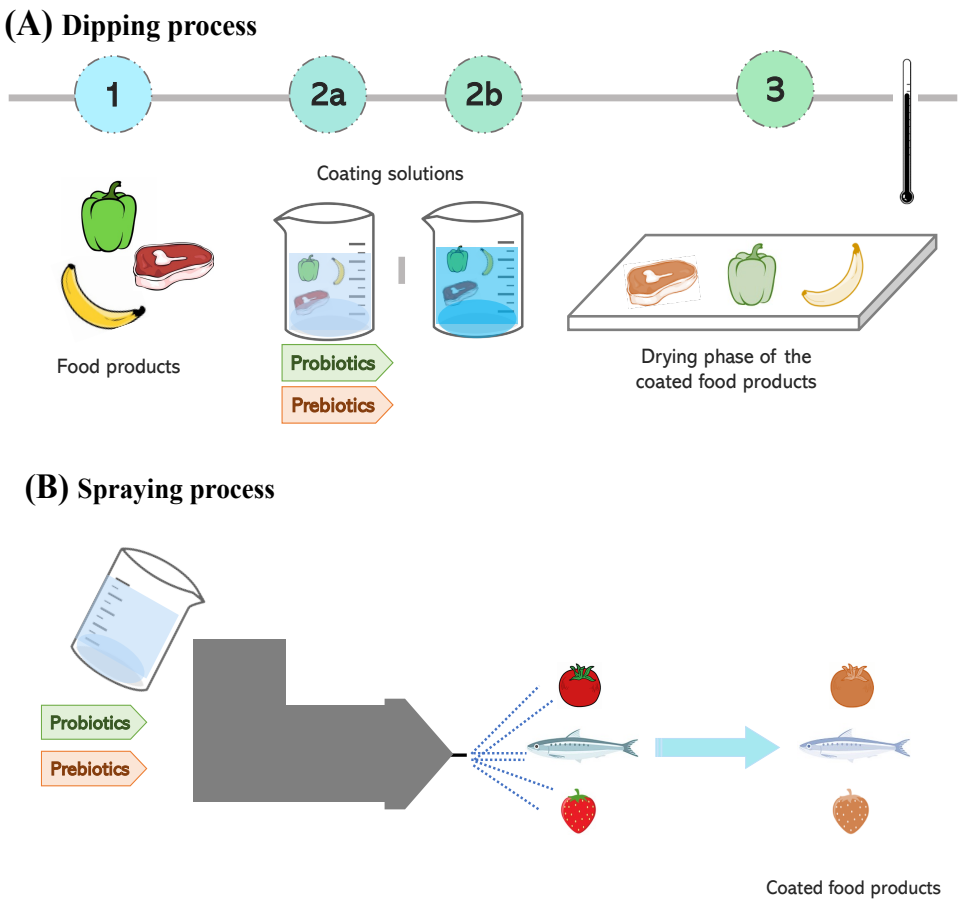


Figure 2.4. *(A)* Schematic diagram of the dipping process. 1-Various food products. 2a-Food products are immersed in the coating-forming solution. 2b- In some cases, it is necessary an immersion in a cross-linking solution. 3-Excess of solution is removed by deposition and drying process can occur at room temperature or by heating. *(B)* Schematic diagram of the spraying process. The coating solution is distributed on the surface of the food pieces from the drops that are formed in the nozzle of the equipment. In both cases, prebiotics and probiotics are added to the coting-forming solutions.

5.2.2. Spraying

The spraying method is the most common in the food industry (Suhag et al., 2020). This method is based on the distribution of the coating solution on the surface of the food in the form of drops with the help of nozzles (Parreidt et al., 2018) due to the development of high pressure spray applicators and air atomizing systems (Silva-Vera et al., 2018) (Figure 2.2-B). There are three different techniques. Two of them use air to induce atomization (air spray atomization and air assisted airless atomization techniques). The third technique, pressure atomization, achieves the atomization of the coating solution through pressure and not the use of air (Suhag et al., 2020). A key factor of this method is that the viscosity of the coating solution should not be high (Ju et al., 2019). In order for the droplet size to be adequate, different parameters must be taken into account, such as the density and surface tension of the coating solution, the operating conditions of the equipment (air flow and pressure), and the system conditions (the spray angle and nozzle size) (Parreidt et al., 2018). The spraying method has the advantages of forming a coating with uniform thickness throughout the food piece and the possibility of applying layers of coatings with different characteristics (Suhag et al., 2020). Besides, the amount of coating solution needed is less, the possibility of contamination is lower and it is possible to work with large food surfaces (Parreidt et al., 2018) (Ribeiro et al., 2021). Some of the recent research using the spraying method is based on studies on fruits (Farina, Passafiume, Tinebra, Palazzolo, & Sortino, 2020) (Jiang et al., 2019) (Lara et al., 2020), meat (Gedarawatte et al., 2021) (Apriliyani et al., 2020), and fish products (Simen Sørbø, 2022) (Kulawik, Jamróz, Zając, Guzik, & Tkaczewska, 2019).

5.3. Incorporation of probiotics and prebiotics in edible films and coatings

As for probiotics, regardless of the production method for both edible films or coatings, the key factor is maintaining their viability throughout the entire process (Zoghi et al., 2020). During the production processes of edible packaging, probiotics can be subjected to different environmental stresses (Figure 2.5), such as high or low temperatures, acid and alkaline pH, oxidative stress, high hydrostatic pressure, osmotic pressure and starvation (Mbye et al., 2020).

In all production methods, the microorganisms are usually added to the film-forming or coating-forming solution, which must have a suitable pH and temperature, depending on the tested probiotic strain. The most important phases in terms of the probiotic viability are the drying stages, where the temperature increases considerably, in addition to the fact that the water activity (a_w) can decrease, compromising the survival of the microorganisms. To mitigate the loss of viability during the drying steps,

cryoprotective plasticizers can be used (Hellebois et al., 2020). Resistance to each of these parameters is strain dependent, so it is important to select appropriately the probiotic to use based on the production method to be employed (Mbye et al., 2020). When developing edible films and coatings with probiotic microorganisms, the most common methods are usually solving casting or dipping techniques (Hellebois et al., 2020). The encapsulation of microorganisms using techniques such as spray drying, and atomization have been extensively studied (Guimarães et al., 2018) (Pop et al., 2020) (Rodrigues, Cedran, Bicas, & Sato, 2020). However, there is very little research on the use of dry methods to make films and coatings with free probiotics. Recently, Wang *et al.* (2021) developed a novel spray-coating strategy using milk powder and sucrose as matrix and *L. salivarius* as probiotic microorganism (Wang et al., 2021). They observed that a drying temperature of 60 °C for up to 30 min was optimal while temperatures higher than 63 °C leaded to a deactivation of the bacterium.

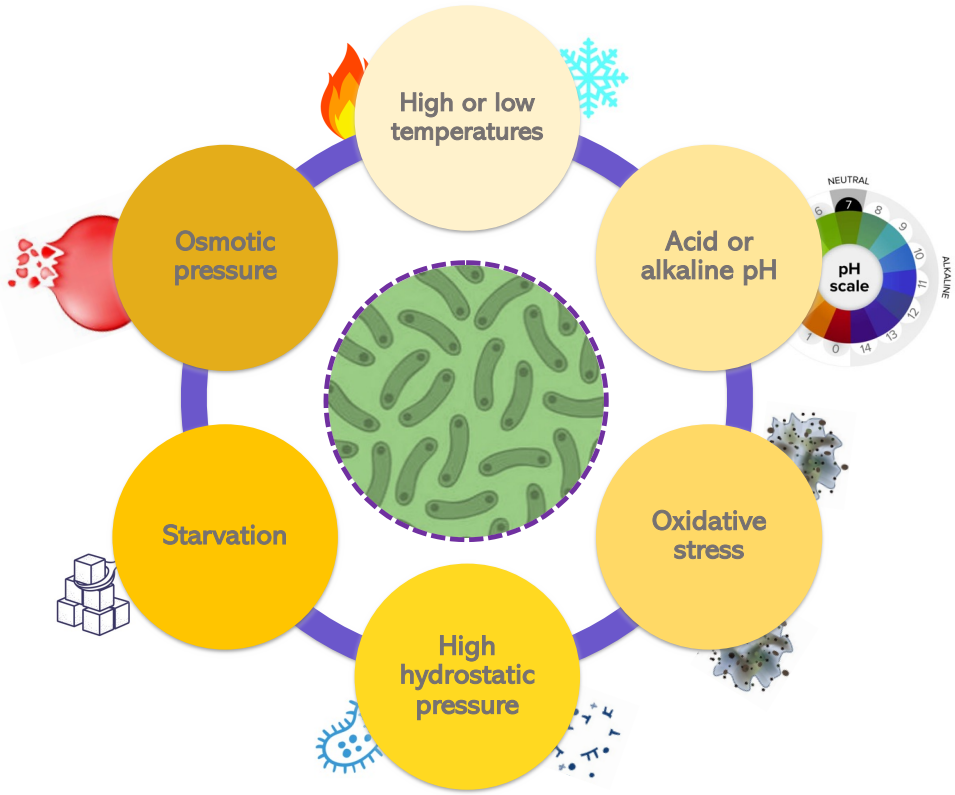


Figure 2.5. Key factors to consider when including probiotics in the formulation of edible films and coatings.

In relation to the addition of prebiotics, they are also added to the film-forming or coating-forming solutions. In this case, the parameters that must be taken into account are the temperature, pH, time of solubilization or the dispersion method (Paulo et al., 2021). The solubilization of the prebiotic will depend on its nature and its compatibility

with the chemical characteristics of the film-forming or coating-forming solutions. However, the addition of prebiotics is simpler and shows fewer difficulties than probiotics, since these are compounds that are not as sensible to physical and chemical factors, and they are hardly degradable.

6. Interaction between biopolymers in film network and the effect of the presence of probiotics and prebiotics

The different biopolymers that conform the matrix of films and coatings have several functional groups, which leads to a great variety of interactions inside the matrices (Chen et al., 2021). In some cases, these interactions may be more important than the physicochemical properties of the individual biopolymers (Zhang et al., 2021). The interactions that occur inside the matrix can be divided into two main groups: covalent bonding and non-covalent interactions.

Covalent bonding can be defined as strong chemical bonds that involve the sharing of electron pairs between atoms. In this group are different covalent interactions such as esterification, etherification, amidation, glycosylation, etc. Generally, these covalent bonds take place between amino (-NH_2, -NRH), hydroxyl (-OH) and carboxyl (-CO_2H) groups in proteins and polysaccharides (W. Chen et al., 2021) (Zhang et al., 2021). The presence of this type of interactions, their number and their nature are linked to the physical properties of the edible packaging developed. Specifically in the case of protein matrix, disulfide bonds have a significant impact in the structure and morphology of edible films (Deng et al., 2020). Disulfide bonds can be defined as a strong covalent bond that can be obtained by the oxidation of a pair of thiol groups (Fass & Thorpe, 2018). Recent studies have focused on this type of interaction to modify the characteristics of edible packaging. Kumari et al. (2021) developed a fenugreek protein-based edible film in an alkaline environment (pH 12). At this pH, these authors observed that films were more stable due to the presence of disulfide bonds, and this also produced a reduced WVP in the films developed. Deng et al. (2020) developed a disulfide bond crosslinked gelatin/ε-polylysine edible film. Results showed that the oxidation of thiol groups leaded to a more compact and denser microstructure. This allowed to achieve an improvement in the water resistance of this protein films (Deng et al., 2020). These results are promising, as they could improve the poor water resistance of films developed exclusively with proteins, since this is their main disadvantage. Covalent crosslinking can also be induced through chemical (alkali treatment, free radical initiated (Chen et al., 2021) and irradiation (Gopalakrishnan et al., 2021)) or enzymatic methods (transglutaminase, laccase and peroxidase, among others

(W. Chen et al., 2021)). Concerning chemical methods, irradiation or photo-crosslinking method is one of the most common (Li, Sheng, Sun, & Wang, 2020). Ben-Fadhel et al. (2021) developed a calcium caseinate edible coating that was treated with γ- irradiation. As a result, these authors observed that γ- irradiation at 32 kGy improved the mechanical and water vapour barrier properties of the calcium caseinate coating. Huang et al. (2020) observed that electron beam irradiation has a positive effect on the tensile strength, opacity values and microstructure of fish gelatine films additivated with antioxidants due to the formation of new bonds inside the matrix. Regarding enzymatic methods, transglutaminase is one of the enzymes commonly used to improve the characteristics of films developed using protein as matrix. Transglutaminase introduces isopeptide bonds between the amino acids glutamine and lysine (Giosafatto, Fusco, Al-Asmar, & Mariniello, 2020). Numerous authors have seen an improvement of the mechanical properties of their films after using this enzyme (Minh et al., 2019) (Ahammed et al., 2021) (Escamilla-García et al., 2019) (Marcet, Sáez, Rendueles, & Díaz, 2017).

As for the non-covalent interactions, they include van der Waals forces, hydrogen bonding, and hydrophobic and electrostatic interactions (Chen et al., 2021) (Zhang et al., 2021). Van der Waals forces do not result from a chemical electronic bond; they are distance-dependent and therefore are weaker. Hydrogen bonding is an electrostatic interaction between hydrogen atoms and negatively charged atoms. In these type of interaction there is always a hydrogen donor and a hydrogen acceptor (Zhang et al., 2021). Hydrogen bonding interactions are very common within film and coating matrices. Several authors have observed the importance of this type of interactions through Fourier transform infrared (FTIR). Dai *et al.* (2019) (Dai, Zhang, & Cheng, 2019) verified the formation of hydrogen bonds between starch and glycerol on starch-based edible films. This bonding made the polymer matrix more structurally integrated and improved the transparency of the films. Nguyen et al. (2020) observed the formation of hydrogen bonds between polyphenol compounds and chitosan on their edible films. These interactions resulted in an improved of the water vapour transmission rate of the films, but the high number of interactions formed caused a decrease of the film plasticity. Davoodi et al. (2020) developed *Salvia macrosiphon*/chitosan edible films. These authors observed hydrogen-bonded networks by FTIR analysis. Hydrogen-bonding enhanced the mechanical and water vapour barrier properties of their edible films. Thus, there are electrostatic interactions inside the biopolymer matrix. They occur between attractive and repulsive forces between groups that are caused by their electric

charges. In this cases, the charge depends on the pH of the system and the presence of salts can affect the net charge (Zhang et al., 2021).

Understanding all these interactions, how they occur and how they can be modified is of vital importance to improve the physical and mechanical properties of the edible films and coatings developed. Besides, all the interactions mentioned above are affected when probiotics and prebiotics are added to the biopolymer matrix.

6.1. Influence of probiotics in the interactions

All the aforementioned non-covalent and covalent interactions are affected by the presence of probiotic microorganisms in the matrix of the packaging. Although some studies have been carried out, the mechanisms and biochemical phenomena of how probiotics are stabilized in the biopolymer matrices are not yet known (Abedinia et al., 2021) (Soukoulis, Behboudi-Jobbehdar, Macnaughtan, Parmenter, & Fisk, 2017). One of the possible ways of interaction or stabilization between the probiotic cells and the matrix would be through the hydrogen bonds via the polar heads of the phospholipid membranes of microorganisms (Abedinia et al., 2021) (Ma, Jiang, Ahmed, Qin, & Liu, 2019). These interactions between the microorganisms and the biopolymers cause changes in the rest of the interactions of the matrix, changing the physical and chemical properties of the edible packaging. One of the changes that arises when adding microorganisms in the biopolymer matrix is the steric hindrance. The probiotic cells occupy a space in the matrix that can interrupt some interactions, both covalent and non-covalent, from taking place. Several authors have noted changes in matrix properties due to this factor. In some cases, various properties have been improved by the presence of microorganisms, while in others they have been worsened. Table 2.5 shows examples of some changes that have been analyzed recently by several authors. Some authors have studied this topic recently. Yan et al. (2021) developed soy protein hydrogels with sugar beet pectin additivated *L. paracasei* LS14. The presence of the probiotic weakened the hardness of the hydrogels, due to the steric hindrance effect, as it prevented the formation of ordered intermolecular aggregates during the gelation process. In addition, they observed that probiotic cells caused changes in the tertiary structure of the proteins. Ma et al. (2019) developed edible films with three different film-forming solutions (sodium alginate, sodium carboxymethylcellulose and collagen) additivated with *L. lactis*. These authors observed changes in WVP values. These values increased when the microorganisms were added to the films. This could be explained because the addition of bacteria would change the spatial structure of the molecules, reducing intermolecular interactions and increasing the intermolecular space, which

would lead to an increase in permeability to water vapour. Karimi et al. (2020) prepared films with whey protein, polydextrose and nanocellulose fibers additivated with *L. plantarum*. The authors attributed the good viability of the probiotic inside the matrix, in addition to the presence of nutrients and free radical scavenging agents, to the interaction between polydextrose and the phospholipids of the bacterial membrane. Otherwise, Li et al. (2020) observed differences in a cassava starch/carboxymethylcellulose matrix depending on the microorganism they added. *L. plantarum* achieved a good dispersion within the matrix, although the probiotic cells were found as discontinuous particles in the matrix, inhibiting the migration of the polymer chain. This strain improved WVP film properties. However, *Pedococcus pentosaceus* did not achieve a homogeneous distribution within the matrix, as observed when morphology was analysed, which made the films to have poor WVP and mechanical properties. Shahrampour et al. (2020) concluded that the compatibility between the probiotic microorganism and the biopolymers of matrix employed is a key factor. They developed alginate/pectin edible films containing *L. plantarum* KMC 45. Through SEM images, they observed that the probiotic cells had not modified the microstructure of the films, which indicated a good compatibility between alginate and pectin. Furthermore, the addition of the probiotic cells partially improved the mechanical properties and the WVP. These authors suggested that, in this specific case, the bacteria were able to reduce the intermolecular space due to hydrogen bonding with the film-forming agent.

From these studies, it can be concluded that the interactions that take place in the edible packaging when probiotic cells are added depend both on the type of microorganism that is added and on the type of biopolymer of matrix, as well as the compatibility between them. Most of the research that has been done focuses on the viability of probiotics within films and coatings and not on the interactions that occur. A better understanding of the mechanisms of interaction and stabilization of microorganisms can provide more information on how to improve the viability of probiotics within edible packaging.

Table 2.5. Recently studies of the last 3 years about changes observed when probiotics were added to the matrix of edible films and coatings.

Probiotic strain	Biopolymers employed as matrix in the edible packaging	Changes observed due to the presence of probiotics	Reference
L. paracasei LS14	Soy protein and sugar beet pectin	• Weaker hydrogels in terms of firmness • Enhance of the swelling ratios • Avoid of the formation of ordered intermolecular aggregates during the gelation process • Disruption of the microstructure of the hydrogels	(Yan et al., 2021)
Lactococcus lactis ATCC 11454	Sodium alginate, sodium carboxymethylcellulose, collagen and glycerol as plasticizer	• Alterations in the colour and luster of the edible films • Higher WVP values	(Ma et al., 2019)
L. plantarum PTCC 1058	Whey protein, polydextrose, and nanocellulose fibers	• No significant changes in physical and mechanical properties • The interaction between the phospholipids of the bacterial membrane with polydextrose increased their viability	(Karimi et al., 2020)
L. plantarum	Cassava starch, carboxymethylcellulose, and glycerol	• Decrease of the light transmittance of the film • Achievement of good dispersion inside the matrix by morphological analysis • Improvement of the WVP properties	(S. Li et al., 2020)
Pedococcus pentosaceus		• Decrease of the light transmittance of the film • Did not achieve a good dispersion inside the matrix by morphological analysis • Higher values of WVP • Poor mechanical properties	
L. plantarum KMC 45	Alginate, pectin, and glycerol and sorbitol as plasticizers	• No modification of the microstructure that showed good compatibility between alginate and pectin • Partially improvement of the mechanical properties and WVP.	(Shahrampour, Khomeiri, Razavi, & Kashiri, 2020)

L. casei 01	Whey protein isolate and glycerol	• Edible films with probiotics were thicker • They showed a higher solubility • Regarding mechanical properties, they were less flexible	(Dianin et al., 2019)
L. casei DN-114001, *B. bifidum* DSMZ 20215, *L. acidophilus* DSM 20079 and *L. rhamnosus* GG E-96666	Citrus pectin and glycerol as plasticizer	• Slightly decreased in the swelling index • Increase of the WVP • Reduction on the clearness if the films • Reduction of the tensile properties of the films	(Nisar et al., 2022)
B. lactis B-1922, *L. acidophilus* CH-2 and *L. casei* B-1922	Chitosan, sodium alginate and carboxymethyl cellulose, and glycerol as plasticizer	• Changes in the microstructure of sodium alginate films due to the aggregation of probiotics • Changes in water vapour transmission rate (WVTR) and water solubility values	(El-Sayed et al., 2021)
L. plantarum CECT 9567	Egg yolk protein and glycerol as plasticizer Lactobionic acid as prebiotic	• Changes in mechanical properties. Puncture deformation values were lower when probiotic was in the film matrix	(Sáez-Orviz, S., Marcet, I., Rendueles, M., Díaz, 2021)
L. plantarum	Sodium alginate and glycerol as plasticizer	• Higher thickness values • Increase of the WVP values	(Akman, Bozkurt, Dogan, Tornuk, & Tamturk, 2021)

6.2. Influence of prebiotics in the interactions

Adding prebiotic compounds influences the interactions that will occur between the biopolymers inside the packaging matrix. The interactions will be reduced or reinforced, depending on the nature of the prebiotic compounds and their compatibility with the biopolymers of the matrix. The type of changes that occur in the matrices when adding different kind prebiotics have been studied by several authors. Recently research is showed in Table 2.6.

Several studies have focused on the interactions of oligosaccharides in edible packaging. Fernandes et al. (2020) studied the influence of the addition of GOS and XOS in whey protein films. These authors found no differences in the microstructure of the films. However, they did observe differences in the mechanical properties. There was a decrease in tensile strength and an increase in elongation at break, depending on the concentration of added prebiotic (at concentrations greater than 20g /100g of film). This change in properties may be due to less formation of strong protein-protein interactions. In addition, the addition of GOS and XOS, with their large size and composition of sugars and hydroxyl molecules, could induce the formation of complex interactions, which would offer more flexibility to these structures. Regarding the WVP properties, the prebiotic films showed lower diffusion and lower permeability, probably by increasing and hindering the path that water vapor molecules must wander. Furthermore, water interacts with GOS and XOS molecules, due their characteristics, forming strong interactions between water and hydroxyl groups. Xu et al. (2019) developed hemicellulose/chitosan-based films reinforced with cellulose nanofiber and XOS. These authors observed differences in WVP values and mechanical properties. The strong interactions between the biopolymer chains (hydrogen bonds and electrostatic interactions) were disrupted by the presence of XOS, making the films less compact, with less tensile strength and accelerating the permeation of water molecules. Research has also been carried out on inulin. Orozco-Parra et al. (2020) developed an edible film based on cassava starch and inulin. As with the oligosaccharides, inulin decreased the tensile strength of the films and increased their elongation. Prebiotics can have a plasticizing effect by reducing strength while increasing elongation. WVP values increased and so did solubility. Inulin is a very hygroscopic and water-soluble compound, so it has a great affinity for water molecules, improving the diffusion of water vapour. Pereira et al. (2019) developed edible films based on alginate and whey protein with inulin in their matrix. These authors only observed significant differences due to the presence of inulin in the tensile strength of the films.

From these investigations it can be concluded that prebiotic compounds can have an effect similar to plasticizers in the matrix of biopolymers (Paulo et al., 2021), therefore, these compounds allow, in general, to achieve more flexible films (Urbizo-Reyes, San Martin-González, Garcia-Bravo, & Liceaga, 2020). Besides, the interactions that take place depend on the compatibility of the nature of the biopolymers with the prebiotic compounds and their concentration. In addition, in some cases they can be used as a reinforcement agent (Paulo et al., 2021).

Regarding the combination of both, probiotic microorganisms and prebiotic compounds, a combination of the interactions explained above takes place. The main characteristic in all cases is the increase in the viability of the probiotics during films and coatings' processing and storage. In the case of inulin, Orozco-Parra et al. (2020) observed a protective effect on the viability of *L. casei* during storage and so observed Pereira et al. (2019), since the films with this prebiotic compound improved the survival of *B. animalis* after 60 days of storage. This same effect has been observed in other prebiotic compounds such as FOS. Oliveira-Alcântara et al. (2020) observed a protective effect of FOS on probiotic bacteria during film drying as synbiotic films exhibited higher bacterial viability that probiotic films.

Table 2.6. Recently studies of the last 4 years about changes observed when prebiotics were added to the matrix of edible films and coatings.

Prebiotic compound	Biopolymers employed as matrix in the edible packaging	Changes observed due to the presence of prebiotics	Reference
Inulin (0.1, 0.5 and 1%, *w/v*)	Cassava starch and glycerol as plasticizer	• Decrease in tensile strength • Increase in elongation at break • Increase in WVP values and water solubility	(Orozco-Parra et al., 2020)
Inulin (2%, *w/v)*	Alginate and whey protein and glycerol as plasticizer	• Decrease in the tensile strength of the films	(Pereira et al., 2019)
Inulin (10 and 20 g/100 g of dry film)	Carboxymethyl cellulose, cellulose nanofiber and glycerol as plasticizer	• The presence of prebiotic increased the thickness of the films • Reduction in the moisture absorption values	(Zabihollahi et al., 2020)
FOS (1 g/ 100 ml film-forming solution)	Nanofibrillated bacterial cellulose and cashew gum	• Decrease in tensile strength • Films were more permeable to water vapour • Increase of the water solubility • Rougher surfaces in SEM micrographs	(Oliveira-Alcântara et al., 2020)
FOS (15 g/L)	Linseed mucilage, sodium alginate and glycerol and polysorbate as plasticizers	• Prebiotic contributed to improve the colour of the edible coatings	(Fábio J. Rodrigues, Cedran, & Garcia, 2018)
GOS (10, 20 and 30 g/ 100 g)	Whey protein isolate and glycerol as plasticizer	• Decrease in tensile strength and increase in elongation at break at concentrations greater than 20 g/100 g of film • Lower diffusion and lower permeability values	(Fernandes et al., 2020)
XOS (10, 20 and 30 g/ 100 g)			
XOS (5, 10, 15 and 20% of dry weight)	Chitosan, hemicelluloses, and cellulose nanofiber	• Less compact films • Less tensile strength • Higher WVP values	(J. Xu et al., 2019)

7. Regulatory aspects in the commercialization of functional packaging

Edible biopolymers and edible packaging is gaining importance in the last times due to their sustainability and versatility (Amin et al., 2021). The regulation regarding food packaging varies from one country to another (Jeevahan et al., 2020). Generally, as they are edible films and coatings, they must adhere to the regulations related to foodstuff. Thus, all the compounds used must be food grade, in terms of food ingredient or additive, and must be approved by the Codex Alimentarius (Debeaufort & Voilley, 2009) and the corresponding food authorities (i.e., EFSA in the case of the EU and FDA in the case of the USA). In the particular case of probiotics, they must have the QPS (Barlow et al., 2007) or GRAS (*FDA.*, 2016) category in order to be used in the food industry. However, there are no special guidelines to control the commercialization of biodegradable and functional packaging materials (Amin et al., 2021). In the case of EU, it is stipulated that all food contact materials must not endanger human health, must not cause intolerable food composition changes, must not cause changes in organoleptic properties, and must be manufactured according to good manufacturing practices (EC No. 1935/2004).

Besides, regulatory aspects usually depend on formulations and application and need to be considered separately (Schmid & Müller, 2018). The continuous research in this area by researchers will help to better understand the behaviour of the edible packaging and its use in foodstuff and, thus, its regulation.

8. Conclusions and future trends

This review highlighted recent research on edible films and coatings additivated with probiotic microorganisms and prebiotic compounds. In recent years, edible films and coatings additivated with prebiotic and probiotics have been the focus of many researchers due to their potential in the edible food packaging field. The inclusion of probiotic microorganisms and prebiotics as bioactive compounds allows the development of edible functional food packaging. Knowing the characteristics of the different production methods of films and coatings allows selecting those that are most optimal for the bioactive compounds that are to be added. Working with probiotic microorganisms is complex since, as they are living bacteria, there are different chemical and physical factors that can minimize their viability. In this case, edible packaging can help to maintain an adequate concentration over time. The physical and mechanical characteristics of films and coatings depend directly on the interactions that

take place between the biopolymers that make up their matrix. When probiotic bacteria and prebiotic compounds are added, these covalent and non-covalent interactions are modified, which leads to changes in the characteristics and properties of the edible packaging. There is still a strong need to understand the relationships among biopolymer interactions, especially when living bacteria are added in edible packaging and how they are stabilized inside the matrix. Edible functional food packaging has great potential in the food industry, but more research is needed to better understand its behaviour in different foodstuffs and to be able to develop more precise rules and regulations by the different food authorities on its use in food products.

Acknowledgements

This work was financially supported by the Spanish Ministry of Science and Innovation, through the project GRUPIN-IDI/20217000055 and by the grant *Programa Severo Ochoa de Ayudas Predoctorales para la investigación y docencia* (grant number BP19-127 to Sáez-Orviz, S.).

9. References

Abdel-Naeem, H. H. S., Zayed, N. E. R., & Mansour, H. A. (2021). Effect of chitosan and lauric arginate edible coating on bacteriological quality, deterioration criteria, and sensory attributes of frozen stored chicken meat. *Lwt*, 150(May), 111928. https://doi.org/10.1016/j.lwt.2021.111928

Abedinia, A., Alimohammadi, F., Teymori, F., Razgardani, N., Asl, M. R. S., Ariffin, F.Nafchi, A. M., Huda, N. & Roslan, J. (2021). Characterization and cell viability of probiotic/prebiotics film based on duck feet gelatin: A novel poultry gelatin as a suitable matrix for probiotics. *Foods*, 10(8). https://doi.org/10.3390/foods10081761

Aguilar-Sánchez, R., Munguía-Pérez, R., Reyes-Jurado, F., Navarro-Cruz, A. R., Cid-Pérez, T. S., Hernández-Carranza, P., Beristain-Bauza, S. d. C., Ochoa-Velasco, c. E. & Avila-Sosa, R. (2019). Structural, Physical, and Antifungal Characterization of Starch Edible Films Added with Nanocomposites and Mexican Oregano (Lippia berlandieri Schauer) Essential Oil. *Molecules*, 24(12). https://doi.org/10.3390/molecules24122340

Aguirre-Joya, J.A.; Álvarez, B.; Ventura, J.M.; García-Galindo, J.O.; De León-Zapata, M.A.; Rojas, R.; Sauceso, S.; Aguilar, C. N. (2016). Edible coatings and films from lipids, waxes and resins. *In Edible Food Packaging. Materials and Processing Technologies*. (pp. 121–152). Crc Press.

Ahammed, S., Liu, F., Wu, J., Khin, M. N., Yokoyama, W. H., & Zhong, F. (2021). Effect of transglutaminase crosslinking on solubility property and mechanical strength of gelatin-zein composite films. *Food Hydrocolloids*, 116 (February), 106649. https://doi.org/10.1016/j.foodhyd.2021.106649

Aisyah, Y., Murlida, E., & Maulizar, T. A. (2022). Effect of the edible coating containing cinnamon oil nanoemulsion on storage life and quality of tomato (Lycopersicum esculentum Mill) fruits. IOP Conference Series: *Earth and Environmental Science*, 951(1). https://doi.org/10.1088/1755-1315/951/1/012048

Akman, P. K., Bozkurt, F., Dogan, K., Tornuk, F., & Tamturk, F. (2021). Fabrication and characterization of probiotic Lactobacillus plantarum loaded sodium alginate edible films. *Journal of Food Measurement and Characterization*, 15(1), 84–92. https://doi.org/10.1007/s11694-020-00619-6

Alvarez, M. V., Bambace, M. F., Quintana, G., Gomez-Zavaglia, A., & Moreira, M. del R. (2021). Prebiotic-alginate edible coating on fresh-cut apple as a new carrier for probiotic lactobacilli and bifidobacteria. *Lwt,* 137(May 2020), 110483. https://doi.org/10.1016/j.lwt.2020.110483

Amin, U., Khan, M. U., Majeed, Y., Rebezov, M., Khayrullin, M., Bobkova, E., Shariati, M. A.,, Chung, I. M. & Thiruvengadam, M. (2021). Potentials of polysaccharides, lipids and proteins in biodegradable food packaging applications. International *Journal of Biological Macromolecules*, 183(May), 2184–2198. https://doi.org/10.1016/j.ijbiomac.2021.05.182

Andreuccetti, C., Carvalho, R. A., Galicia-García, T., Martinez-Bustos, F., González-Nuñez, R., & Grosso, C. R. F. (2012). Functional properties of gelatin-based films containing Yucca schidigera extract produced via casting, extrusion and blown extrusion processes: A preliminary study. *Journal of Food Engineering*, 113(1), 33–40. https://doi.org/10.1016/j.jfoodeng.2012.05.031

Angiolillo, L., Conte, A., Faccia, M., Zambrini, A. V, & Nobile, M. A. Del Mobile (2014). A new method to produce synbiotic Fiordilatte cheese. Innovative *Food Science and Emerging Technologies*, 22, 180–187. https://doi.org/10.1016/j.ifset.2013.09.010

Anukiruthika, T., Sethupathy, P., Wilson, A., Kashampur, K., Moses, J. A., & Anandharamakrishnan, C. (2020). Multilayer packaging: Advances in preparation techniques and emerging food applications. *Comprehensive Reviews in Food Science and Food Safety*, 19(3), 1156–1186. https://doi.org/10.1111/1541-4337.12556

Apriliyani, M. W., Rahayu, P. P., Andriani, R. D., Manab, A., Purwadi, Sawitri, M. E., & Utama, D. T. (2020). Characteristics of Casein-Chitosan Edible Coating and Its Preservative Effect in Meat during Accelerated Storage. IOP Conference Series: *Earth and Environmental Science*, 478(1). https://doi.org/10.1088/1755-1315/478/1/012060

Ashwell, M. (2002). Concepts of functional foods. In IISI Europe Concise Monograph Series. https://doi.org/10.1108/nfs.2004.34.1.47.3

Atieno, L., Owino, W., Ateka, E. M., & Ambuko, J. (2019). Influence of Coating Application Methods on the Postharvest Quality of Cassava. *International Journal of Food Science*, 2019. https://doi.org/10.1155/2019/2148914

Bambace, M. F., Alvarez, M. V., & Moreira, M. del R. (2019). Novel functional blueberries: Fructo-oligosaccharides and probiotic lactobacilli incorporated into alginate edible coatings. *Food*

Research International, 122(December 2018), 653–660. https://doi.org/10.1016/j.foodres.2019.01.040

Barbosa, C. H., Andrade, M. A., Vilarinho, F., Fernando, A. L., & Silva, A. S. (2021). Active Edible Packaging. *Encyclopedia,* 1(2), 360–370. https://doi.org/10.3390/encyclopedia1020030

Barlow, S., Chesson, A., Collins, J. D., Dybing, E., Flynn, A., Fruijtier-Pölloth, C., Hardy, A., Knaap, A., Kuiper, H., Le Neindre, p., Schans, J., Schlatter, J., Silano, V., Skerfving, S. & Vannier, P. (2007). Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA - Opinion of the Scientific Committee. *EFSA Journal*, 5(12), 1–16. https://doi.org/10.2903/j.efsa.2007.587

Barragán-Menéndez, C., Gálvez-López, D., Rosas-Quijano, R., Salvador-Figueroa, M., Ovando-Medina, I., & Vázquez-Ovando, A. (2020). Films of chitosan and aloe vera for maintaining the viability and antifungal activity of lactobacillus paracasei TEP6. *Coatings*, 10(3). https://doi.org/10.3390/coatings10030259

Ben-Fadhel, Y., Cingolani, M. C., Li, L., Chazot, G., Salmieri, S., Horak, C., & Lacroix, M. (2021). Effect of γ-irradiation and the use of combined treatments with edible bioactive coating on carrot preservation. *Food Packaging and Shelf Life*, 28(January 2020), 100635. https://doi.org/10.1016/j.fpsl.2021.100635

Bindels, L. B., Delzenne, N. M., Cani, P. D., & Walter, J. (2015). Opinion: Towards a more comprehensive concept for prebiotics. *Nature Reviews Gastroenterology and Hepatology*, 12(5), 303–310. https://doi.org/10.1038/nrgastro.2015.47

Boch, P.; Chartier, T. (1998). Understanding and Improvement of Ceramic Processes: *The Example of Tape Casting. Materials Science Forum*, 34–36, 813–819.

Cai, J., Xiao, J., Chen, X., & Liu, H. (2020). Essential oil loaded edible films prepared by continuous casting method: Effects of casting cycle and loading position on the release properties. *Food Packaging and Shelf Life*, 26(July), 100555. https://doi.org/10.1016/j.fpsl.2020.100555

Çakmak, H., Özselek, Y., Turan, O. Y., Fıratlıgil, E., & Karbancioğlu-Güler, F. (2020). Whey protein isolate edible films incorporated with essential oils: Antimicrobial activity and barrier properties. *Polymer Degradation and Stability*, 179. https://doi.org/10.1016/j.polymdegradstab.2020.109285

Calva-Estrada, S. J., Jiménez-Fernández, M., & Lugo-Cervantes, E. (2019). Protein-Based Films: Advances in the Development of Biomaterials Applicable to Food Packaging. *Food Engineering Reviews,* (2019), 78–92. https://doi.org/10.1007/s12393-019-09189-w

Cazón, P., Velazquez, G., Ramírez, J. A., & Vázquez, M. (2017). Polysaccharide-based films and coatings for food packaging: A review. *Food Hydrocolloids*, 68, 136–148. https://doi.org/10.1016/j.foodhyd.2016.09.009

Chen, W., Ma, S., Wang, Q., McClements, D. J., Liu, X., Ngai, T., & Liu, F. (2021). Fortification of edible films with bioactive agents: a review of their formation, properties, and application

in food preservation. *Critical Reviews in Food Science and Nutrition*, 0(0), 1–27. https://doi.org/10.1080/10408398.2021.1881435

Chiralt, A., Menzel, C., Hernandez-García, E., Collazo, S., & Gonzalez-Martinez, C. (2020). Use of by-products in edible coatings and biodegradable packaging materials for food preservation. *Sustainability of the Food System: Sovereignty, Waste, and Nutrients Bioavailability,* 101–127. https://doi.org/10.1016/B978-0-12-818293-2.00006-9

Corbo, M. R., Campaniello, D., Speranza, B., Bevilacqua, A., & Sinigaglia, M. (2015). Non-conventional tools to preserve and prolong the quality of minimally-processed fruits and vegetables. *Coatings*, 5(4), 931–961. https://doi.org/10.3390/coatings5040931

Dai, L., Zhang, J., & Cheng, F. (2019). Effects of starches from different botanical sources and modification methods on physicochemical properties of starch-based edible films. International *Journal of Biological Macromolecules*, 132, 897–905. https://doi.org/10.1016/j.ijbiomac.2019.03.197

Davoodi, S., Davachi, S. M., Ghorbani Golkhajeh, A., Shekarabi, A. S., & Abbaspourrad, A. (2020). Development and Characterization of Salvia macrosiphon/Chitosan Edible Films. ACS *Sustainable Chemistry and Engineering*, 8(3), 1487–1496. https://doi.org/10.1021/acssuschemeng.9b05894

De Moraes, J. O., Scheibe, A. S., Sereno, A., & Laurindo, J. B. (2013). Scale-up of the production of cassava starch based films using tape-casting. *Journal of Food Engineering*, 119(4), 800–808. https://doi.org/10.1016/j.jfoodeng.2013.07.009

De Paola, M. G., Mammolenti, D., Lupi, F. R., De Santo, M. P., Gabriele, D., & Calabrò, V. (2021). Formulation and process investigation of glycerol/starch suspensions for edible films production by tape casting. *Chemical Papers*, (0123456789). https://doi.org/10.1007/s11696-021-01956-6

Debeaufort, F., & Voilley, A. (2009). Lipid-Based edible films and coatings. In Edible Films and Coatings for Food Applications (pp. 135–168). https://doi.org/10.1007/978-0-387-92824-1

Deng, L., Li, X., Miao, K., Mao, X., Han, M., Li, D. Changdao, M. & Ge, L. (2020). Development of Disulfide Bond Crosslinked Gelatin/ε-Polylysine Active Edible Film with Antibacterial and Antioxidant Activities. *Food and Bioprocess Technology*, 13(4), 577–588. https://doi.org/10.1007/s11947-020-02420-1

Dianin, I. B., Oliveira, A. G., Pimentel, T. C., Hernandes, N. F., & Costa, G. N. (2019). Edible biofilms formulated with whey protein isolate and L. Casei Probiotic Culture: Characterization and application in tomatoes and grapes. *Chemical Engineering Transactions*, 75(June), 469–474. https://doi.org/10.3303/CET1975079

Divya, K., Smitha, V., & Jisha, M. S. (2018). Antifungal, antioxidant and cytotoxic activities of chitosan nanoparticles and its use as an edible coating on vegetables. *International Journal of Biological Macromolecules*, 114, 572–577. https://doi.org/10.1016/j.ijbiomac.2018.03.130

Dou, L., Li, B., Zhang, K., Chu, X., & Hou, H. (2018). Physical properties and antioxidant activity of gelatin-sodium alginate edible films with tea polyphenols. *International Journal of Biological Macromolecules*, 118, 1377–1383. https://doi.org/10.1016/j.ijbiomac.2018.06.121

El-Sayed, H. S., El-Sayed, S. M., Mabrouk, A. M. M., Nawwar, G. A., & Youssef, A. M. (2021). Development of Eco-friendly Probiotic Edible Coatings Based on Chitosan, Alginate and Carboxymethyl Cellulose for Improving the Shelf Life of UF Soft Cheese. *Journal of Polymers and the Environment*, 29(6), 1941–1953. https://doi.org/10.1007/s10924-020-02003-3

Eom, H., Chang, Y., Lee, E. sil, Choi, H. D., & Han, J. (2018). Development of a starch/gum-based edible coating for rice cakes to retard retrogradation during storage. *Lwt,* 97(April), 516–522. https://doi.org/10.1016/j.lwt.2018.07.044

Escamilla-García, M., Delgado-Sánchez, L. F., Ríos-Romo, R. A., García-Almendárez, B. E., Calderón-Domínguez, G., Méndez-Méndez, J., Amaro-Reyes, A., Di Pierro, P. & Regalado-González, C. (2019). Effect of transglutaminase cross-linking in protein isolates from a mixture of Two Quinoa varieties with chitosan on the physicochemical properties of edible films. *Coatings*, 9(11). https://doi.org/10.3390/coatings9110736

Espitia, P. J. P., Batista, R. A., Azeredo, H. M. C., & Otoni, C. G. (2016). Probiotics and their potential applications in active edible films and coatings. *Food Research International*, 90, 42–52. https://doi.org/10.1016/j.foodres.2016.10.026

Falguera, V., Quintero, J. P., Jiménez, A., Muñoz, J. A., & Ibarz, A. (2011). Edible films and coatings: Structures, active functions and trends in their use. *Trends in Food Science and Technology*, 22(6), 292–303. https://doi.org/10.1016/j.tifs.2011.02.004

FAO. (2008). FAO Technical Meeting on Prebiotics. Advance Analytical Technologies. Rome.

Farina, V., Passafiume, R., Tinebra, I., Palazzolo, E., & Sortino, G. (2020). Use of Aloe vera gel-based edible coating with natural anti-browning and anti-oxidant additives to improve post-harvest quality of fresh-cut "Fuji" apple. *Agronomy*, 10(4). https://doi.org/10.3390/agronomy10040515

Fass, D., & Thorpe, C. (2018). Chemistry and Enzymology of Disulfide Cross-Linking in Proteins. *Chemical Reviews*, 118(3), 1169–1198. https://doi.org/10.1021/acs.chemrev.7b00123

FDA. Code of Federal Regulations, title 21, CFR 20, 25, 170, 184, 186 and 570. Substances Generally Recognized as Safe. (2016). 81(159), 54960–55055.

Fernandes, Leonardo M. Guimaraes Jonas T., Pimentel Tatiana C., Esmerino Erick A., Freitas Mônika Q., Carvalho Carlos Wanderlei P., C. A. G. and S. M. C. (2020). Chapter 7. Edible whey protein films and coatings added with prebiotic ingredients. In *Agri-Food Industry Sytrategies for Healthy Diets and sustainability* (pp. 177–193). https://doi.org/https://doi.org/10.1016/B978-0-12-817226-1.00007-2

Fernandes, L. M., Guimarães, J. T., Silva, R., Rocha, R. S., Coutinho, N. M., Balthazar, C. F., Calvacanti, R. N., Piler, C. W., Pimentel, T. C., Neto, R. P. C., Tavares, M. I. B., Esmerino,

E. A., Freitas, M. Q., Silva, N., C. & Cruz, A. G. (2020). Whey protein films added with galactooligosaccharide and xylooligosaccharide. *Food Hydrocolloids*, 104(February). https://doi.org/10.1016/j.foodhyd.2020.105755

Figueroa-González, I., Quijano, G., Ramírez, G., & Cruz-Guerrero, A. (2011). Probiotics and prebiotics-perspectives and challenges. *Journal of the Science of Food and Agriculture*, 91(8), 1341–1348. https://doi.org/10.1002/jsfa.4367

Food and Agriculture Organization (FAO) of the United Nations. (2002). Guidelines for the Evaluation of Probiotics in Food. Report of a Joint FAO/WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Food, 1–11. https://doi.org/10.5772/63646

Gao, J., Li, X., Zhang, G., Sadiq, F. A., Simal-Gandara, J., Xiao, J., & Sang, Y. (2021). Probiotics in the dairy industry—Advances and opportunities. *Comprehensive Reviews in Food Science and Food Safety*, 20(4), 3937–3982. https://doi.org/10.1111/1541-4337.12755

García, M. P. M., Gómez-Guillén, M. C., López-Caballero, M. E., & Barbosa-Cánovas, G. V. (2016). Edible films and coatings: fundamentals and applications. Crc Press.

Gedarawatte, S. T. G., Ravensdale, J. T., Johns, M. L., Azizi, A., Al-Salami, H., Dykes, G. A., & Coorey, R. (2021). Effectiveness of gelatine and chitosan spray coating for extending shelf life of vacuum-packaged beef. *International Journal of Food Science and Technology*, 56(8), 4026–4037. https://doi.org/10.1111/ijfs.15025

Gedikoğlu, A. P. A. (2022). The effect of Thymus vulgaris and Thymbra spicata essential oils and/or extracts in pectin edible coating on the preservation of sliced bolognas. *Meat Science*, 184(August 2021). https://doi.org/10.1016/j.meatsci.2021.108697

Gibson, G. R., Hutkins, R., Sanders, M. E., Prescott, S. L., Reimer, R. A., Salminen, S. J., Scott, K., Stanton, C., Swanson, K. S., Cani, P. D., Verbeke, K. & Reid, G. (2017). Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nature Reviews Gastroenterology and Hepatology*, 14(8), 491–502. https://doi.org/10.1038/nrgastro.2017.75

Gibson, G. R., & Roberfroid, M. B. (1995). Critical Review Dietary Modulation of the Human Colonic Microbiota: Introducing the Concept of Prebiotics. *Journal of Nutrition*, 125(June 1995), 1401–1412. https://doi.org/10.1093/jn/125.6.1401

Giosafatto, C. V. L., Fusco, A., Al-Asmar, A., & Mariniello, L. (2020). Microbial transglutaminase as a tool to improve the features of hydrocolloid-based bioplastics. *International Journal of Molecular Sciences*, 21(10). https://doi.org/10.3390/ijms21103656

Gómez-Guillén, M. C., Pérez-Mateos, M., Gómez-Estaca, J., López-Caballero, E., Giménez, B., & Montero, P. (2009). Fish gelatin: a renewable material for developing active biodegradable films. *Trends in Food Science and Technology*, 20(1), 3–16. https://doi.org/10.1016/j.tifs.2008.10.002

Gopalakrishnan, S., Xu, J., Zhong, F., & Rotello, V. M. (2021). Strategies for Fabricating Protein Films for Biomaterial Applications. *Advanced Sustainable Systems*, 5(1). https://doi.org/10.1002/adsu.202000167

Gregirchak, N., Stabnikova, O., & Stabnikov, V. (2020). Application of Lactic Acid Bacteria for Coating of Wheat Bread to Protect it from Microbial Spoilage. *Plant Foods for Human Nutrition*, 75(2), 223–229. https://doi.org/10.1007/s11130-020-00803-5

Guilbert, S., Gontard, N., & Gorris, L. G. M. (1996). Prolongation of the Shelf-life of Perishable Food Products using Biodegradable Films and Coatings. *Lebensm. Wiss. Technol*., 29, 10–17.

Guimarães, A., Abrunhosa, L., Pastrana, L. M., & Cerqueira, M. A. (2018). Edible Films and Coatings as Carriers of Living Microorganisms: A New Strategy Towards Biopreservation and Healthier Foods. *Comprehensive Reviews in Food Science and Food Safety*, 17(3), 594–614. https://doi.org/10.1111/1541-4337.12345

Hassan, B., Chatha, S. A. S., Hussain, A. I., Zia, K. M., & Akhtar, N. (2018). Recent advances on polysaccharides, lipids and protein based edible films and coatings: A review. International *Journal of Biological Macromolecules*, 109, 1095–1107. https://doi.org/10.1016/j.ijbiomac.2017.11.097

Hellebois, T., Tsevdou, M., & Soukoulis, C. (2020). Functionalizing and bio-preserving processed food products via probiotic and synbiotic edible films and coatings. *In Advances in Food and Nutrition Research* (1st ed., Vol. 94). https://doi.org/10.1016/bs.afnr.2020.06.004

Huang, T., Fang, Z., Zhao, H., Xu, D., Yang, W., Yu, W., & Zhang, J. (2020). Physical properties and release kinetics of electron beam irradiated fish gelatin films with antioxidants of bamboo leaves. *Food Bioscience*, 36(818), 100597. https://doi.org/10.1016/j.fbio.2020.100597

Hurtado-Romero, A., Del Toro-Barbosa, M., Garcia-Amezquita, L. E., & García-Cayuela, T. (2020). Innovative technologies for the production of food ingredients with prebiotic potential: Modifications, applications, and validation methods. *Trends in Food Science and Technology*, 104(July), 117–131. https://doi.org/10.1016/j.tifs.2020.08.007

Jeya Jeevahan, J., Chandrasekaran, M., Venkatesan, S. P., Sriram, V., Britto Joseph, G., Mageshwaran, G., & Durairaj, R. B. (2020). Scaling up difficulties and commercial aspects of edible films for food packaging: A review. *Trends in Food Science and Technology*, 100(February), 210–222. https://doi.org/10.1016/j.tifs.2020.04.014

Jiang, Y., Yu, L., Hu, Y., Zhu, Z., Zhuang, C., Zhao, Y., & Zhong, Y. (2019). Electrostatic spraying of chitosan coating with different deacetylation degree for strawberry preservation. International *Journal of Biological Macromolecules*, 139, 1232–1238. https://doi.org/10.1016/j.ijbiomac.2019.08.113

Jost, V., Kobsik, K., Schmid, M., & Noller, K. (2014). Influence of plasticiser on the barrier, mechanical and grease resistance properties of alginate cast films. *Carbohydrate Polymers*, 110, 309–319. https://doi.org/10.1016/j.carbpol.2014.03.096

Jotarkar, P. S., Panjagari, N. R., Singh, A. K., & Arora, S. (2018). Effect of whey protein-iron based edible coating on the quality of Paneer and process optimisation. *International Journal of Dairy Technology*, 71(2), 395–407. https://doi.org/10.1111/1471-0307.12478

Ju, J., Xie, Y., Guo, Y., Cheng, Y., Qian, H., & Yao, W. (2019). Application of edible coating with essential oil in food preservation. *Critical Reviews in Food Science and Nutrition*, 59(15), 2467–2480. https://doi.org/10.1080/10408398.2018.1456402

Karimi, N., Alizadeh, A., Almasi, H., & Hanifian, S. (2020). Preparation and characterization of whey protein isolate/polydextrose-based nanocomposite film incorporated with cellulose nanofiber and L. plantarum: A new probiotic active packaging system. *Lwt,* 121(December 2019). https://doi.org/10.1016/j.lwt.2019.108978

Khwaldia, K., Ferez, C., Banon, S., Desobry, S., & Hardy, J. (2004). Milk proteins for edible films and coatings. *Critical Reviews in Food Science and Nutrition*, 44(4), 239–251. https://doi.org/10.1080/10408690490464906

Koshy, R. R., Mary, S. K., Thomas, S., & Pothan, L. A. (2015). Environment friendly green composites based on soy protein isolate - A review. *Food Hydrocolloids*, 50, 174–192. https://doi.org/10.1016/j.foodhyd.2015.04.023

Kulawik, P., Jamróz, E., Zając, M., Guzik, P., & Tkaczewska, J. (2019). The effect of furcellaran-gelatin edible coatings with green and pu-erh tea extracts on the microbiological, physicochemical and sensory changes of salmon sushi stored at 4 °C. *Food Control*, 100(December 2018), 83–91. https://doi.org/10.1016/j.foodcont.2019.01.004

Kumar, N., Pratibha, Neeraj, Petkoska, A. T., Al-Hilifi, S. A., & Fawole, O. A. (2021). Effect of chitosan–pullulan composite edible coating functionalized with pomegranate peel extract on the shelf life of mango (Mangifera indica). *Coatings*, 11(7), 25–40. https://doi.org/10.3390/coatings11070764

Kumar, S. & Bhatnagar, T. (2014). Studies to enhance the shelf life of fruits using Aloe vera based herbal coatings: A review. International *Journal of Agriculture and food Science Technology*, 5, 211-218.

Kumari, N., Bangar, S. P., Petrů, M., Ilyas, R. A., Singh, A., & Kumar, P. (2021). Development and characterization of fenugreek protein-based edible film. *Foods*, 10(9), 1–13. https://doi.org/10.3390/foods10091976

Lara, G., Yakoubi, S., Villacorta, C. M., Uemura, K., Kobayashi, I., Takahashi, C., Nakajima, M. & Neves, M. A. (2020). Spray technology applications of xanthan gum-based edible coatings for fresh-cut lotus root (Nelumbo nucifera). *Food Research International*, 137(June), 1–10. https://doi.org/10.1016/j.foodres.2020.109723

Lashkari, H., Halabinejad, M., Rafati, A., & Namdar, A. (2020). Shelf Life Extension of Veal Meat by Edible Coating Incorporated with Zataria multiflora Essential Oil. *Journal of Food Quality*, 2020. https://doi.org/10.1155/2020/8871857

Li, C., Sheng, L., Sun, G., & Wang, L. (2020). The application of ultraviolet-induced photo-crosslinking in edible film preparation and its implication in food safety. *Lwt,* 131(May), 109791. https://doi.org/10.1016/j.lwt.2020.109791

Li, S., Ma, Y., Ji, T., Sameen, D. E., Ahmed, S., Qin, W., Dai, J., Suqing, L. & Liu, Y. (2020). Cassava starch/carboxymethylcellulose edible films embedded with lactic acid bacteria to extend the shelf life of banana. *Carbohydrate Polymers*, 248 (July), 116805. https://doi.org/10.1016/j.carbpol.2020.116805

Liminana, P., Garcia-Sanoguera, D., Quiles-Carrillo, L., Balart, R., & Montanes, N. (2018). Development and characterization of environmentally friendly composites from poly(butylene succinate) (PBS) and almond shell flour with different compatibilizers. *Composites Part B: Engineering*, 144(March), 153–162. https://doi.org/10.1016/j.compositesb.2018.02.031

Liu, H., Xie, F., Yu, L., Chen, L., & Li, L. (2009). Thermal processing of starch-based polymers. *Progress in Polymer Science* (Oxford), 34(12), 1348–1368. https://doi.org/10.1016/j.progpolymsci.2009.07.001

López de Lacey, A. M., López-Caballero, M. E., & Montero, P. (2014). Agar films containing green tea extract and probiotic bacteria for extending fish shelf-life. *LWT - Food Science and Technology*, 55(2), 559–564. https://doi.org/10.1016/j.lwt.2013.09.028

Ma, D., Jiang, Y., Ahmed, S., Qin, W., & Liu, Y. (2019). Physical and antimicrobial properties of edible films containing Lactococcus lactis. *International Journal of Biological Macromolecules*, 141, 378–386. https://doi.org/10.1016/j.ijbiomac.2019.09.006

Mangaraj, S., Yadav, A., Bal, L. M., Dash, S. K., & Mahanti, N. K. (2019). Application of Biodegradable Polymers in Food Packaging Industry: A Comprehensive Review. *Journal of Packaging Technology and Research*, 3(1), 77–96. https://doi.org/10.1007/s41783-018-0049-y

Marcet, I., Sáez, S., Rendueles, M., & Díaz, M. (2017). Edible films from residual delipidated egg yolk proteins. *Journal of Food Science and Technology*, 54(12), 3969–3978. https://doi.org/10.1007/s13197-017-2861-8

Maringgal, B., Hashim, N., Mohamed Amin Tawakkal, I. S., & Muda Mohamed, M. T. (2020). Recent advance in edible coating and its effect on fresh/fresh-cut fruits quality. *Trends in Food Science and Technology*, 96 (July 2019), 253–267. https://doi.org/10.1016/j.tifs.2019.12.024

Mbye, M., Baig, M. A., AbuQamar, S. F., El-Tarabily, K. A., Obaid, R. S., Osaili, T. M., Al-Nabulsi, A., Turner, M. S., Shah, N. P. & Ayyash, M. M. (2020). Updates on understanding of probiotic lactic acid bacteria responses to environmental stresses and highlights on proteomic analyses. *Comprehensive Reviews in Food Science and Food Safety*, 19(3), 1110–1124. https://doi.org/10.1111/1541-4337.12554

Milovmovic, M. K. P.-J. (2001). Lipids and biopackaging. Usage of lipids in edible films. *Journal of Agricultural Sciences*, 46(1), 79–87.

Minh, N. P., Vo, T. T., Dung, H. T. B., Ngoc, T. T. B., Duong, L. C., & Nghi, T. T. T. (2019). Preservation of jackfruit bulb by transglutaminase crosslinked whey protein / pectin as edible film coating. *Journal of Pharmaceutical Sciences and Research*, 11(4), 1401–1405.

Moghadam, M., Salami, M., Mohammadian, M., Khodadadi, M., & Emam-Djomeh, Z. (2020). Development of antioxidant edible films based on mung bean protein enriched with pomegranate peel. *Food Hydrocolloids,* 104(November 2019), 105735. https://doi.org/10.1016/j.foodhyd.2020.105735

Monteiro, S. S., Silva, W. P. da, Monteiro, S. S., Gomes, J. P., Pereira, E. M., & Ferreira, J. P. de L. (2022). Probiotic coating applied to papaya slices for high quality snack production by convective drying. *Journal of Food Processing and Preservation*, 46(1), 1–17. https://doi.org/10.1111/jfpp.16183

Mozaffarzogh, M., Misaghi, A., Shahbazi, Y., & Kamkar, A. (2020). Evaluation of probiotic carboxymethyl cellulose-sodium caseinate films and their application in extending shelf life quality of fresh trout fillets. *Lwt*, 126(March), 109305. https://doi.org/10.1016/j.lwt.2020.109305

Nayanakanthi, T. P. K., Senanayake, M., & Siranjiv, P. (2021). Development of an Edible Coating from Okra Mucilage to Preserve the Crispiness in Soft Dough Biscuits Upon Storage. 1(2), 307–320.

Neri-Numa, I. A., & Pastore, G. M. (2020). Novel insights into prebiotic properties on human health: A review. *Food Research International*, 131(October 2019), 108973. https://doi.org/10.1016/j.foodres.2019.108973

Nguyen, T. T., Thi Dao, U. T., Thi Bui, Q. P., Bach, G. L., Ha Thuc, C. N., & Ha Thuc, H. (2020). Enhanced antimicrobial activities and physiochemical properties of edible film based on chitosan incorporated with Sonneratia caseolaris (L.) Engl. leaf extract. Progress in Organic *Coatings*, 140(October 2019), 105487. https://doi.org/10.1016/j.porgcoat.2019.105487

Nisar, T., Alim, A., Iqbal, T., Iqbal, M., Tehseen, S., Zi-Chao, W., & Guo, Y. (2022). Functionality of different probiotic strains embedded in citrus pectin based edible films. International *Journal of Food Science and Technology*, 57(2), 1005–1015. https://doi.org/10.1111/ijfs.15460

Ochoa-Yepes, O., Di Giogio, L., Goyanes, S., Mauri, A., & Famá, L. (2019). Influence of process (extrusion/thermo-compression, casting) and lentil protein content on physicochemical properties of starch films. *Carbohydrate Polymers*, 208(December 2018), 221–231. https://doi.org/10.1016/j.carbpol.2018.12.030

Odila Pereira, J., Soares, J., J.P. Monteiro, M., Gomes, A., & Pintado, M. (2018). Impact of whey protein coating incorporated with Bifidobacterium and Lactobacillus on sliced ham properties. *Meat Science*, 139(October 2017), 125–133. https://doi.org/10.1016/j.meatsci.2018.01.016

Oldoni, F. C. A., Bernardo, M. P., Oliveira Filho, J. G., de Aguiar, A. C., Moreira, F. K. V., Mattoso, L. H. C., Colnago, L. A. & Ferreira, M. D. (2021). Valorization of mangoes with internal

breakdown through the production of edible films by continuous solution casting. *Lwt*, 145(March), 111339. https://doi.org/10.1016/j.lwt.2021.111339

Oliveira-Alcântara, A. V., Abreu, A. A. S., Gonçalves, C., Fuciños, P., Cerqueira, M. A., Gama, F. M. P., Pastrana, L. M., Rodrigues, S. & Azeredo, H. M. C. (2020). Bacterial cellulose/cashew gum films as probiotic carriers. *Lwt*, 130(March), 109699. https://doi.org/10.1016/j.lwt.2020.109699

Oliveira de Moraes, J., Scheibe, A. S., Augusto, B., Carciofi, M., & Laurindo, J. B. (2015). Conductive drying of starch-fiber films prepared by tape casting: Drying rates and film properties. *LWT - Food Science and Technology*, 64(1), 356–366. https://doi.org/10.1016/j.lwt.2015.05.038

Orozco-Parra, J., Mejía, C. M., & Villa, C. C. (2020). Development of a bioactive synbiotic edible film based on cassava starch, inulin, and Lactobacillus casei. *Food Hydrocolloids*, 104(January), 105754. https://doi.org/10.1016/j.foodhyd.2020.105754

Ortiz, C. M., de Moraes, J. O., Vicente, A. R., Laurindo, J. B., & Mauri, A. N. (2017). Scale-up of the production of soy (Glycine max L.) protein films using tape casting: Formulation of film-forming suspension and drying conditions. *Food Hydrocolloids*, 66, 110–117. https://doi.org/10.1016/j.foodhyd.2016.12.029

Otoni, C. G., Lodi, B. D., Lorevice, M. V., Leitão, R. C., Ferreira, M. D., Moura, M. R. d., & Mattoso, L. H. C. (2018). Optimized and scaled-up production of cellulose-reinforced biodegradable composite films made up of carrot processing waste. Industrial *Crops and Products*, 121(March), 66–72. https://doi.org/10.1016/j.indcrop.2018.05.003

Parreidt, T. S., Müller, K., & Schmid, M. (2018). Alginate-based edible films and coatings for food packaging applications. *Foods*, 7(10), 1–38. https://doi.org/10.3390/foods7100170

Paulo, A. F. S., Baú, T. R., Ida, E. I., & Shirai, M. A. (2021). Edible coatings and films with incorporation of prebiotics —A review. *Food Research International*, 148(March). https://doi.org/10.1016/j.foodres.2021.110629

Pavli, F., Kovaiou, I., Apostolakopoulou, G., Kapetanakou, A., Skandamis, P., Nychas, G. J. E., Tassou, C. & Chorianopoulos, N. (2017). Alginate-based edible films delivering probiotic bacteria to sliced ham pretreated with high pressure processing. *International Journal of Molecular Sciences*, 18(9). https://doi.org/10.3390/ijms18091867

Pereira, J. O., Soares, J., Costa, E., Silva, S., Gomes, A., & Pintado, M. (2019). Characterization of edible films based on alginate or whey protein incorporated with Bifidobacterium animalis subsp. lactis BB-12 and prebiotics. *Coatings*, 9(8). https://doi.org/10.3390/coatings9080493

Pinzon, M. I., Sanchez, L. T., Garcia, O. R., Gutierrez, R., Luna, J. C., & Villa, C. C. (2020). Increasing shelf life of strawberries (Fragaria ssp) by using a banana starch-chitosan-Aloe vera gel composite edible coating. International *Journal of Food Science and Technology*, 55(1), 92–98. https://doi.org/10.1111/ijfs.14254

Pop, O. L., Pop, C. R., Dufrechou, M., Vodnar, D. C., Socaci, S. A., Dulf, F. V., Minervini, F. & Suharoschi, R. (2020). Edible films and coatings functionalization by probiotic incorporation: A review. *Polymers*, 12(1), 1–15. https://doi.org/10.3390/polym12010012

Rhim, J. W., Mohanty, A. K., Singh, S. P., & Ng, P. K. W. (2006). Effect of the processing methods on the performance of polylactide films: Thermocompression versus solvent casting. *Journal of Applied Polymer Science*, 101(6), 3736–3742. https://doi.org/10.1002/app.23403

Ribeiro, A. M., Estevinho, B. N., & Rocha, F. (2021). Preparation and Incorporation of Functional Ingredients in Edible Films and Coatings. *Food and Bioprocess Technology*, 14(2), 209–231. https://doi.org/10.1007/s11947-020-02528-4

Robledo, N., Vera, P., López, L., Yazdani-Pedram, M., Tapia, C., & Abugoch, L. (2018). Thymol nanoemulsions incorporated in quinoa protein/chitosan edible films; antifungal effect in cherry tomatoes. *Food Chemistry*, 246(August 2017), 211–219. https://doi.org/10.1016/j.foodchem.2017.11.032

Rodrigues, F. J., Cedran, M. F., Bicas, J. L., & Sato, H. H. (2020). Encapsulated probiotic cells: Relevant techniques, natural sources as encapsulating materials and food applications – A narrative review. *Food Research International*, 137(April), 109682. https://doi.org/10.1016/j.foodres.2020.109682

Rodrigues, Fábio J., Cedran, M. F., & Garcia, S. (2018). Influence of linseed mucilage incorporated into an alginate-base edible coating containing probiotic bacteria on shelf-life of fresh-cut yacon (Smallanthus sonchifolius). *Food and Bioprocess Technology*, 11(8), 1605–1614. https://doi.org/10.1007/s11947-018-2128-z

Rodríguez, G. M., Sibaja, J. C., Espitia, P. J. P., & Otoni, C. G. (2020). Antioxidant active packaging based on papaya edible films incorporated with Moringa oleifera and ascorbic acid for food preservation. *Food Hydrocolloids*, 103(November 2019), 105630. https://doi.org/10.1016/j.foodhyd.2019.105630

Rojas-Lema, S., Nilsson, K., Trifol, J., Langton, M., Gomez-Caturla, J., Balart, R., García-García, D. & Moriana, R. (2021). "Faba bean protein films reinforced with cellulose nanocrystals as edible food packaging material." *Food Hydrocolloids*, 121(February), 107019. https://doi.org/10.1016/j.foodhyd.2021.107019

Saad, N., Delattre, C., Urdaci, M., Schmitter, J. M., & Bressollier, P. (2013). An overview of the last advances in probiotic and prebiotic field. *LWT - Food Science and Techn*ology, 50(1), 1–16. https://doi.org/10.1016/j.lwt.2012.05.014

Sáez-Orviz, S., Marcet, I., Rendueles, M., Díaz, M. (2021). Bioactive packaging based on delipidated egg yolk protein edible films with lactobionic acid and Lactobacillus plantarum CECT 9567: characterization and use as coating in a food model. *Food Hydrocolloids*.

Sáez-Orviz, S., Puertas, C., Marcet, I., Rendueles, M., & Díaz, M. (2020). Bioactive synbiotic coatings with lactobionic acid and Lactobacillus plantarum CECT 9567 in the production

and characterization of a new functional dairy product. *Journal of Functional Foods*, 75(October). https://doi.org/10.1016/j.jff.2020.104263

Saleem, M. S., Anjum, M. A., Naz, S., Ali, S., Hussain, S., Azam, M., Sardar, H., Khaliq, G., Canan, I. & Ejaz, S. (2021). Incorporation of ascorbic acid in chitosan-based edible coating improves postharvest quality and storability of strawberry fruits. *International Journal of Biological Macromolecules*, 189(August), 160–169. https://doi.org/10.1016/j.ijbiomac.2021.08.051

Saleem, M. S., Ejaz, S., Anjum, M. A., Nawaz, A., Naz, S., Hussain, S., Ali, S. & Canan, İ. (2020). Postharvest application of gum arabic edible coating delays ripening and maintains quality of persimmon fruits during storage. *Journal of Food Processing and Preservation*, 44(8), 1–13. https://doi.org/10.1111/jfpp.14583

Schmid, M., & Müller, K. (2018). Whey protein-based packaging films and coatings. In *Whey Proteins: From Milk to Medicine*. https://doi.org/10.1016/B978-0-12-812124-5.00012-6

Schmitt, C., Sanchez, C., Desobry-Banon, S., & Hardy, J. (1998). Structure and Technofunctional Properties of Protein-Polysaccharide Complexes : A Review. *Critical Reviews in Food Science and Nutrition*, 38(8), 689–753.

Shahrampour, D., Khomeiri, M., Razavi, S. M. A., & Kashiri, M. (2020). Development and characterization of alginate/pectin edible films containing Lactobacillus plantarum KMC 45. *Lwt*, 118(July 2019), 108758. https://doi.org/10.1016/j.lwt.2019.108758

Shigematsu, E., Dorta, C., Rodrigues, F. J., Cedran, M. F., Giannoni, J. A., Oshiiwa, M., & Mauro, M. A. (2018). Edible coating with probiotic as a quality factor for minimally processed carrots. *Journal of Food Science and Technology*, 55(9), 3712–3720. https://doi.org/10.1007/s13197-018-3301-0

Silva-Vera, W., Zamorano-Riquelme, M., Rocco-Orellana, C., Vega-Viveros, R., Gimenez-Castillo, B., Silva-Weiss, A., & Osorio-Lira, F. (2018). Study of spray system applications of edible coating suspensions based on hydrocolloids containing cellulose nanofibers on grape surface (Vitis vinifera L.). *Food and Bioprocess Technology*, 11(8), 1575–1585. https://doi.org/10.1007/s11947-018-2126-1

Simen Sørbø, J. L. (2022). Effect of edible coating and modified atmosphere packaging on the microbiological and physicochemical stability of retail maki sushi. *Journal of Food Science*.

Siriwardana, J., & Wijesekara, I. (2021). Analysis of the Effectiveness of an Antimicrobial Edible Coating Prepared from Sweet Whey Base to Improve the Physicochemical, Microbiological, and Sensory Attributes of Swiss Cheese. *Advances in Agriculture*, 2021. https://doi.org/10.1155/2021/5096574

Socaciu, M. I., Fogarasi, M., Semeniuc, C. A., Socaci, S. A., Rotar, M. A., Mureşan, V., Pop, O. L. & Vodnar, D. C. (2020). Formulation and characterization of antimicrobial edible films based on whey protein isolate and tarragon essential oil. *Polymers*, 12(8), 1–21. https://doi.org/10.3390/POLYM12081748

Soukoulis, C., Behboudi-Jobbehdar, S., Macnaughtan, W., Parmenter, C., & Fisk, I. D. (2017). Stability of Lactobacillus rhamnosus GG incorporated in edible films: Impact of anionic biopolymers and whey protein concentrate. *Food Hydrocolloids*, 70, 345–355. https://doi.org/10.1016/j.foodhyd.2017.04.014

Soukoulis, C., Yonekura, L., Gan, H. H., Behboudi-Jobbehdar, S., Parmenter, C., & Fisk, I. (2014). Probiotic edible films as a new strategy for developing functional bakery products: The case of pan bread. *Food Hydrocolloids*, 39, 231–242. https://doi.org/10.1016/j.foodhyd.2014.01.023

Strano, M. C., Restuccia, C., De Leo, R., Mangiameli, S., Bedin, E., Allegra, M., Quartieri, A., Cirvilleri, G. & Pulvirenti, A. (2021). Efficacy of an antifungal edible coating for the quality maintenance of Tarocco orange fruit during cold storage. *Crop Protection*, 148(March), 105719. https://doi.org/10.1016/j.cropro.2021.105719

Suhag, R., Kumar, N., Petkoska, A. T., & Upadhyay, A. (2020). Film formation and deposition methods of edible coating on food products: A review. *Food Research International*, 136(July), 109582. https://doi.org/10.1016/j.foodres.2020.109582

Umaraw, P., & Verma, A. K. (2017). Comprehensive review on application of edible film on meat and meat products: An eco-friendly approach. *Critical Reviews in Food Science and Nutrition* (Vol. 57). https://doi.org/10.1080/10408398.2014.986563

Urbizo-Reyes, U., San Martin-González, M. F., Garcia-Bravo, J., & Liceaga, A. M. (2020). Development of chia seed (Salvia hispanica) mucilage films plasticized with polyol mixtures: Mechanical and barrier properties. *International Journal of Biological Macromolecules*, 163, 854–864. https://doi.org/10.1016/j.ijbiomac.2020.07.023

Valerio, F., Volpe, M. G., Santagata, G., Boscaino, F., Barbarisi, C., Di Biase, M., Bavaro, A. R., Lonigro, S. L. & Lavermicocca, P. (2020). The viability of probiotic Lactobacillus paracasei IMPC2.1 coating on apple slices during dehydration and simulated gastro-intestinal digestion. *Food Bioscience*, 34(January), 100533. https://doi.org/10.1016/j.fbio.2020.100533

Vargas, M., Pastor, C., Chiralt, A., McClements, D. J., & González-Martínez, C. (2008). Recent advances in edible coatings for fresh and minimally processed fruits. *Critical Reviews in Food Science and Nutrition*, 48(6), 496–511. https://doi.org/10.1080/10408390701537344

Vedove, T. M. A. R. D., Maniglia, B. C., & Tadini, C. C. (2021). Production of sustainable smart packaging based on cassava starch and anthocyanin by an extrusion process. *Journal of Food Engineering*, 289(October 2019), 110274. https://doi.org/10.1016/j.jfoodeng.2020.110274

Venkatachalam, K., & Lekjing, S. (2020). A chitosan-based edible film with clove essential oil and nisin for improving the quality and shelf life of pork patties in cold storage. *RSC Advances*, 10(30), 17777–17786. https://doi.org/10.1039/d0ra02986f

Vieira, M. G. A., Da Silva, M. A., Dos Santos, L. O., & Beppu, M. M. (2011). Natural-based plasticizers and biopolymer films: A review. *European Polymer Journal*, 47(3), 254–263. https://doi.org/10.1016/j.eurpolymj.2010.12.011

Wang, A., Lin, J., & Zhong, Q. (2021). Spray-coating as a novel strategy to supplement broiler feed pellets with probiotic Lactobacillus salivarius NRRL B-30514. *Lwt*, 137(June 2020), 110419. https://doi.org/10.1016/j.lwt.2020.110419

Warnakulasuriya, S. N., & Nickerson, M. T. (2018). Review on plant protein–polysaccharide complex coacervation, and the functionality and applicability of formed complexes. *Journal of the Science of Food and Agriculture*, 98(15), 5559–5571. https://doi.org/10.1002/jsfa.9228

Wong, C. H., Mak, I. E. K., & Li, D. (2021). Bilayer edible coating with stabilized Lactobacillus plantarum 299v improved the shelf life and safety quality of fresh-cut apple slices. *Food Packaging and Shelf Life*, 30(August), 100746. https://doi.org/10.1016/j.fpsl.2021.100746

Xiong, Y., Chen, M., Warner, R. D., & Fang, Z. (2020). Incorporating nisin and grape seed extract in chitosan-gelatine edible coating and its effect on cold storage of fresh pork. *Food Control*, 110(August 2019), 107018. https://doi.org/10.1016/j.foodcont.2019.107018

Xu, J., Xia, R., Yuan, T., & Sun, R. (2019). Use of xylooligosaccharides (XOS) in hemicelluloses/chitosan-based films reinforced by cellulose nanofiber: Effect on physicochemical properties. *Food Chemistry*, 298(June). https://doi.org/10.1016/j.foodchem.2019.125041

Yan, W., Jia, X., Zhang, Q., Chen, H., Zhu, Q., & Yin, L. (2021). Interpenetrating polymer network hydrogels of soy protein isolate and sugar beet pectin as a potential carrier for probiotics. *Food Hydrocolloids*, 113(October 2020), 106453. https://doi.org/10.1016/j.foodhyd.2020.106453

Yousuf, B., Sun, Y., & Wu, S. (2021). Lipid and Lipid-containing Composite Edible Coatings and Films. *Food Reviews International*, 00(00), 1–24. https://doi.org/10.1080/87559129.2021.1876084

Zabihollahi, N., Alizadeh, A., Almasi, H., Hanifian, S., & Hamishekar, H. (2020). Development and characterization of carboxymethyl cellulose based probiotic nanocomposite film containing cellulose nanofiber and inulin for chicken fillet shelf life extension. *International Journal of Biological Macromolecules*, 160, 409–417. https://doi.org/10.1016/j.ijbiomac.2020.05.066

Zendeboodi, F., Khorshidian, N., Mortazavian, A. M., & da Cruz, A. G. (2020). Probiotic: conceptualization from a new approach. *Current Opinion in Food Science*, 32(April), 103–123. https://doi.org/10.1016/j.cofs.2020.03.009

Zhang, B., Qiao, D., Zhao, S., Lin, Q., Wang, J., & Xie, F. (2021). Starch-based food matrices containing protein: Recent understanding of morphology, structure, and properties. *Trends in Food Science and Technology*, 114(June), 212–231. https://doi.org/10.1016/j.tifs.2021.05.033

Zoghi, A., Khosravi-Darani, K., & Mohammadi, R. (2020). Application of edible films containing probiotics in food products. *Journal of Consumer protection and Food Safety*, 15(4), 307–320. https://doi.org/10.1007/s00003-020-01286-x

Zong, X., Zhang, X., Bi, K., Zhou, Y., Zhang, M., Qi, J., Xu, X., Mei, l., Xiong, G. & Fu, M. (2021). Novel emulsion film based on gelatin/polydextrose/camellia oil incorporated with Lactobacillus pentosus: Physical, structural, and antibacterial properties. *Food Hydrocolloids*, 121(July), 107063. https://doi.org/10.1016/j.foodhyd.2021.10706

Capítulo 3.

Materiales y métodos

3.1. Microorganismos

3.1.1. *Lactobacillus plantarum* CECT 9567

Como microorganismo probiótico se empleó la cepa *Lactobacillus plantarum* CECT 9567 (Colección Española de Cultivos Tipo, Valencia, España). Se trata de una bacteria del ácido láctico (BAL) aislada a partir de kéfir. Es aerobia, Gram-positiva y de amplio uso en la industria alimentaria por su actividad probiótica (De Montijo-Prieto, Castro, Reina, Jimenez-Valera, & Ruiz-Bravo, 2019). Previamente a su uso en los diferentes biomateriales, se comprobó que esta cepa era capaz de emplear ácido lactobiónico como sustrato para su crecimiento, obteniendo un resultado positivo. *L. plantarum* se mantuvo congelada a -20°C en una solución de glicerol al 40% (*v*/*v*). Para su uso, este microorganismo se cultivó en placas de MRS (de Man Rogosa and Sharpe, Sigma-Aldrich) con un 2% (*p*/*v*) de agar (VWR). Las placas se incubaron a 30°C durante 48 h y se almacenaron hasta su uso a 4°C.

Para su adición a los diferentes biomateriales, *L. plantarum* se inoculó en medio MRS líquido, añadiendo 100 mL de sustrato en matraces de 250 mL (relación volumen de trabajo:aire 1:4). Estos cultivos se crecieron en un incubador orbital (modelo G25, New Brunswick Scientific Co., Nueva Jersey, EEUU) a 200 rpm y 30°C durante 24 h.

3.1.2. *Lactobacillus paracasei* CBA L74

Lactobacillus paracasei CBA L74 (microorganismo patentado y proporcionado por Heinz Italia S.p.A) se empleó como bacteria probiótica en el desarrollo de cápsulas sinbióticas. Se trata de una bacteria Gram-positiva, anaerobia facultativa y homo-fermentativa. Al igual que con *L. plantarum*, se comprobó previamente la capacidad de esta cepa para emplear ácido lactobiónico como sustrato para su crecimiento, obteniendo un resultado positivo.

L. paracasei se mantuvo congelada a -20°C en una solución de glicerol al 40% (*v*/*v*). Para su uso, este microorganismo se revivió en 10 mL de animal-free broth (AFB) compuesto por 20 g/L de extracto de levadura bacto (BD Biosciences, Milán, Italia), 0,5 g/L $MgSO_4$ (Sigma-Aldrich), 50 g/L de glucosa (Sigma-Aldrich) y 0,5 g/L de ácido cítrico (Sigma-Aldrich). La cepa se incubó a 37°C durante 24 h y tras este tiempo se empleó para la elaboración de capsulas sinbióticas.

3.1.3. *Pseudomonas taetrolens* LMG 2366

Para la producción de ácido lactobiónico como compuesto prebiótico, se empleó la bacteria *Pseudomonas taetrolens* LMG 2366 (Colección Coordenada Belga de

Microorganismos (BCCM), Gante, Bélgica). Se trata de una bacteria aerobia, Gram-negativa y no patogénica. Es capaz de oxidar la lactosa con un 100% de selectividad para el ácido lactobiónico y con elevados rendimientos de conversión (Alonso, Rendueles, & Díaz, 2011). *P. taetrolens* se mantuvo congelada a -20°C en una solución de glicerol 40% (*v*/*v*). Para su uso, se cultivó en placas de NB (Nutrient Broth (Sigma-Aldrich), compuesto de 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, con un 2% de agar (*p*/*v*) (VWR). Las placas se incubaron a 30°C durante 48 h y se conservaron a 4°C hasta su uso.

3.1.3.1. Fermentación en biorreactor con agitación mecánica para la producción de ácido lactobiónico

Uno de los tipos de biomateriales desarrollados en la presente tesis con ácido lactobiónico se elaboró a partir de la fermentación de suero dulce de leche con *P. taetrolens* en un biorreactor con agitación mecánica.

Para ello, se empleó permeado de ultrafiltración de suero dulce de queso suministrado por Industrias Lácteas Asturianas (ILAS) S.A. (Navia, Asturias, España). Este suero está compuesto principalmente por lactosa (~ 200 g/L) y una pequeña concentración de sales minerales (menos del 5% del permeado de ultrafiltración). El contenido de proteínas de este sustrato es insignificante, tal y como se determinó por el método de Bradford (Bradford M.M., 1976). Este permeado de suero se diluyó con agua destilada hasta alcanzar una concentración de 40 g/L y se ajustó el pH a 6,50 con NaOH 10N (Sigma-Aldrich). El permeado de suero se esterilizó empleando un sistema de microfiltración tangencial, equipado con un *cassette* de membranas de PVDF con un área de membrana de 0,5 m^2 (Pellicom 2 cassette) y con un tamaño de poro de 0,22 µm (Millipore, California, EEUU). La microfiltración se realizó con una presión transmembrana de 1,4 bar. Este permeado microfiltrado se empleó como sustrato en el pre-inóculo y en la fermentación.

Primero, *P. taetrolens* se inoculó en un matraz Erlenmeyer de 500 mL, conteniendo 100 mL de NB (relación volumen de trabajo:aire 1:4) y se incubó a 30°C durante 10 h con una agitación de 250 rpm. Tras este tiempo, se realizó un pre-inóculo al 10% (*v*/*v*) en el permeado microfiltrado bajo las mismas condiciones de incubación. Tras este tiempo, la biomasa se obtuvo por centrifugación (10 000 rpm durante 10 min). Las fermentaciones se escalaron a un biorreactor de agitación mecánica (modelo BioFlo 110, New Brunswick Scientific Co. Inc., Edison, Nueva Jersey, EEUU) con 2 L de capacidad. El volumen de trabajo del biorreactor fue de 1 L que se inoculó al 10% (*v/v)* con el pre-inóculo realizado usando como sustrato permeado microfiltrado. El

biorreactor estaba 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) de tal forma que los valores de pH y oxígeno disuelto se pudieron monitorizar a lo largo de toda la fermentación. Los parámetros de operación empleados fueron de 30°C, 350 rpm y 1 Lpm de aireación suministrado mediante burbujeo, teniendo en cuenta las condiciones optimizadas por Alonso *et al.* (2012) para optimizar la producción de ácido lactobiónico. La formación de espuma se evitó adicionando automáticamente emulsión antiespumante Y-30 (Sigma-Aldrich) diluida 1:10 en agua destilada. Para maximizar la producción de ácido lactobiónico, el pH se dejó libre durante la fase de crecimiento exponencial y tras el comienzo de la fase estacionaria el pH se mantuvo en 6,50 añadiendo NaOH 5N (Sigma-Aldrich) (Alonso et al., 2012). Las fermentaciones terminaron a las 72 h.

3.1.3.2. Detección y eliminación de endotoxinas

Al tratarse de una bacteria Gram-negativa, *P. taetrolens* tiene endotoxinas de forma natural. Las endotoxinas son agregados complejos de lipopolisacáridos (LPS) y son uno de los componentes principales de la membrana externa de las bacterias Gram-negativas. A pesar de estar unidas a la pared bacteriana, los LPS se liberan de forma constante al medio (Magalhães, Lopes, & Priscila, 2007). Los LPS están formados por una región interna compuesta por grupos de ácidos grasos hidrofóbicos (lípido A) y una región central y externa compuesta por polisacáridos hidrofílicos (Heine, Rietschel, & Ulmer, 2001). Los LPS son sustancias pirogénicas, tóxicas para humanos y animales, bastante resistentes a cambios de pH y temperatura, con un tamaño de 10 a 20 kDa y que cuando se encuentran agregadas pueden tener un tamaño de hasta 1 MDa. Además, la presencia de iones Ca^{2+} y Mg^{2+} estabilizan los agregados de LPS (Li & Luo, 1999).

Aunque no existe un límite disponible para la concentración de endotoxinas que puede haber en los productos administrados por vía oral, en la industria farmacéutica este límite está establecido en 5 unidades de endotoxina (EU) por kilogramo de masa corporal (Wassenaar & Zimmermann, 2018). Por tanto, para que los productos obtenidos tras la fermentación con *P. taetrolens* se puedan emplear en el ámbito alimentario, las endotoxinas se deben eliminar. Por ello, tras la fermentación el medio se centrifugó (10000 rpm durante 20 min) y el sobrenadante se microfiltró tangencialmente tal y como se explicó en la *Sección 3.1.3.1*. La presencia de endotoxinas se detectó empleando el kit ToxinSensor™ Gel Clot (GenScript, Piscataway, Nueva Jersey, EEUU). Este kit se usó para detectar la presencia de

endotoxinas antes y después de la microfiltración tangencial. Se siguió el protocolo de detección semi-cuantitativa, en la que la concentración mínima de endotoxinas detectadas es de 0,25 EU/mL.

3.2. Polímeros

Para la elaboración de los distintos biomateriales se emplearon diferentes reactivos químicos comerciales: gelatina, alginato de sodio, maltodextrina, caseinato de sodio, glicerol (todos ellos de Sigma-Aldrich, Steinheim, Alemania) y goma arábiga (Panreac S.A., Barcelona, España). Además, como compuesto prebiótico se empleó ácido lactobiónico y lactobionato de calcio (su forma de sal más habitual), ambos con un 98% de pureza y de Sigma-Aldrich.

3.2.1. Obtención de gránulos de yema delipidada

Los gránulos delipidados de la yema de huevo se obtuvieron siguiendo el protocolo de Marcet *et al.* (2014) con algunas modificaciones. La yema se separó de la clara y se secó con papel secante. La vitelina se rompió para conseguir exclusivamente el contenido de la yema del huevo. Ésta se mezcló con agua destilada en una proporción 1:1,5 (*v/v*). El pH de esta solución se ajustó a 7,0 usando NaOH 0,1M (Sigma-Aldrich). La solución se centrifugó a 10,000 rpm durante 45 min. El sedimento, compuesto mayoritariamente por los gránulos, se recogió y liofilizó (0,1 mBar, -70°C durante 24 h) (Tesltar Cryodos, Barcelona, España). Los gránulos de yema de huevo liofilizados se delipidaron empleando etanol absoluto (99%, VWR, VWR Chemicals, Pensilvania, EEUU) en una proporción 50:1 (*p/v*) Esta solución se mantuvo en agitación a temperatura ambiente durante 2 h. Tras este tiempo, los gránulos se recuperaron filtrándolos con una bomba de vacío y papel Whatman no 1. Los gránulos delipidados de proteína de yema de huevo fueron secados a 40°C durante 24 h en una estufa y se mantuvieron a -20°C hasta su uso. El peso seco de los gránulos delipidados obtenidos fue del 96% (*p/p*).

3.3. Preparación de biomateriales

3.3.1. *Films*

En la presente tesis se desarrollaron *films* empleando dos proteínas diferentes como matriz: proteína de yema de huevo delipidada y gelatina.

Para elaborar los *films* de proteína de yema de huevo delipidada se disolvieron 3 g de estos en 75,5 mL de agua destilada. A continuación, se añadieron 1,5 ml de NaOH 1M. La mezcla se mantuvo en agitación durante 20 min a 65°C hasta que se observó una

apariencia homogénea en la solución. Después, la mezcla se centrifugó a 10 000 rpm durante 10 min y se recuperó el sobrenadante. Se añadió glicerol (Panreac S.A., Barcelona, España) en una concentración del 50% (*p*/*p*) con respecto al peso total de proteína añadido. Se prepararon *films* que fueron tratados con transglutaminasa (TG) para analizar si se mejoraban las propiedades mecánicas de los mismos. En este caso, el pH se ajustó a 8,6 con HCl 1M y se añadieron 10 U de TG por gramo de proteína (Probind TX, BDF ingredients, Girona, España). La reacción se mantuvo en un horno a 45°C durante 90 min. Tras este tiempo se subió el pH a 10,0 con NaOH 1M. De otra manera, también se prepararon *films* probióticos (PRO) conteniendo *L. plantarum*, prebióticos (PRE) conteniendo ácido lactobiónico y sinbióticos (SYN) conteniendo ambos. La composición de cada uno de ellos se muestra en la Tabla 3.1. El valor de pH final de las soluciones formadoras de *film* PRO, PRE y SYN fue de 11,6.

Tabla 3.1. Composición de los cuatro tipos diferentes de *films* elaborados con proteína delipidada de yema de huevo como matriz.

Film	Ácido lactobiónico	*L. plantarum* CECT 9567
Control	-	-
Prebiótico (PRE)	10 g/L	-
Probiótico (PRO)	-	8 $\log_{10}$ UFC/mL
Sinbiótico (SYN)	10 g/L	8 $\log_{10}$ UFC/mL

Tabla 3.2. Composición de los diferentes *films* elaborados empleando fermentado de suero microfiltrado.

Film	Gelatina (g/100 mL de solución formadora de *film*)	LBA (g/100 mL de solución formadora de *film*)	*L. plantarum*
Control (C45)	1,8	-	
Control (C90)	3,6	-	
LBA45	1,8	~ 4	
LBA90	3,6	~ 4	
LP45	1,8	~ 4	8 $\log_{10}$ UFC/mL
LP90	3,6	~ 4	8 $\log_{10}$ UFC/mL

Los *films* empleando gelatina como matriz se desarrollaron partiendo del fermentado de permeado de suero microfiltrado. Teniendo en cuenta la concentración de ácido lactobiónico del medio fermentado, se añadieron dos concentraciones diferentes de gelatina (0,45 y 0,9 g gelatina/ g ácido lactobiónico). La mezcla se calentó a 40°C hasta

que se obtuvo una disolución homogénea. Con esta solución formadora de *film* se elaboraron seis *films* diferentes (Tabla 3.2), teniendo en cuenta la cantidad de gelatina añadida y la presencia o no de *L. plantarum* como microorganismo probiótico.
En ambos casos y para obtener los *films*, 20 mL de las soluciones formadoras de *film* se añadieron a placas Petri y se secaron en una estufa a 40°C durante 24 h. Tras este tiempo, los *films* se pudieron despegar de las placas sin que fueran pegajosos o quebradizos.

3.3.2. *Coatings*

Los *coatings* prebióticos, probióticos y sinbióticos se emplearon para recubrir queso fresco de cabra. Estos se elaboraron a partir de leche fresca de cabra de una granja local (San Martín del Rey Aurelio, Asturias, España). La leche se pasteurizó siguiendo un procedimiento LTLT (*low temperatura-long time*), calentándola a 60°C durante 25 min. Tras la pasteurización, la leche se enfrió a 34°C y se añadió renina en una concentración de 0,0025 g/L (Chy-Max®, CHR-Hansen, Dinamarca). No se emplearon cultivos lácticos iniciadores ya que el proceso de pasteurización LTLT permite la supervivencia de las BAL. Tras incubar la leche a 34°C durante 40 min, la cuajada se cortó múltiples veces para facilitar la sinéresis. Los quesos frescos se elaboraron con un peso de 10 g y se mantuvieron en la nevera a 4°C durante 1 h.
El *coating* se elaboró disolviendo alginato de sodio (20 g/L) a 70°C con agitación, hasta lograr una solución homogénea. A continuación, se añadió glicerol (15 g/L). Antes de añadir ácido lactobiónico como prebiótico y/o el *L. plantarum* como probiótico, la disolución se enfrío a unos 30°C. Los diferentes recubrimientos elaborados se muestran en la Tabla 3.3.

Tabla 3.3. Composición de los diferentes *coatings* elaborados.

Queso	*Coating*	Ácido lactobiónico	*L. plantarum*
Control negativo 1	No	-	-
Control negativo 2	Yes	-	-
Prebiótico 1	Yes	20 g/L	-
Prebiótico 2	Yes	40 g/L	-
Probiótico	Yes	-	9 $\log_{10}$ UFC/mL
Sinbiótico 1	Yes	20 g/L	9 $\log_{10}$ UFC/mL
Sinbiótico 2	Yes	40 g/L	9 $\log_{10}$ UFC/mL

Los quesos se recubrieron por medio de la técnica de inmersión o *dipping*. Los quesos se sumergieron durante 2 min en la solución formadora de *coating* y se dejaron en reposo durante 1 min a temperatura ambiente para eliminar el exceso de recubrimiento. Con el objetivo de fortalecer el coatings, los quesos se sumergieron en una solución endurecedora de $CaCl_2$ (5%, *p/v*) durante 2 min a temperatura ambiente. Finalmente, los quesos recubiertos se dejaron 30 min a temperatura ambiente y se conservaron a 4°C en recipientes estériles hasta que se llevaron a cabo los diferentes análisis.

3.3.3. Cápsulas

Para desarrollar las cápsulas se emplearon cuatro formulaciones diferentes (Tabla 3.4), utilizando ácido lactobiónico como compuesto prebiótico y *L. paracasei* como microorganismo probiótico. *L. paracasei* se creció en AFB con ácido lactobiónico como fuente única de carbono (50 g/L) incubándose a 37°C durante 24 h. Sobre este medio de cultivo se añadió alginato de sodio en una concentración de 20 g/L (Sigma-Aldrich) hasta que se obtuvo una solución homogénea. A continuación, esta mezcla se añadió gota a gota a una distancia de 10 cm con ayuda de una jeringuilla estéril de 1 mL en una solución de $CaCl_2$ (0,1 o 0,5 M, Sigma-Aldrich). Las cápsulas se mantuvieron en la solución de $CaCl_2$ en agitación y a temperatura ambiente durante 10 o 30 min (Tabla 3.4). Algunas de las cápsulas fueron reforzadas además en una solución de chitosano (4,42 g/L, 0,44% de ácido acético glacial, pH 5,7-6,0 NaOH 1 M, todos ellos de Sigma-Aldrich), en la que se mantuvieron en agitación y a temperatura ambiente durante 40 min. Una vez elaboradas, las cápsulas se recuperaron y mantuvieron a 4°C hasta que se realizaron el resto de los análisis de las cápsulas frescas.

Tabla 3.4. Detalle de las cuatro formulaciones empleadas para elaborar las cápsulas sinbióticas.

Cápsulas	Solución de $CaCl_2$	Solución de chitosano
A	0,1 M + 10 min	-
B	0,1 M + 30 min	-
C	0,1 M + 30 min	40 min
D	0,5 M + 10 min	40 min

Por otro lado, las cápsulas también se secaron utilizando dos técnicas diferentes: la liofilización y el secado térmico. La liofilización se realizó usando un liofilizador Christ Alpha 1-2LDplus (Martin Christ, Osterode am Harz, Alemania). El secado térmico se realizó usando una máquina de secado (CL252, Trevi, Rimini, Italia) con un flujo de aire de 0,1 a 0,3 m/s y una temperatura de 37°C durante 12 h. Estas cápsulas se

almacenaron en un lugar seco y a temperatura ambiente hasta su uso en el resto de análisis.

3.3.4. Micropartículas

Para elaborar las micropartículas se utilizaron como agentes encapsulantes maltodextrina (12 y 16%, *p/p*), goma arábiga (4 y 8%, *p/p*), caseinato de sodio, gelatina y mezclas de goma arábiga y maltodextrina (75:25, 50:50 y 25:75). Estas soluciones se mezclaron con lactobionato de calcio como prebiótico (0,05 g/mL) en una proporción 1:3. Además, las mezclas de caseinato de sodio y gelatina fueron tratadas con TG con el objetivo de conocer si se podía obtener una mejora de las propiedades de las micropartículas con este tratamiento. Para llevar a cabo este tratamiento, se ajustó el pH de estas soluciones a 7,0 y se añadió TG en una concentración de 0,33 g/L (50 U/g de proteína, Probind TX, BDF ingredientes). Las soluciones se incubaron a 45°C durante 90 min. Tras este tiempo, la temperatura se subió a 70°C durante 10 min para desactivar la enzima.

Todas las mezclas se congelaron a -80°C. Las micropartículas se elaboraron empleando la técnica de liofilización (0,1 mBar, -70°C, Telstar Cryodos). Tras la liofilización, las micropartículas se tamizaron con un tamiz de tamaño de poro de 355 µm y fueron almacenadas en una atmósfera seca hasta su empleo en posteriores análisis. Por otro lado, las micropartículas también se añadieron a quesos frescos de cabra (elaborados como se detalló en la *Sección 3.3.2*) como modelo alimentario.

3.4. Métodos analíticos

3.4.1. Propiedades mecánicas

Para el análisis de las propiedades mecánicas de los diferentes biomateriales desarrollados, se empleó un texturómetro TA. XT plus Texture Analyser (Stable Microsystems, Reino Unido).

En el caso de los *films*, este equipo se empleó para evaluar la fuerza de punción (*Puncture Strenght*, PS) y la deformación por punción (*Puncture Deformation*, PD). Los parámetros PS y PD se calcularon de acuerdo con las siguientes ecuaciones (Otero-Pazos et al., 2016):

$$PS = \frac{Fm}{Th} \qquad \text{(Ecuación 3.1)}$$

$$PD = \frac{\sqrt{D^2 + R^2} - R}{\mathrm{R}} \qquad \text{(Ecuación 3.2)}$$

siendo *Fm* la máxima fuerza aplicada antes de la ruptura del *film*, expresada en Newtons (N); *Th* el grosor de los *films* expresado en mm; *D* la distancia expresada en mm que recorre la sonda desde que entra en contacto con el *film* hasta que se rompe y *R* el radio del orificio circular (expresado en mm) que presentan los *films* una vez rotos. El grosor de los *films* se determinó utilizando un micrómetro digital (Mitutoyo, Japón). Las medidas se realizaron en diez puntos diferentes, en zonas exteriores e interiores de los *films*. Los resultados de grosor de los *films* que se muestran en la presente tesis se corresponden con la media de estos valores. Para llevar a cabo el ensayo con el texturómetro, los *films* se cortaron en cuadrados y se colocaron en la plataforma del equipo. Las muestras fueron sometidas a un test de penetración a temperatura ambiente, empleando una sonda esférica P/5 S a una velocidad de 2,0 mm/s y empleando una célula de carga de 5 kg.

En el caso de los quesos recubiertos con *coatings* y los quesos con micropartículas, el análisis con el texturómetro permitió conocer los valores de firmeza y pegajosidad, ambos parámetros expresados en gramos. Para llevar a cabo el ensayo, los quesos recubiertos fueron sometidos a un test de penetración a temperatura ambiente, empleando una sonda esférica SMS P/0.5S a una velocidad de 2,0 mm/s y una célula de carga de 5 kg.

Para la caracterización mecánica de las cápsulas se realizó un test de Bloom. El ensayo de Bloom se llevó a cabo siguiendo la normativa ISO 9665 (Intenational Standard ISO 9665, 1998). El parámetro de Bloom es una medida de la fuerza que se debe aplicar para provocar una deformación en un gel. El ensayo se realizó a temperatura ambiente empleando la sonda P/0.5 1/2'', a una velocidad de 0,5 mm/s, con una distancia de penetración de 4 mm, una tasa de adquisición de datos de 200 pps y con una célula de carga de 5 kg. Los resultados se expresan en grados Bloom, que se definen como la masa en gramos necesaria para oprimir el gel 4 mm sin romperlo. Se considera que la fuerza del gel es baja para valores de Bloom inferiores a 120 g, media en el intervalo de 120 a 200 g y alta para valores superiores a 200 g.

En todos los casos, los experimentos se realizaron siempre en muestras independientes y por triplicado y los valores mostrados se corresponden con la media.

3.4.2. Eficacia de encapsulación

La eficacia de encapsulación (EE) se define como la proporción del compuesto activo encapsulado frente a la cantidad de compuesto activo total empleado. La EE se calculó con la siguiente ecuación (Cilek, Luca, Hasirci, Sahin, & Sumnu, 2012):

$$EE = \frac{EAC}{TAC} x\ 100 = \frac{TAC - SAC}{TAC}\ x\ 100 \qquad \text{(Ecuación 3.3)}$$

siendo TAC la cantidad total de compuesto activo; EAC la cantidad de compuesto activo encapsulado y SAC la cantidad de compuesto activo que se encuentra en la superficie de las micropartículas. Para calcular SAC, se añadió 1 mL de agua destilada sobre 0,005 g de micropartículas y se mantuvieron en agitación y temperatura ambiente durante 10 min. Las muestras fueron centrifugadas a 13 200 rpm durante 5 minutos y el sobrenadante se analizó como se describe en la *Sección 3.4.12*.

3.4.3. Solubilidad en agua

Para analizar la solubilidad en agua de los *films* fragmentos homogéneos de estos fueron sumergidos en 20 mL de agua destilada con un 2% de tampón Tris-HCl (*p/p*) pH 7,0 (Sigma-Aldrich). Las muestras se mantuvieron a temperatura ambiente durante 24 h. Los fragmentos de *films* que no se disolvieron se recuperaron filtrando dichas soluciones con papel Whatman no 1 (Sigma-Aldrich) y se secaron en un horno a 105°C durante 24 h. Por otro lado, otros fragmentos de los *films* fueron secados en las mismas condiciones sin ser disueltos previamente. La solubilidad se calculó empleando la siguiente ecuación (Blanco-Pascual, Montero, & Gómez-Guillén, 2014):

$$WS\ (\%) = \frac{m1 - m2}{m1}\ x\ 100 \qquad \text{(Ecuación 3.4)}$$

siendo *m1* es el peso en g de los fragmentos de film secados en un horno a 105°C durante 24 h y *m2* es el peso en g de los fragmentos de film, que no se disolvieron en agua, una vez que fueron secados. Todos los experimentos se realizaron por triplicado y los resultados que se muestran se corresponden con la media.

En el caso de las micropartículas, se añadió 1 mL de agua destilada a 0,005 g de cada tipo de micropartícula. Las muestras se incubaron a 20°C con una agitación de 300 rpm durante 30 minutos (Thermomixer, Eppendorf, Hamburgo, Alemania). Tras este tiempo, las muestras se centrifugaron (13 200 rpm durante 5 min) y se analizó el sobrenadante como se describe en la *Sección 3.4.12*.

3.4.4. Cinética de rehidratación

Se comprobó la habilidad de rehidratación de las cápsulas secas en dos condiciones: por un lado, empleando una solución de NaCl al 0,9% a 37°C y por otro, usando yogurt natural (Danone, Danone Group SA, Francia) a 4°C. Las cápsulas se analizaron en ambas condiciones y se recogieron a determinados tiempos (0,025 min, 6 min, 60 min,

180 min y 1440 min). El contenido en agua se midió empleando un titulador volumétrico Karl Fisher HI 903 (Hanna Instruments, Padovana, Italia).

La capacidad de rehidratación de las cápsulas se calculó como la proporción entre el contenido en agua de las cápsulas secas y el contenido en agua de las cápsulas frescas, tal y como se describe en la siguiente ecuación:

$$Capacidad\ de\ rehidratación = \frac{W_{rehidratación}}{W_{frescas}}\ x\ 100 \qquad \text{(Ecuación 3.5)}$$

siendo $W_{rehidratación}$ los g de agua absorbidos por las cápsulas secas durante la rehidratación con NaCl al 0,9% o con yogurt y $W_{frescas}$ los g de agua presentes en las cápsulas frescas antes de ser secadas. Todos los experimentos se realizaron por triplicado y los resultados se corresponden con la media.

3.4.5. Transmitancia óptica y transparencia

Las propiedades de barrera frente a la luz visible y ultravioleta (UV) de los *films* se analizaron en el rango de longitud de onda de 200 a 800 nm. Para ello se empleó un espectrofotómetro (Helios gamma, Thermo Fischer Scientific, EEUU). Para ello, se cortó un fragmento de cada *film* que se introdujo en una cubeta de cuarzo. Como blanco se empleó la cubeta de cuarzo vacía. La transparencia de los *films* se calculó con la siguiente ecuación:

$$Transparencia = \frac{A_{600}}{x} \qquad \text{(Ecuación 3.6)}$$

siendo A_{600} es la absorbancia de los films a 600 nm y *x* es el grosor de los films en mm. Todos los experimentos se llevaron a cabo por triplicado y los resultados mostrados se corresponden con la media de dichos valores.

3.4.6. Propiedades del color

El color de los *films* se analizó empleando un equipo Lovibond® LC 100 Spectrocolorimeter (Tintometer® Group, Lovibond House, Reino Unido). Se midieron tres parámetros de color: L* (que mide la luminosidad o brillo), a* (que mide el enrojecimiento y verdor) y b* (que mide la amarillez y el tono azul). La diferencia total de color (ΔE) se calculó con la siguiente ecuación (Zabihollahi, Alizadeh, Almasi, Hanifian, & Hamishekar, 2020):

$$\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \qquad \text{(Ecuación 3.7)}$$

ΔL*, Δa* y Δb* se corresponden con la diferencia de color entre las muestras de los *films* y la referencia estándar. Además, los parámetros b^* y L^* también se encuentran directamente relacionados con la tonalidad amarilla (*yellowness index*, YI) que se puede calcular específicamente con la siguiente ecuación (Francis F. J. & Clydesdale F. M., 1977):

$$YI = \frac{142{,}86\ x\ b^*}{L^*} \qquad \text{(Ecuación 3.8)}$$

Todas las medidas se realizaron por triplicado y los resultados mostrados se corresponden con la media de dichos valores.

3.4.7. Morfología

La morfología de los *films* y micropartículas se analizó empleando microscopía electrónica de barrido (*scanning electron microscopy*, SEM; JSM-6610LV, JEOL, EEUU).

En el caso de los *films*, éstos se cortaron con una cuchilla quirúrgica. Los fragmentos se colocaron en los soportes y se recubrieron con oro. Se analizó siempre el corte transversal de los *films* utilizando diferentes aumentos.

En el caso de las micropartículas, éstas se colocaron directamente en los soportes y se recubrieron con oro, analizándose usando un aumento 100x. Por otro lado, también se estudió la morfología de los quesos con micropartículas. En este caso, fragmentos de los quesos fueron previamente liofilizados (0,1 mBar, -70°C durante 24 h).

3.4.8. Estereomicroscopía de fluorescencia

La caracterización visual de las cápsulas se llevó a cabo empleando un estereomicroscopio de fluorescencia LEICA M205Fa (Leica Microsystems Inc., Heidelberg, Alemania). Las cápsulas no se sometieron a ningún tratamiento especial, se colocaron sobre el portaobjetos y se empleó una magnificación 12x. Para el procesamiento de las imágenes y la determinación del tamaño de las cápsulas se empleó el software Leica Application Suite v4.0.

La forma de las cápsulas se caracterizó empleando el factor de esferificación (spherification factor, SF). El parámetro SF se calculó con la siguiente ecuación:

$$SF = \frac{d_{max} - d_{min}}{d_{max} + d_{min}} \qquad \text{(Ecuación 3.9)}$$

siendo d_{max} el diámetro mayor de la cápsula y d_{min} el diámetro menor de la cápsula perpendicular a d_{max}. Teniendo en cuenta este factor, 0 es el valor de para una esfera perfecta y el valor de 1 se correspondería a un objeto de forma alargada.

La caracterización visual de las cápsulas es importante para detectar la presencia de grietas y observar los cambios que se producen tras los procesos de secado, rehidratación y las pruebas de digestión *in vitro* simulada.

3.4.9. Microscopía confocal con fluorocromos

En algunos casos, la viabilidad de los probióticos dentro de los *films* fue analizada empleando fluorocromos y microscopía confocal. Para ello, los *films* se tiñeron con dos fluorocromos diferentes: SYBRGreen y yoduro de propidio (ambos de Sigma-Aldrich). La solución de SYBRGreen se preparó diluyéndola en una proporción 1: 10 000 en un tampón TE (10 mM Tris-HCl y 1 mM EDTA a pH 8,0; todo de Sigma-Aldrich). La solución de yoduro de propidio se preparó en agua destilada en una concentración de 5 µg/mL. Los films se introdujeron primero en la solución de SYBRGreen y después en la de yoduro de propidio, en ambos casos durante 20 min, a temperatura ambiente y en oscuridad. Tras el periodo de incubación, las muestras se analizaron con un microscopio láser confocal (Confocal Laser Microscope SP8-Leica TCS-SP2-AOBS, Leica Microsystems, Alemania), equipado con una lente de cámara de 63x/1.400iL. Se empleó un láser de luz blanca con una $\lambda_{excitación}$ de 488 nm y $\lambda_{emisión}$ de 588-531 nm para el fluorocromo SYBRGreen, y de $\lambda_{excitación}$ de 434 nm y $\lambda_{emisión}$ de 663-759 que para el yoduro de propidio. Para detectar la fluorescencia se empleó un fotomultiplicador.

La viabilidad y distribución del probiótico también fue analizada en el interior de los *coatings*. En este caso, los fragmentos de los *coatings* se lavaron con agua destilada un par de veces y se depositaron durante 3 min en una solución con un 0,1% de naranja de acridina (Sigma-Aldrich), previamente disuelta en un tampón fosfato 67 mM (pH 6,0). Los fragmentos se lavaron con el mismo tampón fosfato durante 1 min y se colocaron en una solución de $CaCl_2$ 100 mM durante 30 s. Tras ser secadas, los fragmentos de *coating* se obervaron con un microscopio confocal Leica TCS-SP-AOBS ($\lambda_{excitación}$ 480 nm; $\lambda_{emisión}$ 508-603 nm).

3.4.10. Determinación de la densidad óptica

Para conocer la concentración de bacterias que se usó de manera inicial en los diferentes biomateriales se determinó la densidad óptica (DO) La biomasa separada mediante centrifugación (13 200 rpm durante 5 min) se resuspendió en solución salina (NaCl, 0,7%, *p*/*v*) que a su vez fue empleada como blanco en la medición. La

absorbancia de las muestras se midió a 600 nm en un espectrofotómetro (modelo 1203, Shimazdu UV-Vis, Kioto, Japón). La densidad óptica de cada cepa bacteriana se relacionó con las UFC/mL (unidades formadoras de colonia) por medio de curvas de crecimiento.

3.4.11. Recuento de microorganismos viables y cultivables en medio sólido

La concentración de células viables y cultivables se determinó mediante el recuento de UFC en placas en medio sólido. Las muestras tomadas periódicamente a lo largo de los experimentos de viabilidad se centrifugaron a 13 200 rpm durante 5 min con el objetivo de separar la biomasa del medio líquido. La biomasa se resuspendió en solución salina (NaCl, 0,7%, *p/v*) y se homogeneizó con el *vórtex* a fin de evitar la sedimentación de las células. A partir de esta muestra se realizaron diluciones seriadas 1:10 en NaCl (0,7% *p/v*), sembrándose en placa las diluciones pertinentes. Las placas se incubaron a 30°C durante 48 h. Transcurrido el tiempo de incubación, se realizó el recuento de las UFC correspondientes a las diluciones estadísticamente significativas (de 20 a 200 colonias). El resultado promedio se expresó como UFC/g de biomaterial o producto alimentario.

3.4.12. Cromatografía líquida de alta eficacia (HPLC)

La concentración de ácido lactobiónico se analizó mediante un cromatógrafo líquido de alta eficacia (High Performance Liquid Chromatography, HPLC). Previamente a ser analizadas, las muestras fueron diluidas con agua miliQ (cuando fue necesario) y todas ellas fueron filtradas con filtros de PVDF de 0,45 µm.

El equipo empleado fue un cromatógrafo de líquidos de la marca Agilent (modelo serie 1200, California, EEUU). En la determinación y cuantificación analítica se empleó una columna Coregel ION 300 (Teknocroma, Barcelona, España) acoplada a un detector de índice de refracción (RID) con una temperatura fijada de 40°C. Como método de análisis se empleó el descrito por Alonso *et al.* (2013) con algunas modificaciones. Como fase móvil se empleó una solución de ácido sulfúrico 0,450 mM (pH 3,1; Sigma-Aldrich), un flujo de 0,3 mL/min y una temperatura de columna de 75°C. El ácido lactobiónico fue cuantificado empleando un patrón externo de grado HPLC (Sigma-Aldrich). La adquisición y análisis de datos fue realizada con el software de Agilent ChemStation.

3.4.13. Pruebas de digestión *in vitro* simulada

Las pruebas de digestión *in vitro* simulada se hicieron con el objetivo de comprobar la capacidad de protección de los biomateriales desarrollados en la supervivencia y viabilidad de *L. plantarum* y *L. paracasei*, así como de la posible capacidad protectora del ácido lactobiónico. El ácido lactobiónico funciona como una fibra por lo que no es digerible y es resistente a las enzimas digestivas humanas (Cardoso, Marques, Dagostin, & Masson, 2019).

Las pruebas de digestión *in vitro* realizadas constan de 3 etapas diferentes: la fase oral, gástrica e intestinal. Las condiciones de cada una de las etapas fueron llevadas a cabo de acuerdo con el protocolo desarrollado por Minekus *et al.* (2014) con algunas modificaciones. La composición de sales y electrolitos de los fluidos simulados salivares (*simulated salivary fluid*, SSF), gástricos (*simulated gastric fluid*, SGF) e intestinales (*simulated intestinal fluid*, SIF) se recoge en la Tabla 3.5.

Tabla 3.5. Composición de sales y electrolitos de los fluidos simulados salivares (SSF), gástricos (SGF) e intestinales (SIF). Todos los reactivos empleados son de Sigma-Aldrich.

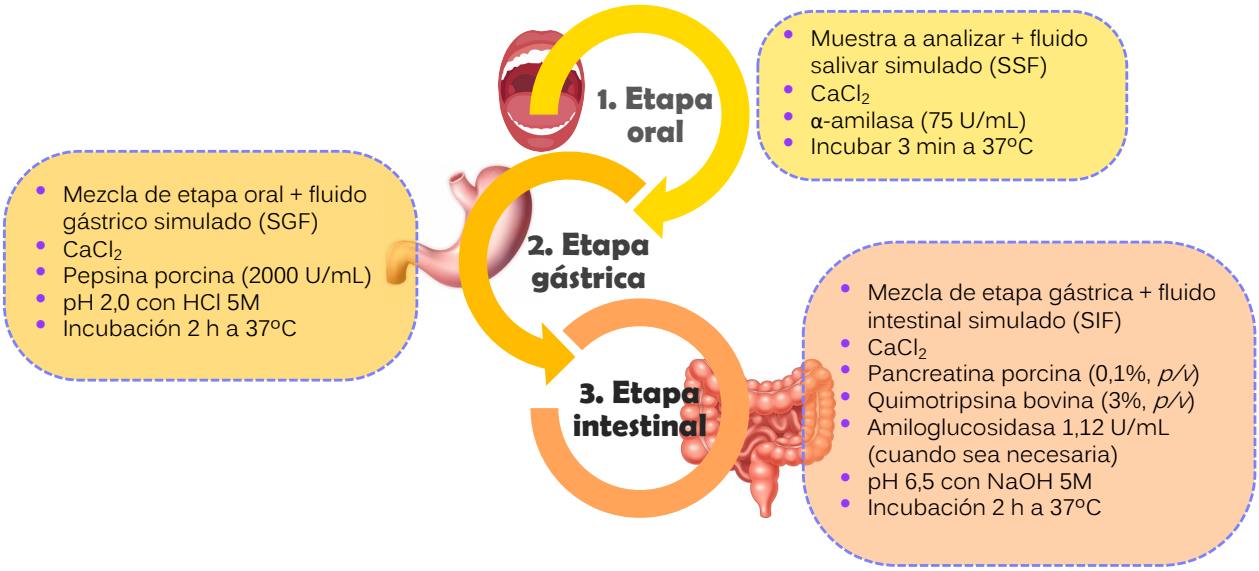
	***Simulated salivary fluid* (SSF) (g/L)**	***Simulated gastric fluid* (SGF) (g/L)**	***Simulated intestinal fluid* (SIF) (g/L)**
KCl	1,12	0,51	0,51
KH_2PO_4	0,50	0,12	0,11
$NaHCO_3$	1,14	2,10	7,14
$MgCl_2(H_2O)_6$	0,031	0,020	0,067
$(NH_4)_2CO_3$	0,0058	0,048	-
NaCl	-	2,76	2,24

El procedimiento de la digestión *in vitro* simulada se muestra esquemáticamente en la Figura 3.1.

En función del biomaterial que se fuese a analizar o el propio modelo alimentario completo se tomaron diferentes cantidades de muestra que se añadieron a la etapa oral. Para la etapa oral se mezclaron 25 mL de SSF con $CaCl_2$ (Sigma-Aldrich) y α-amilasa (1333 U/mg proteína, CAS 900-90-2, Sigma-Aldrich) hasta alcanzar una concentración final de 0,083 g/L y 75 U/mL, respectivamente. Esta etapa tuvo una duración de 3 min y se realizó a una temperatura de 37°C. Para la etapa gástrica se añadieron 25 mL de SGF sobre la mezcla de la etapa oral. En la fase gástrica se añadió además pepsina porcina (2500 U/mg proteína, CAS 9001-75-6, Sigma-Aldrich) y $CaCl_2$ hasta alcanzar una concentración de 2000 U/mL y 0,0083 g/L en el volumen final. Una

vez bien mezclado, se ajustó el pH a 2,0 con HCl 5M (Sigma-Aldrich) y se incubó a 37°C durante 2 h. Finalmente, para la etapa intestinal se añadieron 50 mL sobre la mezcla de la etapa anterior, alcanzando un volumen final de 100 mL. Sobre esta mezcla se añadió pancreatina porcina (0,1% (*p/v*), CAS 9049-47-6, Sigma-Aldrich), quimotripsina bovina (3% (*p/v*), CAS 9004-07-3, Sigma-Aldrich) y $CaCl_2$ (0,033 g/L). Cuando el biomaterial analizado contuvo alginato de sodio, se añadió además amiloglucosidasa (260 U/mL CAS 9032-08-0) hasta alcanzar una concentración en el volumen final de 1,12 U/mL (Warren, Zhang, Waltzer, Gidley, & Dhital, 2015). El pH de esta mezcla se ajustó a 6,5 con NaOH 5M (Sigma-Aldrich) y se incubó a 37°C durante 2 h.

Figura 3.1. Esquema del procedimiento de digestión simulada *in vitro*.



La supervivencia y viabilidad del probiótico se comprobó tomando muestras a diferentes tiempos. En función del biomaterial que se estuviese analizando se tomaron determinados gramos de *film*, *coating*, cápsulas o modelos alimentarios completos. Las muestras se homogeneizaron con NaCl (0,7%, (*p/v*)) empleando un Stomacher™ (Seward, Reino Unido) 2 min y a máxima velocidad. El recuento de microorganismos viables y cultivables en medio sólido se realizó como se detalla en la *Sección 3.4.11*.

3.5. Análisis estadísticos

Para los análisis estadísticos se llevaron a cabo análisis de la varianza (ANOVA). Para determinar diferencias estadísticas entre los datos se realizó un test de Fischer (Fischer's Least Significant Difference, LSD). Un nivel de $p < 0.05$ fue considerado como significante. Todos los análisis estadísticos se llevaron a cabo empleando el software estadístico Statgraphics 18® Centurion.

Referencias

Alonso, S., Rendueles, M., & Díaz, M. (2011). Efficient lactobionic acid production from whey by Pseudomonas taetrolens under pH-shift conditions. *Bioresource Technology*, *102*(20), 9730–9736. https://doi.org/10.1016/j.biortech.2011.07.089

Alonso, S., Rendueles, M., & Díaz, M. (2012). Role of dissolved oxygen availability on lactobionic acid production from whey by Pseudomonas taetrolens. *Bioresource Technology*, *109*, 140–147. https://doi.org/10.1016/j.biortech.2012.01.045

Alonso, S., Rendueles, M., & Díaz, M. (2013). Feeding strategies for enhanced lactobionic acid production from whey by Pseudomonas taetrolens. *Bioresource Technology*, *134*, 134–142. https://doi.org/10.1016/j.biortech.2013.01.145

Blanco-Pascual, N., Montero, M. P., & Gómez-Guillén, M. C. (2014). Antioxidant film development from unrefined extracts of brown seaweeds Laminaria digitata and Ascophyllum nodosum. *Food Hydrocolloids*, *37*, 100–110.https://doi.org/10.1016/j.foodhyd.2013.10.021

Bradford M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, (72), 248–254. https://doi.org/10.1016/j.cj.2017.04.003

Cardoso, T., Marques, C., Dagostin, J. L. A., & Masson, M. L. (2019). Lactobionic Acid as a Potential Food Ingredient: Recent Studies and Applications. *Journal of Food Science*, *84*(7), 1672–1681. https://doi.org/10.1111/1750-3841.14686

Cilek, B., Luca, A., Hasirci, V., Sahin, S., & Sumnu, G. (2012). Microencapsulation of phenolic compounds extracted from sour cherry pomace: Effect of formulation, ultrasonication time and core to coating ratio. *European Food Research and Technology*, *235*(4), 587–596. https://doi.org/10.1007/s00217-012-1786-8

De Montijo-Prieto, S., Castro, D. J., Reina, J. C., Jimenez-Valera, M., & Ruiz-Bravo, A. (2019). Draft genome sequence of Lactobacillus plantarum C4 (CECT 9567), a potential probiotic strain isolated from kefir. *Archives of Microbiology*, *201*(3), 409–414. https://doi.org/10.1007/s00203-019-01629-9

Francis F. J. & Clydesdale F. M. (1977). *Food Colorimetry: Theroy and Applications*. Retrieved from https://doi.org/10.1002/food.19770210122

Heine, H., Rietschel, E. T., & Ulmer, A. J. (2001). The biology of endotoxin. *Applied Biochemistry and Biotechnology - Part B Molecular Biotechnology*, *19*(3), 279–296. https://doi.org/10.1385/MB:19:3:279

Intenational Standard ISO 9665. (1998). *Adhesives-Animal glues-Methods of sampling and testing*. *1998*.

Li, L., & Luo, R. G. (1999). Quantitative determination of Ca2+ effects on endotoxin removal and protein yield in a two-stage ultrafiltration process. *Separation Science and Technology*, *34*(9), 1729–1741. https://doi.org/10.1081/SS-100100735

Magalhães, P. O., Lopes, A. M., & Priscila, G. (2007). *Methods of Endotoxin Removal from*

Biological Preparations : a Review. *10*(3), 388–404.

Marcet, I., Álvarez, C., Paredes, B., & Díaz, M. (2014). Inert and Oxidative Subcritical Water Hydrolysis of Insoluble Egg Yolk Granular Protein, Functional Properties, and Comparison to Enzymatic Hydrolysis. *Journal of Agricultural and Food Chemistry*, *62*(32), 8179–8186.

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carrière, F., Boutrou, R., Corredig, M., Dupont, D., Dufour, C., Egger L., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A., Marze, S., McClements, D.J., Ménard, O., Recio, I., Santos, C.N., Singh, R.P., Vegarud, G. E., Wickham, M.S.J., Weiteschies, W. & Brodkorb, A. (2014). A standardised static in vitro digestion method suitable for food-an international consensus. Food and Function, 5(6), 1113–1124. https://doi.org/10.1039/c3fo60702jOtero-Pazos, P., Sendón, R., Blanco-Fernandez, B., Blanco-Dorado, S., Alvarez-Lorenzo, C., Concheiro, A., Rodríguez-Bernaldo de Quirós, A. (2016). Preparation of antioxidant active films based on chitosan: diffusivity study of α-tocopherol into food simulants. *Journal of Food Science and Technology*, *53*(6), 2817–2826. https://doi.org/10.1007/s13197-016-2256-2

Warren, F. J., Zhang, B., Waltzer, G., Gidley, M. J., & Dhital, S. (2015). The interplay of α-amylase and amyloglucosidase activities on the digestion of starch in in vitro enzymic systems. *Carbohydrate Polymers*, *117*, 185–191. https://doi.org/10.1016/j.carbpol.2014.09.043

Wassenaar, T. M., & Zimmermann, K. (2018). Lipopolysaccharides in food, food supplements, and probiotics: should we be worried? *European Journal of Microbiology and Immunology*, *8*(3), 63–69. https://doi.org/10.1556/1886.2018.00017

Zabihollahi, N., Alizadeh, A., Almasi, H., Hanifian, S., & Hamishekar, H. (2020). Development and characterization of carboxymethyl cellulose based probiotic nanocomposite film containing cellulose nanofiber and inulin for chicken fillet shelf life extension. *International Journal of Biological Macromolecules*, *160*, 409–417. https://doi.org/10.1016/j.ijbiomac.2020.05.066

Capítulo 4.
Desarrollo de materiales con proteínas como matriz

En este capítulo se recogen las investigaciones llevadas a cabo empleando proteínas como matriz en el desarrollo de materiales funcionales de aplicación alimentaria, concretamente en la elaboración de films comestibles.

Las proteínas son uno de los principales biopolímeros que se emplean en el desarrollo de materiales alimentarios comestibles (Amin et al., 2021). Estas proteínas pueden ser de origen animal o vegetal y, además, también se pueden obtener de los diferentes subproductos de la industria alimentaria, con el objetivo de revalorizarlos (Ribeiro, Estevinho, & Rocha, 2021) (Chiralt, Menzel, Hernandez-García, Collazo, & Gonzalez-Martinez, 2020). De manera general, los biomateriales desarrollados usando proteínas como matriz presentan buenas propiedades mecánicas y de barrera frente a la pérdida de aromas y compuestos volátiles. Además, se trata de un tipo de matriz adecuada para añadir compuestos bioactivos en su formulación. Varios autores han estudiado su idoneidad para añadir microorganismos probióticos y compuestos prebióticos (Calva-Estrada, Jiménez-Fernández, & Lugo-Cervantes, 2019) (Gopalakrishnan, Xu, Zhong, & Rotello, 2021) (Fernandes et al., 2020). Por tanto, se trata de una matriz apropiada para estudiar la influencia y el comportamiento del ácido lactobiónico como prebiótico en la formulación de films y su combinación con Lactobacillus plantarum CECT 9567. Uno de los inconvenientes que presentan los films de proteínas es que, en la mayoría de los casos, son altamente solubles en agua, lo que dificulta su empleo en la industria alimentaria, ya que la mayor parte de los alimentos tiene un alto grado de humedad (Gopalakrishnan et al., 2021).

Este capítulo está dividido en dos apartados. En el primero, se expone la investigación llevada a cabo empleando gelatina como matriz y en el segundo se exponen los tres trabajos elaborados empleando como matriz proteína de yema de huevo delipidada.

4.1. Gelatina

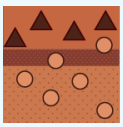
En este apartado se muestra el trabajo elaborado empleando gelatina como matriz en el desarrollo de *films* con ácido lactobiónico y *L. plantarum* CECT 9567 como microorganismo probiótico.

4.1.1. Desarrollo de *films* comestibles con *Lactobacillus plantarum* CECT 9567 y ácido lactobiónico producido mediante fermentación de suero dulce

En este subapartado, el ácido lactobiónico empleado se produjo a través de la fermentación por parte de *P. taetrolens* LMG 2336 usando como sustrato suero dulce desproteneizado. Esta bacteria es capaz de oxidar la lactosa presente en el subproducto de la industria lechera en ácido lactobiónico por medio de la enzima lactosa deshidrogenasa (Alonso et al., 2013). Al tratarse de una bacteria Gram negativa, *P. taetrolens* produce endotoxinas, las cuales son agregados complejos de LPS, que deben ser eliminadas, ya que son sustancias pirogénicas, tóxicas para humanos y animales (Wassenaar & Zimmermann, 2018). En este caso, las endotoxinas producidas por *P. taetrolens* fueron eliminadas por microfiltración tangencial para que el fermentado se pudiese utilizar de forma segura en el ámbito alimentario.

Con este trabajo se quería aprovechar y lograr partir de un residuo de la industria alimentaria para obtener un biomaterial con capacidad prebiótica al que se le pudiese añadir *L. plantarum* CECT 9567 como microorganismo probiótico. En este caso y una vez obtenido el ácido lactobiónico a partir de la síntesis biológica, se seleccionó la gelatina como matriz para desarrollar los films por ser una proteína fácil de incorporar y ser una de las más estudiadas en el ámbito alimentario (Nilsen-Nygaard et al., 2021) (Calva-Estrada et al., 2019). Los *films* elaborados fueron caracterizados atendiendo a sus propiedades físicas, químicas y mecánicas. Además, también se analizó la viabilidad del probiótico y el consumo por su parte de ácido lactobiónico como sustrato durante el tiempo de almacenamiento.

- **Publicación:** Sáez-Orviz, S.; Marcet, I.; Rendueles, M.; Díaz, M. 2022. Preparation of edible films with *Lactobacillus plantarum* and lactobionic acid produced by sweet whey fermentation. *Membranes*, 12, 115, (https://doi.org/10.3390/membranes12020115).
- **Estado:** publicado
- **Índice de impacto:** 4,106 (2020)



membranes

Preparation of edible films with *Lactobacillus plantarum* and lactobionic acid produced by sweet whey fermentation

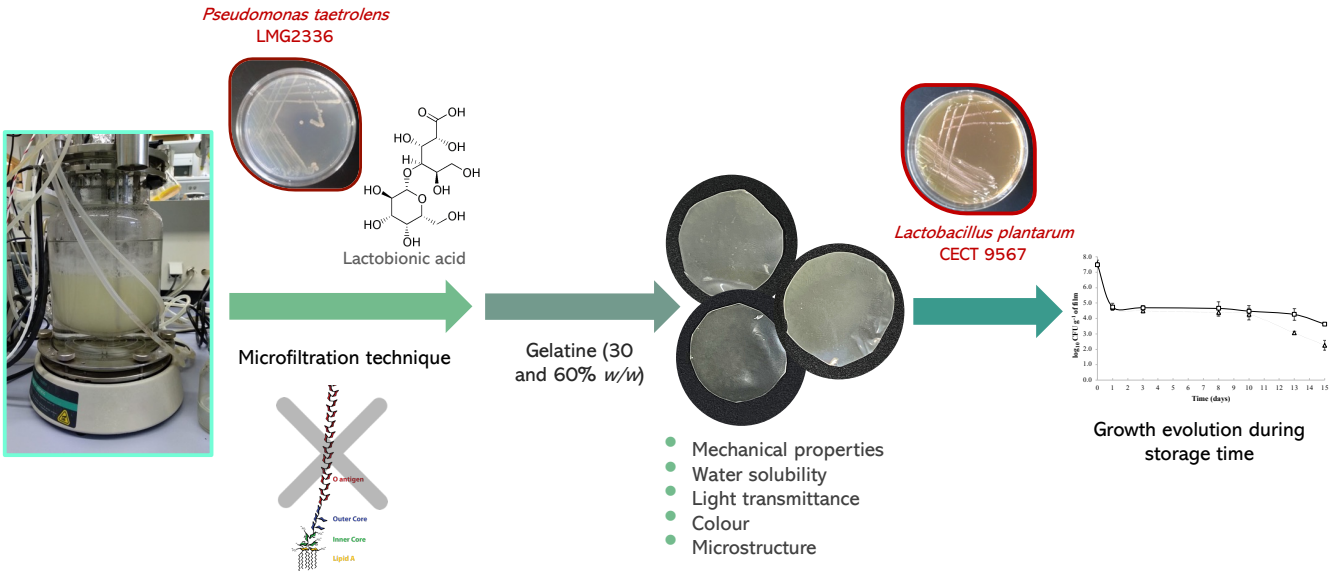
S. Sáez-Orviz [1], I. Marcet [1], M. Rendueles [1*] and M. Díaz [1]

[1] Department of Chemical and Environmental Engineering, University of Oviedo. C/Julián Clavería 8, 33006. Oviedo, Spain.

* Corresponding author

E-mail address: mrenduel@uniovi.es (Manuel Rendueles)

Graphical abstract



Abstract

Cheese whey is one of the most abundant by-products from the dairy industry that causes economic losses and pollution problems. In this study, ultrafiltered sweet whey permeate was fermented by *Pseudomonas taetrolens* LMG 2336 to produce a prebiotic compound (lactobionic acid, LBA). Endotoxins produced by these microorganisms were successfully removed using microfiltration techniques, allowing the fermented whey to be used in the food industry. The fermented whey was used to develop prebiotic edible films by adding gelatine in two different concentrations (30 and 60% (w/w), LBA30 and LBA60). *Lactobacillus plantarum* CECT 9567 was added as a probiotic microorganism (LP30 and LP60), containing both a prebiotic and a probiotic. The mechanical properties, water solubility, light transmittance, colour, and microstructure of the films were fully characterised. LBA and probiotic concentration

inside LP30 and LP60 were monitored under storage conditions. The strength and water solubility of the films were affected by the presence of LBA, and all these films were homogeneous but slightly opaque. The presence of LBA as prebiotic improved the viability of *L. plantarum* during cold storage, compared to the control. Therefore, these films could be used in the food industry to coat different foodstuff to obtain functional products.

Keywords: ultrafiltered sweet whey permeate; lactobionic acid; prebiotic; probiotic; edible film

1. Introduction

Cheese whey is one of the most abundant by-products from the dairy industry. It is estimated that about 50% of the whey generated is disposed of directly into water systems, causing economic losses and pollution problems (Fernandes, Leonardo M. Guimaraes Jonas T., Pimentel Tatiana C., Esmerino Erick A., Freitas Mônika Q., Carvalho Carlos Wanderlei P., 2020). Thus, the food field has great interest to look for specific uses for the generated cheese whey to reduce environmental problems and economic costs. Cheese whey can be deproteinized, microfiltered and ultrafiltered to generate concentrate whey protein (Janine Beucler, 2004) that can be used in different food products (Fernandes, Leonardo M. Guimaraes Jonas T., Pimentel Tatiana C., Esmerino Erick A., Freitas Mônika Q., Carvalho Carlos Wanderlei P., 2020). The liquid obtained after micro and ultrafiltration is the whey permeate. Whey permeate is composed mainly of lactose (5%), water (93%) and minerals (0.53%) with a minimal amount of proteins (0.85%) and fats (0.36%) (Janine Beucler, 2004). Because of this poor composition, there are few ways to revalue this by-product of the dairy industry. One of them is the production by microbial fermentation of different compounds, such as oligosaccharides (Janine Beucler, 2004), lactic acid and lactobionic acid (LBA) (Alonso, Rendueles, & Díaz, 2013a).

LBA has recently generated considerable attention in the food industry. This compound has many characteristics that make it interesting, such as its antioxidant (Cardoso, Marques, Sotiles, Dagostin, & Masson, 2019), antimicrobial (Kang et al., 2020) and prebiotic properties (Alonso et al., 2013a), as it is resistant to the digestive environment and enzymes and can be metabolized by gastrointestinal microflora (Saarela, Hallamaa, Mattila-Sandholm, & Mättö, 2003) (Schaafsma, 2008). Although LBA has only been approved by the FDA for use in the form of calcium salt (calcium lactobionate) (*FDA. Code of Federal Regulations, Title 21, 21 CFR 172.720. US Food and Drug*

Administration, 2017) (Cardoso, Marques, Dagostin, & Masson, 2019a), approval is under consideration by other food authorities and is expected in the short term. LBA is produced mostly by chemical synthesis (Alonso et al., 2013a), but it can be also produced by microbial fermentation. Obtaining LBA through biological synthesis has several advantages over chemical synthesis, as no undesirable products are generated, and no expensive metal catalysts are used [3]. Through biological fermentation, numerous wastes from the food industry, such as cheese whey, can be used as substrate to obtain products with high added value. This fermentation can be carried out by several bacteria such as *Zymomonas mobilis* or *Burkholderia cepacia* (Alonso et al., 2013a), but one of the most studied is *Pseudomonas taetrolens* (García, Rendueles, & Díaz, 2017) (Alonso, Rendueles, & Díaz, 2011). The LBA obtained has prebiotic capacity and therefore can be used to develop and improve foodstuffs and sustainable packaging materials.

Nowadays, conventional packaging materials developed using non-renewable origin are being replaced by more sustainable alternatives, such as casein (Marcet, Sáez, Rendueles, & Díaz, 2017), sodium alginate (Yerramathi et al., 2021) or gelatine (Pellá et al., 2020) edible films. The development of these type of films has attracted the attention of researchers and new types of materials have recently emerged (Galus, Kibar, Gniewosz, & Kraśniewska, 2020). Some of these new materials include whole grain flours (such as amaranth (Chandla, Saxena, & Singh, 2017), quinoa (Pająk, Przetaczek-Rożnowska, & Juszczak, 2019) and chia (Charles-Rodríguez, A.V.; Rivera-Solís, L.L.; Martins, J.T.; Genisheva, Z.; Robledo-Olivo, A.; González-Morales, S.; López-Guarin, G.; Martínez-Vázquez, D.G.; Vicente, A.A.; Flores-López, 2020)), fruit and vegetable residues (such as orange, lettuce and carrot (Andrade, Ferreira, & Gonçalves, 2016)) and root plants (such as starch from yam (Gutiérrez, Tapia, Pérez, & Famá, 2015)). In addition, consumers are increasingly demanding food products that not only meet their nutritional demands but also have additional benefits, improving their health and reducing risk of certain diseases. In this sense, the inclusion of bioactive compounds is a new trend in the development of packaging materials. Some of the bioactive compounds that can be added to films include antioxidants (Tanwar et al., 2021), antimicrobials (Shapi'i, Othman, Nordin, Kadir Basha, & Nazli Naim, 2020) and natural preservatives (Sapper & Chiralt, 2018), as well as prebiotics. Thus, fermented whey permeate can be used to develop bioactive edible films with LBA as prebiotic. Furthermore, if the prebiotic is combined with a probiotic, a synbiotic packaging can be obtained. When both are combined, prebiotics can increase the survival and growth of

probiotic microorganisms (Adebola, Corcoran, & Morgan, 2014). Regarding probiotics, the most commonly used belong to the genera *Bifidobacterium* and *Lactobacillus* (Espitia, Batista, Azeredo, & Otoni, 2016).

Therefore, the aim of this study is to use a waste product from the food industry such as sweet whey as substrate to develop an edible film with bioactive properties. For that purpose, an ultrafiltered sweet whey permeate will be used as substrate and fermented by *P. taetrolens* LMG 2336, which converts the lactose in the substrate into LBA, resulting in a bioactive and high value-added product with prebiotic capacity. To be used in the food field, endotoxins produced by *P. taetrolens* (a Gram-negative microorganism) need to be removed, so the fermented whey will be microfiltrated. Gelatine will be added to the fermented whey to be the protein matrix for the films. The bioactive edible films produced will be characterised regarding their mechanical and physical properties. In addition, *Lactobacillus plantarum* CECT 9567 will be added as a probiotic microorganism to analyse its viability inside the LBA edible films under storage conditions.

2. Materials and Methods

2.1. Microorganism, inoculum, substrate, and fermentation conditions

2.1.1. Microorganism and growth conditions

Pseudomonas taetrolens LMG 2336 (from the Belgian Coordinated Collection of Microorganisms, Ghent, Belgium) was used. The microorganism was inoculated on Nutrient Broth (NB, containing 1 g L^{-1} meat extract, 2 g L^{-1} yeast extract, 5 g L^{-1} peptone and 5 g L^{-1} NaCl, all from Sigma-Aldrich, Steinheim, Germany) agar (20 g L^{-1}, VWR Chemicals, PA, USA) plates and incubated at 30 ºC for 48 h. A loopful from a fresh NB agar plate was inoculated in a 500 mL Erlenmeyer flask containing 100 mL of NB broth (Sigma-Aldrich) (ratio medium air 1:4). The inoculum was incubated in an orbital shaker (Model G25; New Brunswick Scientific Co., NJ, USA) at 250 rpm and 30 ºC for 10 h. After this time, biomass was separated by centrifugation (10 000 rpm, 10 min) and was subsequently used as a bulk starter.

2.1.2. Sweet whey preparation

Ultrafiltrated sweet whey permeate (supplied by ILAS S.A., Asturias, Spain) with an initial concentration of approximately 200 g L^{-1} of lactose was diluted in distilled water to reach a concentration of 40 g $L^{-1}$of lactose. pH was adjusted to 6.5 by adding NaOH 10 N (Sigma-Aldrich). Then, it was sterilized employing a tangential microfiltration unit

equipped with a PVDF membrane-cassette with a pore size of 0.22 µm (Millipore, Massachusetts, USA). The permeate was used for the rest of the experiments.

2.1.3. Culture in a stirred tank bioreactor

Biomass of *P. taetrolens* LMG 2336 (obtained as explained in Section 2.1.1.) was inoculated (at 10% [*v/v*]) in a 500 mL Erlenmeyer flask containing 100 mL of sterile ultrafiltrated sweet whey permeate and it was incubated 12 h at 30 ºC and with an agitation of 250 rpm. After this time, the culture was again centrifuged (10 000 rpm, 10 min) and the biomass obtained was employed to inoculate the 2-L bioreactor (BioFlo 110; New Brunswick Scientific Co., NJ, USA), employing again a 10% (*v/v*) of inoculum. The working volume of the bioreactor was of 1 L with mechanical agitation. The operating parameters used were 30 ºC, 350 rpm and 1 Lpm aeration, following the conditions optimised by Alonso *et al.* (2012) (Alonso, Rendueles, & Díaz, 2012) to maximize the production of LBA. Foaming was avoided by automatic addition of diluted (1:10) Y-30 emulsion (Sigma-Aldrich). The bioreactor was equipped with a pH-meter (Mettler Toledo, Greifensee, Switzerland). To maximize the LBA production, pH was left uncontrolled during the exponential growth phase and then with the automatic addition of 5M NaOH (Sigma-Aldrich) was maintained at a value of 6.5 (Alonso et al., 2012). Bioreactor fermentation process lasted a total of 72 hours.

2.1.4. Detection and elimination of bacterial endotoxins

P. taetrolens LMG 2336, as other Gram-negative bacteria, produces endotoxins. These endotoxins must be eliminated so that the products obtained can be used in the food field. To this end, after fermentation, the medium was centrifuged (10 000 rpm, 20 minutes) and tangentially microfiltered, employing the same equipment described in Section 2.1.2. ToxinSensor™ Gel Clot kit (GenScript, Piscataway, NJ, USA) was used to detect the presence of endotoxins, employing the semi-quantitative detection protocol (minimum detected concentration of endotoxins of 0.25 EU mL^{-1} (EU, endotoxin units)).

2.2. Film preparation

Four different type of film-forming solutions (Table 4.1) were prepared as follows. After being tangentially microfiltered, gelatine (Sigma-Aldrich) was added to the fermented whey permeate fraction. Preliminary tests were carried out without adding gelatine and films could not be properly peeled off from the Petri dishes. Therefore, two different concentrations of gelatine (30 and 60% w/w) were used. The mixture was heated at

40ºC to dissolve the gelatine and to obtain a homogeneous solution. LBA30 and LBA60 were directly obtained from this solution. To prepare LP30 and LP60, *Lactobacillus plantarum* CECT 9567 (from the Spanish Type Culture Collection, Valencia, Spain) was added to the film-forming solution until a concentration of 8 $\log_{10}$ CFU mL^{-1} was reached. *L. plantarum* was previously growth in MRS Broth (de Man, Rogosa and Sharpe, Sigma-Aldrich) in aerobic conditions, at 30ºC for 24 h.

To obtain the dried films, 20 mL of each film-forming solution were cast in a Petri dish and dried in an oven at 40ºC for 24 h. Finally, the films could be removed from the Petri dishes without being sticky or brittle. Two controls only with gelatine (30 and 60% (*w/w*)) were also prepared.

Table 4.1. Composition of the different types of film-forming solutions prepared.

Samples	Percentage of gelatine (*w/w*) added	LBA (g L^{-1})	*Lactobacillus plantarum* CECT 9567
Control (C30)	30	-	-
Control (C60)	60	-	-
LBA30	30	~ 40	-
LBA60	60	~ 40	-
LP30	30	~ 40	8 $\log_{10}$ CFU mL^{-1}
LP60	60	~ 40	8 $\log_{10}$ CFU mL^{-1}

2.3. Film characterization

2.3.1. Composition of the film

To check the amount of LBA in the films, High Performance Liquid Chromatography (HPLC) was employed according to Sáez-Orviz et al. (2019) (Sáez-Orviz, Camilleri, Marcet, Rendueles, & Díaz, 2019). 0.5 g of each film sample were dissolved in 1 mL of distilled water and was filtered (0.22 µm, Whatman, Sigma-Aldrich) before HPLC analysis. The chromatography equipment used (Agilent 1200, Agilent Technologies Inc., Santa Clara, CA, USA) was fitted with Coregel ION 300 column (Teknocroma, Barcelona, Spain) coupled to a refractive index detector (at a temperature of 40ºC). Sulfuric acid solution (0.450 mM L^{-1}, pH 3.1) was used as a mobile phase with a flow rate of 0.3 mL min^{-1} and a column temperature of 75ºC. Data acquisition and analysis were performed with ChemStation software (Agilent).

2.3.2. Thickness and mechanical properties

The thickness of the films was measured using a digital micrometer (Mitutoyo, Japan). The analysis was performance at ten different points, both inside and outside the films. Film thickness is reported as the average of these measurements.

Mechanical characterisation was performed using a TA.XTplus Texture Analyzer (Stable Systems, Godalming, Surrey, UK). Films were cut into squares and were submitted to a penetration test, at room temperature, employing a spherical probe SMS P/5S with a test speed of 2.0 mm s^{-1} and a 5 kg load cell. Results are expressed in terms of puncture strength (PS) and puncture deformation (PD). Both parameters were calculated according to the following equations:

$$\mathrm{PS} = \mathrm{Fm}/\mathrm{Th}$$

$$\mathrm{PD} = (\sqrt{\mathrm{D}^2 + \mathrm{R}^2} - \mathrm{R})/\mathrm{R}$$

Fm is the maximum force applied before the breakage of the film (N), Th is the film thickness (mm), D is the distance covered by the probe while it is in contact with the film until the film is broken (mm) and R is the radius of the orifice in the plates (mm). Experiments were carried out in triplicate and reported results correspond to the mean value.

2.3.3. Water solubility (WS)

Water solubility (WS) was measured as follows. Films were cut into circumferences of 40 mm of diameter and were immersed in 20 mL of distilled water with 2% (*w/w*) Tris-HCl pH 7.0 (Sigma-Aldrich). Samples were kept for 24 h at room temperature. After this time, the mixture was filtered using a vacuum pump and Whatman no 1 paper (Sigma-Aldrich) to recover the insolubilized pieces of films and they were dried in an oven at 105ºC for 24 h. WS was calculated as follows (Blanco-Pascual, Montero, & Gómez-Guillén, 2014):

$$\mathrm{WS}(\%) = (\mathrm{m1} - \mathrm{m2})/\mathrm{m1}\ \mathrm{x}\ 100$$

where m1 is the weight (g) of the dry films and m2 is the weight (g) of the solubilized and dry films. Experiments were carried out in triplicate and reported results correspond to the mean value.

2.3.4. Optical transmittance, transparency, and opacity index

Optical transmittance and transparency of the films were measured as follows. Rectangular pieces of each film were cut and place into a spectrophotometer test cell. Wavelengths from 200 to 800 nm were tested in a Helios gamma spectrophotometer (Thermo Fischer Scientific, USA). An empty test cell was used as reference. Transparency of the films was calculated as follows (Sáez-Orviz, Marcet, Rendueles, & Díaz, 2021):

$$\text{Transparency} = \text{A}_{600}/\text{x}$$

where A_{600} is the film absorbance at 600 nm and x is the thickness of the film (mm).

2.3.5. Colour properties

Colour of the film samples was measured employing a Lovibond® LC100 Spectrocolorimeter (Tintometer® Group, Lovibond house, UK). Three parameters were measured: L^* (lightness/brightness), a^* (redness/greenness) and b^* (yellowness). A white standard plate was tested. The total colour difference ($\Delta\mathbf{E}$) was calculated as follows (Zabihollahi, Alizadeh, Almasi, Hanifian, & Hamishekar, 2020):

$$\Delta\text{E} = \sqrt{(\Delta\text{L}^*)^2 + (\Delta\text{a}^*)^2 + (\Delta\text{b}^*)^2}$$

where ΔL*, Δa* and Δb* are the difference between the colour of the white standard plate (L*= 94.00, a*= 0.8 and b*=1.1) and film samples. The b* and L* parameters are linked to yellowness index (YI) according to the following equation (Francis F. J. & Clydesdale F. M., 1977):

$$\text{YI} = (142.86\ \text{x}\ \text{b}^*)/\text{L}^*$$

Experiments were carried out in triplicate and reported results correspond to the mean value.

2.3.6. Scanning electron microscopy (SEM)

Surface and cross-section film morphology was observed employing scanning electron microscopy (SEM) (JSM-6610LV, JEOL, USA). Films samples were freeze-dried (0.1 mBar, -70ºC for 24 h) and cut into squares employing a surgical blade. The film squares were mounted around stubs and coated with gold. Cross-section morphology of the films was observed with magnifications of 500x.

2.3.7. Viability of *Lactobacillus plantarum* CECT 9567 and lactobionic acid evolution inside the films under storage conditions

The evolution of the probiotic growth and LBA concentration was followed for 15 days. In addition to viability on LP30 and LP60 films, two controls were also carried out in C30 and C60 films additivated with *L. plantarum* (C30LP and C60LP), with the aim to analyse the effect of LBA on the viability of the microorganism. Samples were taken at 0, 1, 3, 8, 10, 13 and 15 days. For both measurements, 0.1 g of LP30 and LP60 film samples were placed in a Stomacher™ bag (Seward, UK) with 1 mL of NaCl 0.7% (*w/v*) (Sigma-Aldrich) and were homogenized with a Stomacher™ device (Seward, UK) at medium speed for 60 s to dissolve the film. Microbial growth was analyzed by preparing serial dilutions (1:10) and incubating on MRS agar plates for 48 h at 30ºC. Results were expressed in $\log_{10}$ CFU g^{-1}. LBA concentration was also measured as explained in Section 2.3.1. In both cases, each sample was performanced in triplicate.

2.4. Statistical analysis

Analysis of variance (ANOVA) was applied. Fisher's Least Significant Difference (LSD) procedure was performed to determine significant differences between the data and a level of $p < 0.05$ was considered significant. Statgraphics 18® Centurion software was used for the analysis.

3. Results and discussion

3.1. Analysis of fermented whey composition and removal of endotoxins

The presence of endotoxins was checked after fermentation in the bioreactor. The result with the ToxinSensor™ Gel Clot kit was positive, indicating the presence of a concentration of endotoxins higher than 0.25 EU mL^{-1} in the fermented whey. After centrifugation and tangential microfiltration of the fermented whey, the endotoxin assay was performed again. The results were negative, so the concentration of endotoxins was lower than 0.25 EU mL^{-1} in the permeate from ultrafiltration of fermented whey. As far as it is known, there is no limit available for endotoxin present in orally administered products (Wassenaar & Zimmermann, 2018). Despite this, the concentration is far below the values required in the pharmaceutical industry (5 EU per kg of body mass) (Wassenaar & Zimmermann, 2018). Therefore, the product obtained with this microfiltered fermented whey can be considered safe. Endotoxins tend to form globules or aggregates in aqueous solutions (Taylor, Li, & Luo, 1982). The globules can have a variety of shapes (Magalhães, Lopes, & Priscila, 2007) and they are larger and bigger in

the presence of different ions, such as Ca^{2+}, Mg^{2+} and Na^{+} (Taylor et al., 1982). Individual endotoxin molecules have a molecular weight between 10 and 20 kDa, but these larger aggregates can have a molecular weight as high as 1 MDa (Taylor et al., 1982). The sweet whey permeate has a considerable concentration of calcium (Nishanthi, Chandrapala, & Vasiljevic, 2017). Thus, the presence of Ca^{2+} allows a greater aggregation of endotoxins to be retained in the tangential microfiltration process, which facilitates their removal to an acceptable concentration. This is an important point for the potential use of LBA in the food field produced from a by-product of the food industry, as none of the research on whey fermentation to produce LBA by *P. taetrolens* has enquired this problem (Alonso et al., 2011) (Alonso, Rendueles, & Díaz, 2013b) (Alonso, Rendueles, & Díaz, 2013c) (García, Bautista, Rendueles, & Díaz, 2019).

Regarding the composition of the fermented whey, only lactose and LBA were measured as the permeate has a very low concentration of fat (0.36%) and proteins (0.85%) (Janine Beucler, 2004). LBA concentration after the fermentation was of 44.69 ± 5.84 g L^{-1} and the lactose concentration was of 7.36 ± 0.57 g L^{-1}. After the microfiltration process to remove endotoxins, the concentration of both compounds in the permeate was again analysed, yielding 40.92 ± 1.09 and 6.32 ± 0.84 g L^{-1} for LBA and lactose, respectively. The microfiltered fermented permeate whey was diluted with water to a concentration of 15 g L^{-1} of LBA. There is currently no established legal concentration for human consumption (*FDA. Code of Federal Regulations, Title 21, 21 CFR 172.720. US Food and Drug Administration*, 2017), but the LBA concentration used to make the films is much lower than the values that would cause similar effects to lactose intolerance (24 g LBA per day (Cardoso, Marques, Dagostin, & Masson, 2019b)).

3.2. Characterization of the films

3.2.1. Thickness and mechanical properties of the films

The thickness of the edible films is shown in Table 4.2. Three groups were distinguished with significant differences ($p < 0.05$): control samples (C30 and C60), edible films with 30% of gelatine (*w*/*w*) (LBA30 and LP30) and edible films with 60% of gelatine (*w*/*w*) (LBA60 and LP60). The presence of LBA made the films thicker compared to the control films. This may be because LBA molecules are very hygroscopic (Cardoso, Marques, Sotiles, et al., 2019) and have a higher capacity to retain water, making the films thicker. Furthermore, the more gelatine added in LBA films, the thicker they became. Regarding the presence of probiotics, it did not influence the thickness of the films.

Regarding mechanical properties, LBA affected this parameter (Table 4.2). PS values obtained with the same amount of gelatine (LBA60 and LP60) were lower than C60 and similar to the control with less amount of gelatine (C30). LBA30 and LP30 showed the lowest PS values. Thus, the presence of LBA in the edible films made them less resistant. LBA molecules are very hygroscopic (54 g mol^{-1}) comparing with other agents such as glycerol (33 g mol^{-1}) or sorbitol (14 g mol^{-1}) (Grimes, Green, Wildnauer, & Edison, 2004) and have a greater capacity to retain water (Cardoso, Marques, Sotiles, et al., 2019). Due to these characteristics, the presence of LBA makes the films thicker but mechanically weaker.

Table 4.2. Puncture strength (PS), puncture deformation (PD), thickness, water solubility (WS) and transparency index of the four edible film samples. Different letters in the same column indicate significant differences ($p < 0.05$).

Sample	PS (N mm^{-1})	PD (%)	Thickness (µm)	WS (%)	Transparency index
C30	82.0 ± 12.2 ^{a}	7.8 ± 2.4 ^{a}	55.3 ± 15.7 ^{a}	20.4 ± 0.9 ^{a}	1.05 ± 0.15 ^{a}
C60	130.3 ± 13.2 ^{b}	13.7 ± 7.9 ^{b}	63.6 ± 15.8 ^{a}	21.1 ± 2.3 ^{a}	1.68 ± 0.07 ^{a}
LBA30	31.9 ± 3.6 ^{c}	11.8 ± 2.7 ^{b}	165.4 ± 38.5 ^{b}	63.5 ± 3.4 ^{b}	3.18 ± 0.29 ^{b}
LBA60	82.5 ± 11.3 ^{a}	12.6 ± 4.1 ^{b}	212.0 ± 36.9 ^{c}	66.8 ± 4.0 ^{b}	3.45 ± 0.24 ^{c}
LP30	27.7 ± 2.0 ^{c}	28.5 ± 2.8 ^{c}	194.1 ± 22.9 ^{b}	60.8 ± 3.5 ^{b}	2.87 ± 0.03 ^{b}
LP60	73.7 ± 11.4 ^{a}	33.8 ± 3.5 ^{c}	230.3 ± 28.9 ^{c}	61.0 ± 4.6 ^{b}	3.48 ± 0.07 ^{c}

In the case of PD, significant differences were found when the probiotic was added, since LP30 and LP60 showed the highest values (Table 4.2), so, the addition of *L. plantarum* made the films stickier. This can be explained by the fact that the microbial mass increases the dampness of the film-forming solution and thus of the films, resulting in higher PD values. Other authors also observed that the addition of microorganisms affected the elongation break parameter (Shahrampour, Khomeiri, Razavi, & Kashiri, 2020) (Gagliarini, Diosma, Garrote, Abraham, & Piermaria, 2019). For all the other films, the values obtained were similar, apart from C30 which showed the lowest value, probably due to its thin thickness. Statistically, the values found for C60, LBA30 and LBA60 were similar (Table 4.2), so the presence of LBA and the percentage of gelatine did not influence the PD parameter.

3.2.2. Water solubility (WS)

WS of LBA and LP edible films at room temperature was higher than the gelatine control films (Table 4.2). Due to the chemical characteristics of LBA it has a high interaction with water which makes this compound to be easily soluble in aqueous media. In a

recent study, 400 mg mL^{-1} of LBA were completely dissolved at room temperature (Cardoso, Marques, Sotiles, et al., 2019). Despite their high solubility, WS values are lower than for other films such as casein films (Marcet et al., 2017), soybean polysaccharides (Liu et al., 2020) or sodium alginate (Yerramathi et al., 2021). It was also observed that the presence of probiotic microorganisms did not alter the solubility of the films. This result was also observed by other authors (Shahrampour et al., 2020) (Khodaei, Hamidi-Esfahani, & Lacroix, 2020).

3.2.3. Light transmittance, transparency, and opacity index

The light transmittance of the edible films was analysed in a wavelength range between 200 and 800 nm (Figure 4.1). A low transmission in the UV range is a desirable property for food packaging material, since the 98% of UV radiation (315 to 400 nm) reaches the earth (Criado, Fraschini, Salmieri, & Lacroix, 2020), favouring the oxidation of foods with high lipid content (Marcet et al., 2017). For UVC (100-280 nm) and UVB (280-320 nm) regions, the transmittance of all the film samples were very low. In the UVA (320-400 nm) range, there was an increase in the optical transmittance only for the control samples (C30 and C60).

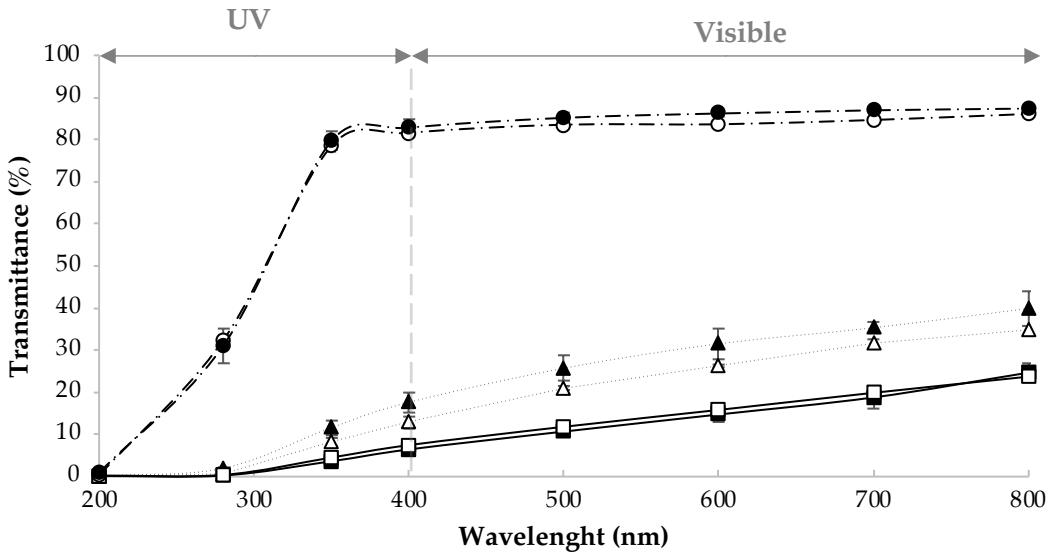


Figure 4.1. Light transmittance (%) of the edible films at different wavelengths (200-800 nm). (●) C60, (○) C30, (▲) LBA30, (△) LP30, (■) LBA60 and (□) LP60. Statistically ($p < 0.05$), three different groups were observed: control samples (C30, C60), LBA30-LP30 and LBA60-LP60.

For wavelengths in the visible region, the optical transmittance for the LBA edible films was very low compared with the control edible films (Figure 4.1). The maximum transmittance, reached at 800 nm, were of 39.97%, 34.65%, 24.60% and 23.67% for LBA30, LP30, LBA60 and LP60 edible film samples, respectively. There were no

significant differences between the controls with both percentages of gelatine (C30 and C60) in the transmittance values but there were significant differences between the LBA-LP edible film samples, being LBA60 and LP60 the ones with the lower transmittance values. The results obtained were consistent with the transparency index (Table 2) since control films were more transparent than LBA-LP films. Due to these differences, it can be concluded that the composition of the fermented whey affected the transmittance parameter. Although the sweet whey permeate is a very poor medium, it may be that some minerals (0.53%) (Janine Beucler, 2004) or the gelatine itself that interact with the LBA resulting in more opaque films, as the sweet whey permeate is transparent after fermentation. Besides that, the addition of probiotic microorganism did not influence this parameter, as LBA30-LP30 and LBA60-LP60 groups did not show significant differences.

3.2.4. Colour properties

Colour properties are important in the development of food packaging as the visual appearance of food products is a key factor that directly influences consumer acceptability. The edible film colour properties are shown in Table 4.3. Significant differences ($p < 0.05$) were found in all parameters measured (L*, a*, b*, ΔE and YI) only between control and LBA edible films samples, regardless of whether they had probiotic microorganisms or not. All LBA films were slightly yellowish, as *YI* parameter showed (Table 3). This yellowish hue may be due to the natural colour of the sweet whey permeate (Janine Beucler, 2004), which remains intact after microbial fermentation.

Table 4.3. Colour of the edible film samples. L* (lightness/brightness), a* (redness/greenness), b* (yellow/blueness), ΔE (total colour difference), YI (yellowness index) and opacity of the edible film samples. Different letters in the same column indicate significant differences ($p < 0.05$).

Sample	L*	a*	b*	ΔE	YI
C30	92.1 ± 0.6 [a]	0.5 ± 0.2 [a]	1.7 ± 0.6 [a]	2.3 [a]	2.69 [a]
C60	92.3 ± 0.2 [a]	0.17 ± 0.3 [a]	1.2 ± 0.8 [a]	2.1 [a]	1.79 [a]
LBA30	93.9 ± 0.7 [b]	0.5 ± 0.2 [b]	7.3 ± 0.7 [b]	6.3 [b]	11.16 [b]
LBA60	92.6 ± 0.9 [b]	0.3 ± 0.1 [b]	7.6 ± 0.4 [b]	6.8 [b]	11.98 [b]
LP30	94.0 ± 0.5 [b]	0.4 ± 0.2 [b]	7.9 ± 0.5 [b]	6.9 [b]	12.01 [b]
LP60	92.5 ± 1.0 [b]	0.4 ± 0.1 [b]	7.6 ± 0.4 [b]	6.7 [b]	11.74 [b]

These data are reinforced by the visual appearance of the films (Figure 4.2), where it was observed that the controls were completely transparent and colourless while the edible LBA films had opaquer and yellower appearance. Despite these differences, edible films were homogeneous in all cases.

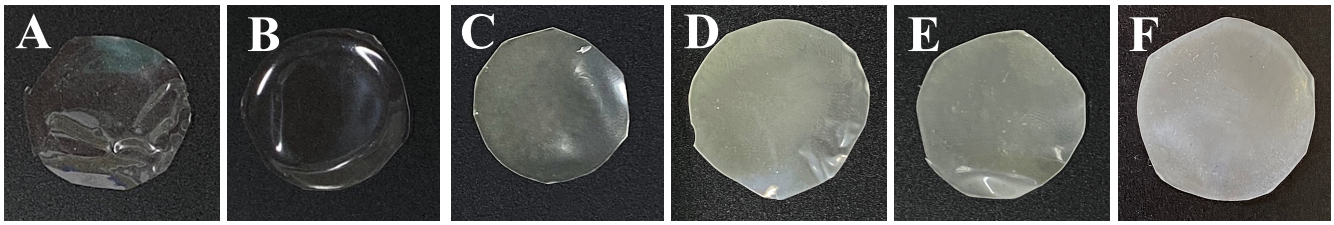


Figure 4.2. Visual appearance of the edible films. (A) C30, (B) C60, (C) LBA30, (D) LBA60, (E) LP30 and (F) LP60 edible films.

3.2.5. Scanning electron microscopy

The surface microstructure of all tested films was homogeneous, without pores and with no difference between them (data not shown). Micrographs of the cross-section of the edible films are shown in Figure 4.3. The presence of LBA and *L. plantarum* did not produce noticeable changes in the microstructure of the films. However, the percentage of gelatine did. LBA60 and LP60 edible films showed a more compact structure with less agglomerated proteins than LBA30 and LP30 films. This agrees with the results obtained for the PS values (Table 4.2), where films with 60% gelatine obtained better values than those with 30% gelatine. In the case of the controls, protein agglomerations were observed in both cases.

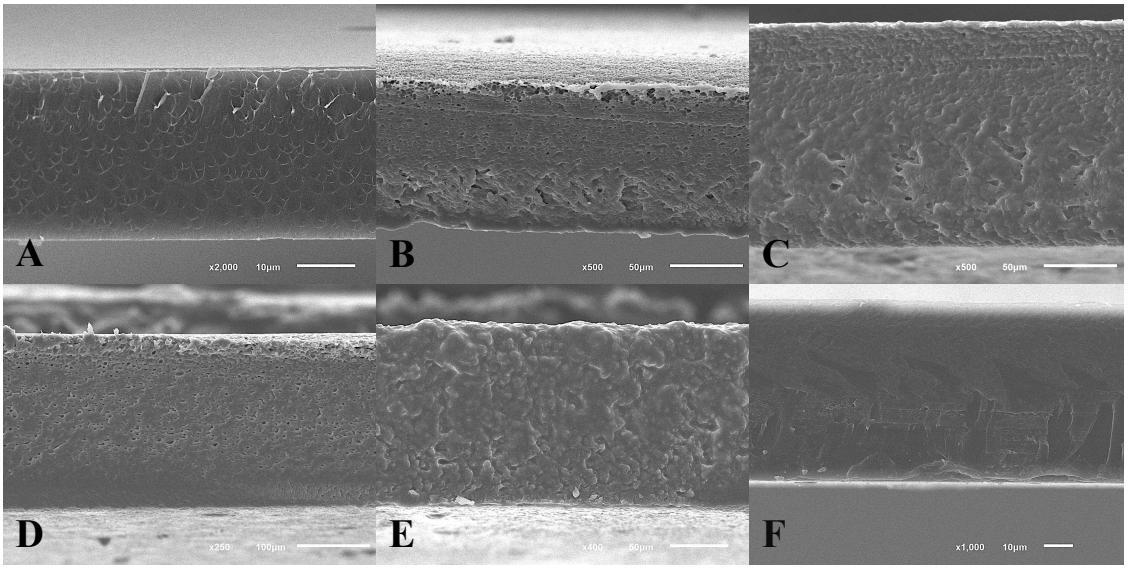


Figure 4.3. SEM images of the edible film cross-sections. (A) C30 (2000x); (B) C60 (500x); (C) LBA30 (500x); (D) LBA60 (250x); (E) LP30 (400x) and (F) LP60 (1000x).

3.2.6. Viability of *Lactobacillus plantarum* CECT 9567 and lactobionic acid evolution inside the films under storage conditions

The probiotic growth inside LP edible films is shown in Figure 4.4. In all cases, there was a rapid decrease in the *L. plantarum* concentration between the initial time and the first day, with a reduction of three logarithmic units in the samples. This result indicated that the process conditions have had an impact on bacteria survival. *L. plantarum* was removed from the culture medium to the nutrient-poor film-forming solution and was dry at 40ºC, which may have caused a rapid loss of moisture and thus viability of the microorganisms (Tripathi & Giri, 2014). In the case of the C30LP and C60LP films, the viability was much lower due to the presence of only gelatine in the composition of the film. The presence of LBA in the composition improved the viability of *L. plantarum*, as shown in Figure 4. In films LP30 and LP60, after the initial decrease, a concentration of approximately 4.5 $\log_{10}$ CFU g^{-1} of film remained constant until day 13, when the microbial concentration decreased again, reaching a value of 2.30 and 3.64 $\log_{10}$ CFU g^{-1} of LP30 and LP60 film, respectively. This may be due to the lack of nutrients that can be used as substrate for the maintenance and growth of the probiotic bacteria. As other authors also point out, the storage temperature (4ºC) could also affect the probiotic survival (Melchior, Marino, Innocente, Calligaris, & Nicoli, 2020) (Pescuma, Hébert, Mozzi, & Font de Valdez, 2010), as the optimum temperature for *L. plantarum* growth is 30°C.

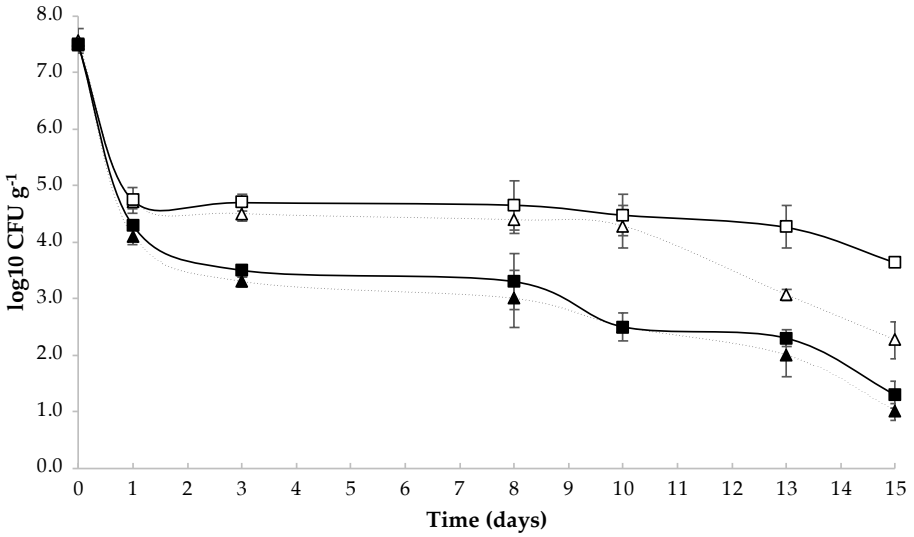


Figure 4.4. Evolution of growth of *L. plantarum* in LP30 (△), LP60 (□), C30LP (▲) and C60LP (■) edible films over 15 days of storage. The amount of microorganism is represented as $\log_{10}$ CFU per g of film. C30LP and C60LP did not show significant differences between them, and significant differences were found between LBA30 and LBA60 from day 13 to 15. Experiments were performanced in triplicated and reported results correspond to the mean value.

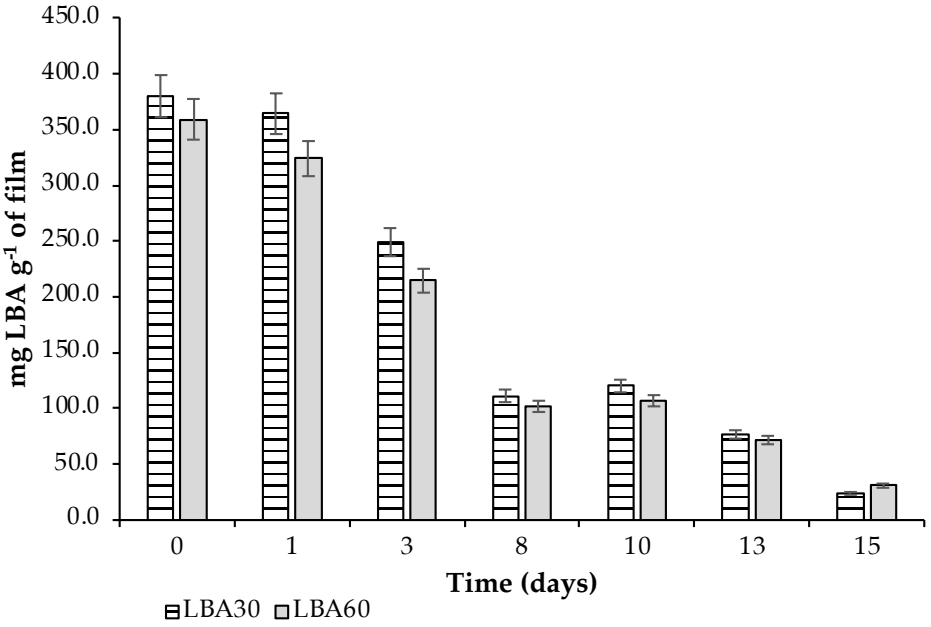


Figure 4.5. Evolution of LBA in LP30 and LP60 edible films over 15 days of storage. LBA concentration is represented as mg of LBA per g of film. There were no significant differences ($p > 0.05$). Experiments were performanced in triplicated and reported results correspond to the mean value.

The probiotic viability results agreed with the analysis of the concentration of LBA in the edible films during 15 days of storage (Figure 4.5). It was observed that the concentration of LBA decreased. On day 13, there was a reduction in LBA concentration of 79.0% for LBA30 films and 79.97% for LBA60 films, so the probiotic was using the prebiotic compound as substrate. At the final point, the reduction in prebiotic concentration was 93.82% and 91.48% for LBA30 and LBA60, respectively. Thus, the decrease in probiotic concentration during the last days is due to the absence of nutrients in the film. In consequence, the presence of LBA favoured the viability of *L. plantarum* during cold storage. Similar studies using LBA as prebiotic compound have shown an enhance in the viability of probiotic microorganisms (Sáez-Orviz, Passannanti, et al., 2021) (Sáez-Orviz, S., Marcet, I., Rendueles, M., Díaz, 2021) (Sáez-Orviz, Puertas, Marcet, Rendueles, & Díaz, 2020). Improvement of microbial viability during cold storage in the presence of prebiotics has also been found with other compounds such as inulin (Orozco-Parra, Mejía, & Villa, 2020) and fructo-oligosaccharides (Bambace, Alvarez, & Moreira, 2019). Therefore, films developed from a by-product of the food industry could be used in the food industry to coat different dairy products, such as cheese, providing them an added value by containing a prebiotic and a probiotic microorganism, which would make it possible to obtain a functional food product.

4. Conclusions

Edible films enriched in LBA employing fermented whey and gelatine as protein matrix were develop and characterised successfully. The ultrafiltrated sweet whey permeate was fermented with *P. taetrolens* and endotoxins were successfully removed after the microfiltration process, allowing its safe use in the food field. Control, LBA30, LP30, LBA60 and LP60 edible films showed adequate mechanical properties, although PS parameter was influenced by the presence of LBA and PD by the presence of the probiotic microorganism. The edible films showed medium water solubility, largely because the chemical characteristics of LBA made them very soluble in water. Films were homogeneous and slightly opaques, as LBA interact somehow with the minerals present in the sweet whey or with the added gelatine itself. Regarding the addition of *L. plantarum* as probiotic microorganism, results were similar between LP30 and LP60 indicating that the amount of gelatine did not influence in the viability of the bacteria. But the presence of LBA did have an influence as this prebiotic compound favoured the maintenance of *L. plantarum* viability during cold storage by being consumed as substrate. A future line of research is to study the behaviour of these films in food products as well as the possible protective capacity for the probiotics of the films themselves in *in vitro* digestion tests.

Author Contributions: Conceptualization, S.S.-O., and I. M.; methodology, formal analysis, investigation, writing-original draft preparation, S. S.-O.; writing-review and editing, I.M. and M. R.; supervision, M. R.; funding acquisition, M. D. All authors have read and agreed to the published version of the manuscript.

Funding: This work was financially supported by the Principality of Asturias, by the project GRUPIN- IDI/2021/000055 and by the grant *Programa Severo Ochoa de Ayudas Predoctorales para la Investigación y Docencia* (grant number BP19-127 to Sáez-Orviz, S.).

Acknowledgements: The authors thank ILAS S.A., for providing the ultrafiltrated sweet whey permeate necessary for the fermentation experiment.

Conflicts of Interest: The authors declare no conflict of interest.

5. References

Adebola, O. O., Corcoran, O., & Morgan, W. A. (2014). Synbiotics: The impact of potential prebiotics inulin, lactulose and lactobionic acid on the survival and growth of lactobacilli probiotics. *Journal of Functional Foods*, *10*, 75–84. https://doi.org/10.1016/j.jff.2014.05.010

Alonso, S., Rendueles, M., & Díaz, M. (2011). Efficient lactobionic acid production from whey by Pseudomonas taetrolens under pH-shift conditions. *Bioresource Technology*, *102*(20), 9730–9736. https://doi.org/10.1016/j.biortech.2011.07.089

Alonso, S., Rendueles, M., & Díaz, M. (2012). Role of dissolved oxygen availability on lactobionic acid production from whey by Pseudomonas taetrolens. *Bioresource Technology*, *109*, 140–147. https://doi.org/10.1016/j.biortech.2012.01.045

Alonso, S., Rendueles, M., & Díaz, M. (2013a). Bio-production of lactobionic acid: Current status, applications and future prospects. *Biotechnology Advances*, *31*(8), 1275–1291. https://doi.org/10.1016/j.biotechadv.2013.04.010

Alonso, S., Rendueles, M., & Díaz, M. (2013b). Feeding strategies for enhanced lactobionic acid production from whey by Pseudomonas taetrolens. *Bioresource Technology*, *134*, 134–142. https://doi.org/10.1016/j.biortech.2013.01.145

Alonso, S., Rendueles, M., & Díaz, M. (2013c). Selection method of pH conditions to establish Pseudomonas taetrolens physiological states and lactobionic acid production. *Applied Microbiology and Biotechnology*, *97*(9), 3843–3854. https://doi.org/10.1007/s00253-012-4607-x

Andrade, R. M. S., Ferreira, M. S. L., & Gonçalves, É. C. B. A. (2016). Development and Characterization of Edible Films Based on Fruit and Vegetable Residues. *Journal of Food Science*, *81*(2), E412–E418. https://doi.org/10.1111/1750-3841.13192

Bambace, M. F., Alvarez, M. V., & Moreira, M. del R. (2019). Novel functional blueberries: Fructo-oligosaccharides and probiotic lactobacilli incorporated into alginate edible coatings. *Food Research International*, *122*(December 2018), 653–660. https://doi.org/10.1016/j.foodres.2019.01.040

Blanco-Pascual, N., Montero, M. P., & Gómez-Guillén, M. C. (2014). Antioxidant film development from unrefined extracts of brown seaweeds Laminaria digitata and Ascophyllum nodosum. *Food Hydrocolloids*, *37*, 100–110. https://doi.org/10.1016/j.foodhyd.2013.10.021

Cardoso, T., Marques, C., Dagostin, J. L. A., & Masson, M. L. (2019a). Lactobionic Acid as a Potential Food Ingredient: Recent Studies and Applications. *Journal of Food Science*, *84*(7), 1672–1681. https://doi.org/10.1111/1750-3841.14686

Cardoso, T., Marques, C., Dagostin, J. L. A., & Masson, M. L. (2019b). Lactobionic Acid as a Potential Food Ingredient: Recent Studies and Applications. *Journal of Food Science*, *84*, 1672–1681. https://doi.org/10.1111/1750-3841.14686

Cardoso, T., Marques, C., Sotiles, A. R., Dagostin, J. L. A., & Masson, M. L. (2019). Characterization of lactobionic acid evidencing its potential for food industry application. *Journal of Food Process Engineering*, *42*(7), 1–11. https://doi.org/10.1111/jfpe.13277

Chandla, N. K., Saxena, D. C., & Singh, S. (2017). Amaranth (Amaranthus spp.) starch isolation, characterization, and utilization in development of clear edible films. *Journal of Food Processing and Preservation*, *41*(6). https://doi.org/10.1111/jfpp.13217

Charles-Rodríguez, A.V.; Rivera-Solís, L.L.; Martins, J.T.; Genisheva, Z.; Robledo-Olivo, A.;

González-Morales, S.; López-Guarin, G.; Martínez-Vázquez, D.G.; Vicente, A.A.; Flores-López, M. . (2020). Edible Films Based on Black Chia (Salvia hispanica L.) Seed Mucilage Containing Rhus microphylla Fruit Phenolic Extract. *Coatings*, *10*(326). https://doi.org/https://doi.org/10.3390/coatings10040326

Criado, P., Fraschini, C., Salmieri, S., & Lacroix, M. (2020). Cellulose nanocrystals (CNCs) loaded alginate films against lipid oxidation of chicken breast. *Food Research International*, *132*(October 2019), 109110. https://doi.org/10.1016/j.foodres.2020.109110

Espitia, P. J. P., Batista, R. A., Azeredo, H. M. C., & Otoni, C. G. (2016). Probiotics and their potential applications in active edible films and coatings. *Food Research International*, *90*, 42–52. https://doi.org/10.1016/j.foodres.2016.10.026

FDA. Code of Federal Regulations, Title 21, 21 CFR 172.720. US Food and Drug Administration. (2017). Retrieved from https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=172.720

Fernandes, Leonardo M. Guimaraes Jonas T., Pimentel Tatiana C., Esmerino Erick A., Freitas Mônika Q., Carvalho Carlos Wanderlei P., C. A. G. and S. M. C. (2020). Chapter 7. Edible whey protein films and coatings added with prebiotic ingredients. In *Agri-Food Industry Sytrategies for Healthy Diets and sustainability* (pp. 177–193). https://doi.org/https://doi.org/10.1016/B978-0-12-817226-1.00007-2

Francis F. J. & Clydesdale F. M. (1977). *Food Colorimetry: Theroy and Applications*. Retrieved from https://doi.org/10.1002/food.19770210122

Gagliarini, N., Diosma, G., Garrote, G. L., Abraham, A. G., & Piermaria, J. (2019). Whey protein-kefiran films as driver of probiotics to the gut. *Lwt*, *105*(February), 321–328. https://doi.org/10.1016/j.lwt.2019.02.023

Galus, S., Kibar, E. A. A., Gniewosz, M., & Kraśniewska, K. (2020). Novel materials in the preparation of edible films and coatings-A review. *Coatings*, *10*(7), 1–14. https://doi.org/10.3390/coatings10070674

García, C., Bautista, L., Rendueles, M., & Díaz, M. (2019). A new synbiotic dairy food containing lactobionic acid and Lactobacillus casei. *International Journal of Dairy Technology*, *72*(1), 47–56. https://doi.org/10.1111/1471-0307.12558

García, C., Rendueles, M., & Díaz, M. (2017). Microbial amensalism in Lactobacillus casei and Pseudomonas taetrolens mixed culture. *Bioprocess and Biosystems Engineering*, *40*(7), 1111–1122. https://doi.org/10.1007/s00449-017-1773-3

Grimes, P. E., Green, B. A., Wildnauer, R. H., & Edison, B. L. (2004). The use of polyhydroxy acids (PHAs) in photoaged skin. *Cutis*, *73*(2 Suppl), 3–13.

Gutiérrez, T. J., Tapia, M. S., Pérez, E., & Famá, L. (2015). Structural and mechanical properties of edible films made from native and modified cush-cush yam and cassava starch. *Food Hydrocolloids*, *45*, 211–217. https://doi.org/10.1016/j.foodhyd.2014.11.017

Janine Beucler, M. D. and E. A. F. (2004). Design of a beverage from whey permeate. *Journal of Food Science*, *69*(9).

Kang, S., Kong, F., Shi, X., Han, H., Li, M., Guan, B., Yue, X. (2020). Antibacterial activity and

mechanism of lactobionic acid against Pseudomonas fluorescens and Methicillin-resistant Staphylococcus aureus and its application on whole milk. *Food Control*, *108*(September 2019), 106876. https://doi.org/10.1016/j.foodcont.2019.106876

Khodaei, D., Hamidi-Esfahani, Z., & Lacroix, M. (2020). Gelatin and low methoxyl pectin films containing probiotics: Film characterization and cell viability. *Food Bioscience*, *36*(June 2019). https://doi.org/10.1016/j.fbio.2020.100660

Liu, C., Huang, J., Zheng, X., Liu, S., Lu, K., Tang, K., & Liu, J. (2020). Heat sealable soluble soybean polysaccharide/gelatin blend edible films for food packaging applications. *Food Packaging and Shelf Life*, *24*(February), 100485. https://doi.org/10.1016/j.fpsl.2020.100485

Magalhães, P. O., Lopes, A. M., & Priscila, G. (2007). *Methods of Endotoxin Removal from Biological Preparations : a Review*. *10*(3), 388–404.

Marcet, I., Sáez, S., Rendueles, M., & Díaz, M. (2017). Edible films from residual delipidated egg yolk proteins. *Journal of Food Science and Technology*, *54*(12), 3969–3978. https://doi.org/10.1007/s13197-017-2861-8

Melchior, S., Marino, M., Innocente, N., Calligaris, S., & Nicoli, M. C. (2020). Effect of different biopolymer-based structured systems on the survival of probiotic strains during storage and in vitro digestion. *Journal of the Science of Food and Agriculture*, *100*(10), 3902–3909. https://doi.org/10.1002/jsfa.10432

Nishanthi, M., Chandrapala, J., & Vasiljevic, T. (2017). Compositional and structural properties of whey proteins of sweet, acid and salty whey concentrates and their respective spray dried powders. *International Dairy Journal*, *74*, 49–56. https://doi.org/10.1016/j.idairyj.2017.01.002

Orozco-Parra, J., Mejía, C. M., & Villa, C. C. (2020). Development of a bioactive synbiotic edible film based on cassava starch, inulin, and Lactobacillus casei. *Food Hydrocolloids*, *104*(January), 105754. https://doi.org/10.1016/j.foodhyd.2020.105754

Pająk, P., Przetaczek-Rożnowska, I., & Juszczak, L. (2019). Development and physicochemical, thermal and mechanical properties of edible films based on pumpkin, lentil and quinoa starches. *International Journal of Biological Macromolecules*, *138*, 441–449. https://doi.org/10.1016/j.ijbiomac.2019.07.074

Pellá, M. C. G., Silva, O. A., Pellá, M. G., Beneton, A. G., Caetano, J., Simões, M. R., & Dragunski, D. C. (2020). Effect of gelatin and casein additions on starch edible biodegradable films for fruit surface coating. *Food Chemistry*, *309*(March 2019), 125764. https://doi.org/10.1016/j.foodchem.2019.125764

Pescuma, M., Hébert, E. M., Mozzi, F., & Font de Valdez, G. (2010). Functional fermented whey-based beverage using lactic acid bacteria. *International Journal of Food Microbiology*, *141*(1–2), 73–81. https://doi.org/10.1016/j.ijfoodmicro.2010.04.011

Saarela, M., Hallamaa, K., Mattila-Sandholm, T., & Mättö, J. (2003). The effect of lactose derivatives lactulose, lactitol and lactobionic acid on the functional and technological properties of potentially probiotic Lactobacillus strains. *International Dairy Journal*, *13*(4), 291–302. https://doi.org/10.1016/S0958-6946(02)00158-9

Sáez-Orviz, S., Marcet, I., Rendueles, M., Díaz, M. (2021). Bioactive packaging based on delipidated egg yolk protein edible films with lactobionic acid and Lactobacillus plantarum CECT 9567: characterization and use as coating in a food model. *Food Hydrocolloids*.

Sáez-Orviz, S., Camilleri, P., Marcet, I., Rendueles, M., & Díaz, M. (2019). Microencapsulation of Calcium Lactobionate for Protection From Microorganisms in a Solid Phase Food. *Biochemical Engineering Journal*, *150*(June), 107281. https://doi.org/10.1016/j.bej.2019.107281

Sáez-Orviz, S., Marcet, I., Rendueles, M., & Díaz, M. (2021). Bioactive packaging based on delipidated egg yolk protein edible films with lactobionic acid and Lactobacillus plantarum CECT 9567: Characterization and use as coating in a food model. *Food Hydrocolloids*, *119*(January). https://doi.org/10.1016/j.foodhyd.2021.106849

Sáez-Orviz, S., Passannanti, F., Gallo, M., Cante, R. C., Nigro, F., Budelli, A. L., Rendueles, M., Nigro, R. & Díaz, M. (2021). Lactic Acid Bacteria Co-Encapsulated with Lactobionic Acid : Probiotic Viability during In Vitro Digestion. *Appl. Sci.*, *11*(11404). https://doi.org/https://doi.org/ 10.3390/app112311404

Sáez-Orviz, S., Puertas, C., Marcet, I., Rendueles, M., & Díaz, M. (2020). Bioactive synbiotic coatings with lactobionic acid and Lactobacillus plantarum CECT 9567 in the production and characterization of a new functional dairy product. *Journal of Functional Foods*, *75*(July). https://doi.org/10.1016/j.jff.2020.104263

Sapper, M., & Chiralt, A. (2018). Starch-based coatings for preservation of fruits and vegetables. *Coatings*, *8*(5). https://doi.org/10.3390/coatings8050152

Schaafsma, G. (2008). Lactose and lactose derivatives as bioactive ingredients in human nutrition. *International Dairy Journal*, *18*(5), 458–465. https://doi.org/10.1016/j.idairyj.2007.11.013

Shahrampour, D., Khomeiri, M., Razavi, S. M. A., & Kashiri, M. (2020). Development and characterization of alginate/pectin edible films containing Lactobacillus plantarum KMC 45. *Lwt*, *118*(October 2019), 108758. https://doi.org/10.1016/j.lwt.2019.108758

Shapi'i, R. A., Othman, S. H., Nordin, N., Kadir Basha, R., & Nazli Naim, M. (2020). Antimicrobial properties of starch films incorporated with chitosan nanoparticles: In vitro and in vivo evaluation. *Carbohydrate Polymers*, *230*(November 2019), 115602. https://doi.org/10.1016/j.carbpol.2019.115602

Tanwar, R., Gupta, V., Kumar, P., Kumar, A., Singh, S., & Gaikwad, K. K. (2021). Development and characterization of PVA-starch incorporated with coconut shell extract and sepiolite clay as an antioxidant film for active food packaging applications. *International Journal of Biological Macromolecules*, *185*(May), 451–461. https://doi.org/10.1016/j.ijbiomac.2021.06.179

Taylor, P., Li, L., & Luo, R. G. (1982). *Separation Science and Technology Quantitative Determination of Ca Effects on Endotoxin Removal and Protein Yield in a Two-Stage Ultrafiltration Process Quantitative Determination of Ca 2 ¿ Effects on Endotoxin Removal and Protein Yield in a Two-Stage Ult*. (October 2014), 37–41. https://doi.org/10.1081/SS-100100735

Tripathi, M. K., & Giri, S. K. (2014). Probiotic functional foods: Survival of probiotics during

processing and storage. *Journal of Functional Foods*, *9*(1), 225–241. https://doi.org/10.1016/j.jff.2014.04.030

Wassenaar, T. M., & Zimmermann, K. (2018). Lipopolysaccharides in food, food supplements, and probiotics: should we be worried? *European Journal of Microbiology and Immunology*, *8*(3), 63–69. https://doi.org/10.1556/1886.2018.00017

Yerramathi, B. B., Kola, M., Annem Muniraj, B., Aluru, R., Thirumanyam, M., & Zyryanov, G. V. (2021). Structural studies and bioactivity of sodium alginate edible films fabricated through ferulic acid crosslinking mechanism. *Journal of Food Engineering*, *301*(October 2020), 110566. https://doi.org/10.1016/j.jfoodeng.2021.110566

Zabihollahi, N., Alizadeh, A., Almasi, H., Hanifian, S., & Hamishekar, H. (2020). Development and characterization of carboxymethyl cellulose based probiotic nanocomposite film containing cellulose nanofiber and inulin for chicken fillet shelf life extension. *International Journal of Biological Macromolecules*, *160*, 409–417. https://doi.org/10.1016/j.ijbiomac.2020.05.066

4.2. Proteína de yema de huevo

En este apartado se muestran los tres trabajos elaborados en torno a la proteína de yema de huevo delipidada. La proteína de yema de huevo es una fracción poco valorizada en la industria alimentaria (Marcet, Álvarez, Paredes, & Díaz, 2014), debido en parte a su baja solubilidad en agua (Anton, 2013), lo que reduce ampliamente sus aplicaciones. Sin embargo, esta característica hace que sea una matriz ideal para el desarrollo de biomateriales con fines alimentarios. Es por ello, que en este apartado, se empleó esta proteína como matriz en el desarrollo y preparación de *films*, que fueron caracterizados y estudiados antes de añadir añadido ácido lactobiónico como compuesto prebiótico y *L. plantarum* CECT 9567 como bacteria probiótica.

4.2.1. Avances recientes en la aplicación alimentaria de los gránulos de la yema de huevo y la fosvitina

En este subapartado se expone una revisión bibliográfica sobre las características de los gránulos de la yema de huevo y la fosvitina como dos de los componentes de la yema de huevo con un alto valor añadido y diversas aplicaciones en la industria alimentaria. En relación con los gránulos de la yema de huevo, se trata de una fracción con un bajo contenido en lípidos y colesterol y un alto contenido en proteínas. Es por ello que esta fracción puede emplearse como agente emulsificante y espumante, como agente encapsulante y como matriz en el desarrollo de *films*. Cabe destacar que en la elaboración de *films* muestra una muy baja solubilidad en agua, lo que hace que sea una proteína idónea para ser usada en recubrimientos alimentarios, ya que la gran mayoría de productos alimentarios tiene un alto contenido en agua. Además, es una importante fuente de folatos. En cuanto a la fosvitina, se describen los nuevos procedimientos de extracción desarrollados en las últimas investigaciones, su capacidad de ser una fuente de fosfolípidos y sus propiedades nutricionales y antimicrobianas.

- **Publicación:** Marcet, I., Sáez-Orviz, S., Rendueles, M., Díaz, M. 2022. Egg yolk granules and phosvitin. Recent advances in food technology and applications. *LWT*, 153, 112442, ISSN 0023-6438, (https://doi.org/10.1016/j.lwt.2021.112442).
- **Estado:** publicada
- **Índice de impacto:** 4,952 (2020)

Egg yolk granules and phosvitin: recent advances respecting their application in food technology

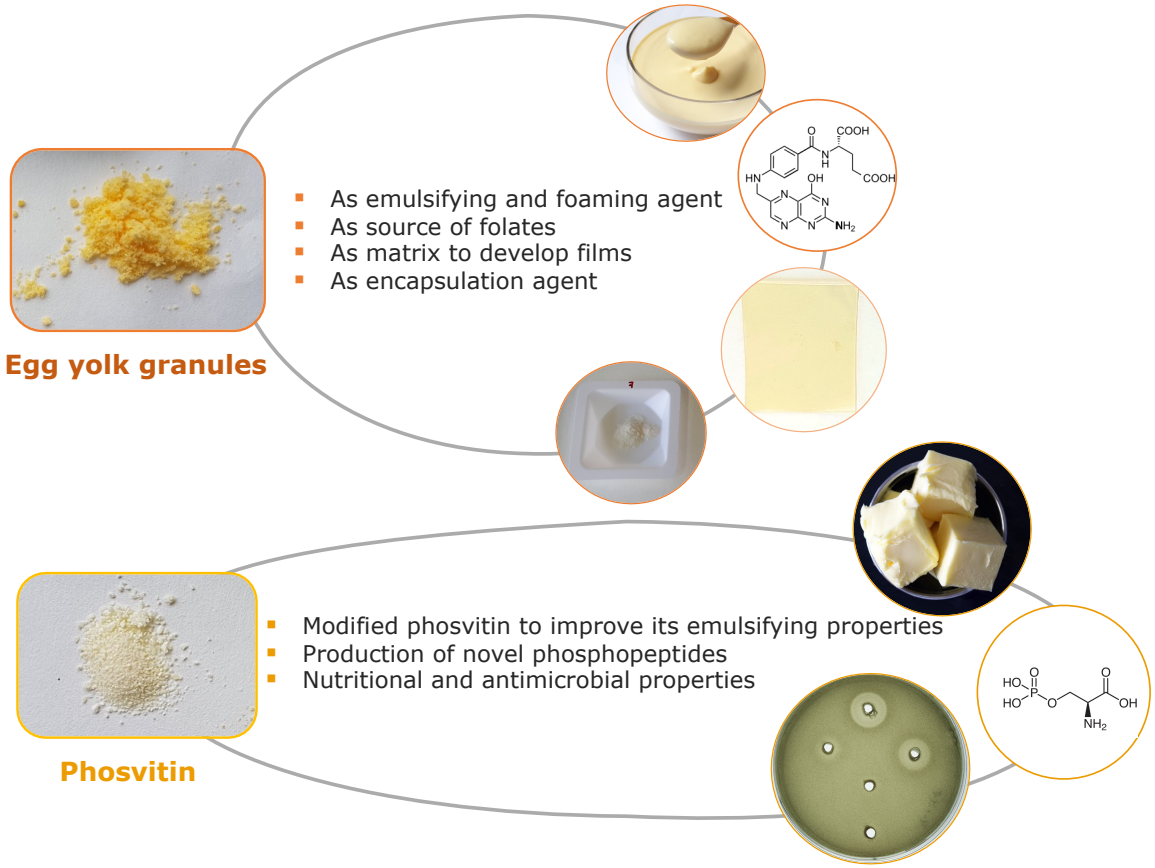
I. Marcet[a], S. Sáez-Orviz[a], M. Rendueles[a*], M. Díaz[a]

[a]Department of Chemical and Environmental Engineering, University of Oviedo, C/Julián Clavería 8, 33006, Oviedo, Spain.

*Corresponding author

E-mail address: mrenduel@uniovi.es (M. Rendueles)

Graphical abstract



Abstract

Egg yolk is consumed all around the world because of its nutritional and functional properties. It can easily be separated by centrifugation into two fractions, the egg yolk granules and plasma fractions. In comparison with the plasma fraction, the granular fraction has a low content of lipids and cholesterol and a high content of proteins; on the other hand, the lipid-rich plasma fraction has gelling and emulsifying properties similar to those of whole egg yolk. Therefore, taking into consideration their different

composition and functional properties, it would be advantageous to increase the value of whole egg yolk by making different use of each of its fractions. In this sense, while the egg yolk plasma could, to some extent, be used as a substitute for whole egg yolk, the range of applications for the granules is more reduced at present and further research into this fraction is required to increase the interest of the food industry in the egg yolk fractionation process. The egg yolk granular fraction is mainly composed of globular proteins, namely lipovitellins or high-density lipoproteins, linked to phosvitin by phosphocalcium bridges. Phosvitin is the most phosphorylated protein found in nature, and shows remarkable metal chelating, antioxidant, emulsifying and antimicrobial properties and, owing to these capacities, it has also the potential to be isolated and incorporated by the food industry as a functional ingredient. Thus, the aim of the present review is to show the most recent advances in the food-related applications developed by the research community for egg yolk granules and their isolated components.

Keywords: egg yolk, granules, high-density lipoproteins, phosvitin, lipovitellins.

1. Introduction

Egg yolk can be divided into two different fractions that can be obtained by dilution of the egg yolk in water, followed by centrifugation. The supernatant is the plasma fraction, which contains 75-81% of the egg yolk dry matter and is mainly composed of low-density lipoproteins (LDL), while the sediment, making up 19-25% of the egg yolk dry matter, is the granular fraction, mainly comprised of high-density lipoproteins (HDL) and phosvitin (Sirvente et al., 2007) (Figure 4.6). LDLs possess a more conventional micelle-like structure, with a layer of phospholipids and proteins surrounding a core of triglycerides and cholesterol. Unlike LDLs, HDLs have a more proteinaceous structure, being a dimeric protein with a relatively small cavity in their structure where lipids can be found. In addition, the structure of the granules has an additional level of complexity, since HDLs are linked to one another, and with phosvitin through phosphocalcic bridges, forming insoluble aggregates of proteins with a diameter between 0.3 and 2.0 μm. In fact, the egg yolk granules can only be effectively dissolved in solutions with a high ionic strength (Anton, 2013).

Looking now at the lipid and protein content of these two fractions, the plasma fraction contains 32% proteins and 64% lipids, within which cholesterol contributes 5%; while the composition of the granular fraction is 64% proteins and 31% lipids, within which cholesterol represents 5%. Therefore, the granular fraction is low in lipids and

cholesterol and shows a high proportion of protein compared to the plasma fraction (Anton, 2007).

These differences in microstructure and composition between the granular and the plasma fractions lead to differences in their functional properties. The plasma fraction is soluble no matter what the pH or the ionic strength of the medium are, and it has a high phospholipid composition and emulsifying and gelling properties similar to those for the whole egg yolk (Kiosseoglou & Paraskevopoulou, 2005; Le Denmat, Anton, & Beaumal, 2000). However, the low solubility of the egg yolk granules limits their applicability, and they have poor functional properties if they are not properly dissolved in the medium, although their nutritional composition could be considered to be healthier owing to their low lipid and cholesterol content. In addition, as was mentioned above, the HDLs are linked together through phosvitin, which is the most phosphorylated protein found in nature and represents 16% of the total dry content of the egg yolk granules. Due to its chemical composition, phosvitin is capable of easily capturing Ca^{2+} and Fe^{2+} ions and it possesses good preservative, antioxidant and emulsifying properties (Lesnierowski & Stangierski, 2018).

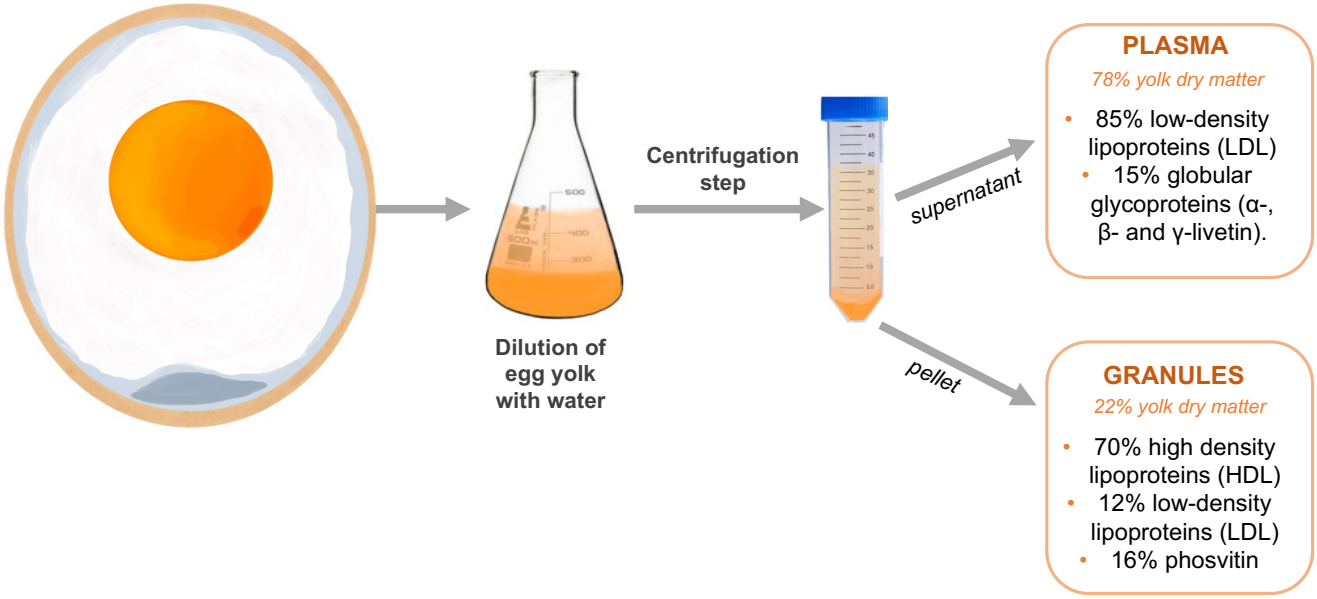


Figure 4.6. Egg yolk granules: formation and composition.

Given these different qualities, the egg yolk fractionation process enables the two fractions to be used separately and each with a different purpose, which would enhance the value of whole egg yolk for the food industry. This, however, is only worthwhile if the two fractions can be commercially exploited in valuable food-related applications. Research into the applicability of egg yolk granules in the food industry is an ongoing

process. They have been used as an emulsifier in the preparation of mayonnaise (Laca, Sáenz, Paredes, & Díaz, 2010), and their emulsification properties after hydrolysis have been tested (Orcajo, Marcet, Paredes & Díaz, 2013). They were treated with phospholipase A1 in order to increase their functional properties (Jin, Huang, Ding, Ma, & Oh, 2011), used as a source of folates (Naderi, House & Pouliot, 2016) or as a source to obtain phosvitin (Ko, Nam, Jo, Lee, & Ahn, 2011). However, an all-embracing study of the most recent research into the improvement of the functional properties of egg yolk granules and their possible exploitation by the food industry is lacking in the current literature.

In this context, the aim of this article is to provide a comprehensive overview of the research papers published over the last five years about the improvement and characterisation of the functional properties of egg yolk granules, as well as their possible practical utilization by the food industry. In addition, the most recent studies concerning phosvitin in the food sector will be highlighted.

2. Egg yolk granules applications

Recent papers on food-related applications for egg yolk granules explore their emulsifying properties, after being previously modified or not, their use as a source of folates, their use as a raw material to prepare edible films or their performance as an encapsulation agent. All these novel studies are summarized in Figure 4.7.

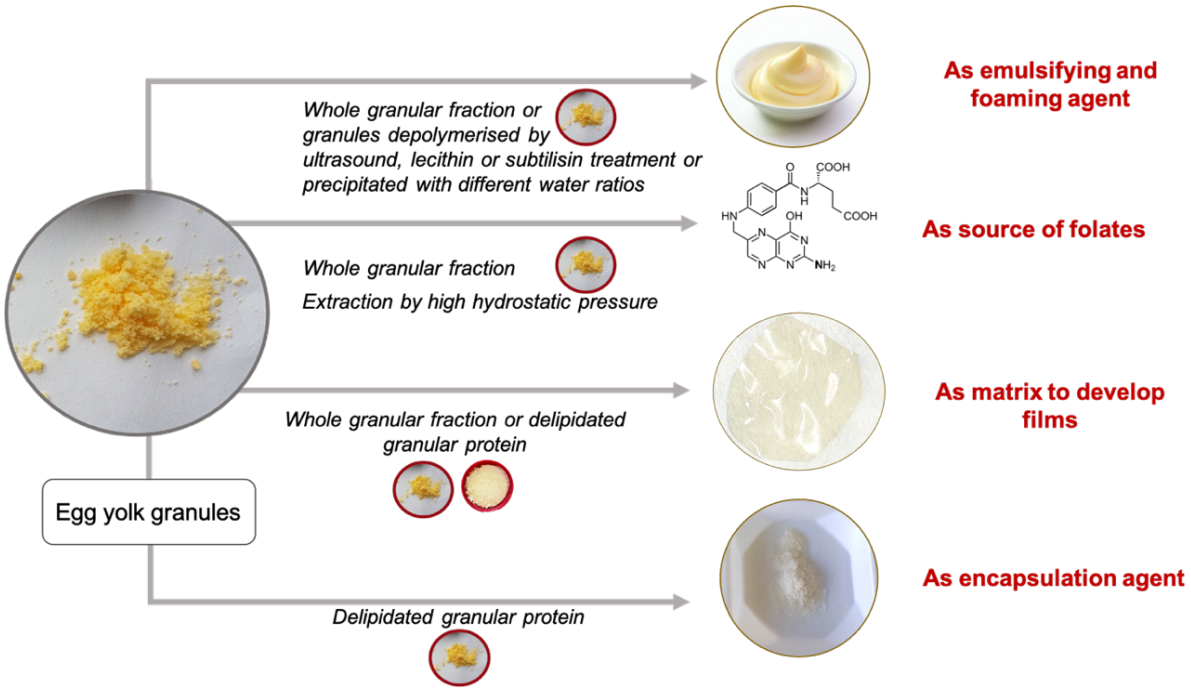


Figure 4.7. Diagram showing egg yolk granules-related applications found in the literature.

2.1. Emulsifying and foaming agent

The emulsifying properties of surface-active compounds are based on their capacity to reach the oil/water interface and cover it properly, and in the case of proteins, those with an extended morphology and high enough flexibility have better emulsifying performance. In this regard, native egg yolk granules have several disadvantages, since their main compounds are relatively large aggregates of globular proteins connected by phosvitin, so their previous solubilisation is necessary to improve their emulsification properties, and as can be observed, several strategies have been explored in recent research (Table 4.4).

Geng, Xie, Wang & Wang (2021) investigated the depolymerisation of the egg yolk granules by high-intensity ultrasound. Granules were dispersed in PBS buffer at pH 7.4 and subjected to ultrasound treatment at several ultrasonic powers for 10 min. According to their results, at the most effective conditions tested, the granule size decreased from 289.4 nm to 181.4 nm, which suggests at least a partial depolymerization of the granules. The emulsifying activity of these treated granules was slightly better than that of native granules. In a similar context, Shen et al. (2020) studied the dissociation of the granular particles by increasing the pH and/or introducing lecithin into the mixture. The addition of 1% lecithin at pH 7.0 led to the solubilisation of up to 72.53% of the granular protein, while the same amount of lecithin at pH 9.0 produced the solubilisation of 97.45%. As would be expected, if the granular protein was previously solubilised, the emulsifying properties of this fraction were greatly improved. Therefore, disrupted granules at pH 9.0 and with 0.25% lecithin produced stable emulsions, but adding more than 0.50% lecithin produced unstable emulsions due to surfactant-induced depletion flocculation. Li et al. (2021) took advantage of these lecithin-egg yolk granule emulsions to prepare spray-dried powders with algal oil. These researchers claim that the stabilised algal oil powder shows excellent physical properties and oxidative stability. Furthermore, the presence of phosvitin in the emulsion enhanced the antioxidant properties of the resulting powder. In another study, Li et al. (2020) hydrolysed granules enzymatically with subtilisin and tested their emulsifying properties, comparing them to the same properties of untreated granules. The emulsions prepared using hydrolysed egg yolk granules showed much higher stability and less creaming than the emulsions prepared using untreated granules in a dose-dependent manner. Thus, the best stability and the lowest creaming values were obtained at the highest concentration of hydrolysed granules tested (0.1%, w/v). Furthermore, according to these authors, the treated granules behaved as a Pickering-

type emulsifier, owing to the structural changes induced by the enzyme in the granular proteins.

In addition, several authors have recently studied the surface-active properties of the native egg yolk granules in order to better understand their emulsifying properties, which could provide valuable information with respect to their possible industrial exploitation.

Gmach, Bertsch, Bilke-Krause & Kulozik (2019) studied the properties of egg yolk granules as an emulsifying agent for preparing oil-in-water emulsions (80/20) with rapeseed oil, peanut oil and corn oil at different pHs. Depending on the pH assessed, authors found a difference of between 10% and 30% in the median drop size. In addition, the smallest emulsion droplets were found when vegetable oils with higher surface were used. In another article, Wang, Xiao, Wang, Li & Wang (2020) studied the possibility of using egg yolk granules as a Pickering-type emulsifying agent at several different pHs. Although the stability of the emulsions was poor at pH > 4.0, it was excellent at pH = 4.0. In addition, when the egg yolk granule size was smaller than 500 nm they behaved as a Pickering-type emulsifier. Li et al. (2019b) also tested the influence of the egg yolk fractions on egg white based foams. Adding egg yolk granules was found to be less detrimental to the foam capacities of the egg white than adding the whole egg yolk and plasma, in part owing to the higher concentration of protein present in the granular fraction. These proteins enhance the formation of a protein film at the air-water interface, increasing the foam stability. In addition, the granular fraction has a lower lipid content than the plasma or the whole egg yolk, it being possible that the excess of lipid affects the surface proteins at the interface, and hence, the foam stability. Motta-Romero, Zhang, Tien, Schlegel & Zhang (2017) separated the egg yolk granules using two egg yolk:water ratios and as a third alternative 0.17 M NaCl, testing the emulsifying capacities of these granules by preparing a mayonnaise. In this respect, the highest granule yield was obtained by diluting the egg yolk with water at a ratio of 1:2, but the best emulsifying properties were obtained with granules separated using a 1:1 proportion of 0.17 M NaCl. All in all, egg yolk granules showed a similar emulsifying capacity to the whole egg yolk, owing to an increase in the viscosity of the mayonnaise and the better emulsion stabilizing properties of the granules in comparison with the whole egg yolk.

It is important to note that although the emulsification properties of egg yolk granules are improved when they are at least partially solubilised, the emulsifying properties of the native granules have been described as similar to those of whole egg yolk when

they are used to prepare mayonnaise. For this application in particular, the good performance of the granules may be due to the low pH of this emulsion, usually around pH 4.0 (McWhorter, Khan, Sexton, Moyle, & Chousalkar, 2021), which facilitates the role of the egg yolk granules as a Pickering-type emulsifier (Wang et al., 2020). In this respect, the granule emulsification capacity is not only limited by the pH of the emulsion, but also by the oil droplet size, since the oil droplet must be big enough to be effectively surrounded by the native egg yolk granule microparticles. Therefore, it is possible that these desirable emulsification properties of untreated egg yolk granules when tested as an emulsifier for mayonnaise cannot be extrapolated to other food emulsions at neutral pH and with a smaller oil droplet size.

Table 4.4. Summary of recent studies into the use of egg yolk granules as an emulsifying agent.

Emulsifier	pH tested	Emulsion/Foam	Particle size of emulsions	Highlighted results	Reference
Granules depolymerised by ultrasound	7.4	30 mL of a solution of treated egg yolk granules (20 mg/mL dry matter content) and 10 mL of soybean oil	NA	The ultrasound treatment produced the partial depolymerisation of the egg yolk granules, which improved their emulsifying properties.	Geng et al. 2021
Granules disrupted by lecithin	7.0 and 9.0	Several protein concentrations up to 1% (w/v). Preparation of oil/water emulsions with 10% of Arowana sunflower oil.	For a lecithin concentration of 0.25% (w/v), around 400 nm at pH 7.0 and around 200 nm at pH 9.0	An increase in the concentration of lecithin produced an increase in granule solubilisation. The dissociation of the granules' aggregate structure improved their emulsifying properties.	Shen et al. 2020
Granules disrupted by lecithin	7.0 and 9.0	10% oil-in-water emulsion with both egg yolk granules and lecithin dispersed in the water phase. The oil phase was docosahexaenoic acid algal oil.	NA	After spray drying of the emulsion, the resulting powder showed good physical properties and oxidative stability.	Li et al. 2021
Egg yolk granules treated with subtilisin	pH 7.0	Oil-in-water emulsion prepared with 9 mL soybean oil and 21 mL of granule solutions with four different protein concentrations.	12.6 μm	Enzymatic hydrolysis produced changes in the granule structure. The treated egg yolk granules behaved as a Pickering emulsifier.	Li et al. 2020
Native granules	3.0, 4.0, 6.5, 8.0 and 10.0	Oil-in-water emulsion (80/20) with 2% protein content and sunflower oil, rapeseed oil, peanut oil and corn oil.	From 40 μm at pH 3.0 to around 15 μm at pH 10.0.	The pH of the granule solution and the surface activity of the vegetable oils were important factors in decreasing the droplet size of the emulsions.	Gmach et al. 2019
Native granules	pH 2.0, 3.0, 3.7, 4.0, 4.5, 5.5, 6.5, 7.0, and 9.0	20 mL of 1% egg yolk granules dispersion and 20 mL of soybean oil.	NA	The emulsions prepared showed a high stability at pH 4.0.	Wang et al. 2020
Native granules	NA	Foams prepared with several concentrations of egg yolk granules and egg white	NA	The characteristics of the foams were influenced by the proportions of plasma and granules. Granules together with egg white showed better foaming properties than whole egg yolk with egg white	Li et al., 2019b
Native granules precipitated at several yolk:water ratios.	Acid pH	Mayonnaise recipe	NA	A higher granules yield was obtained at the yolk:water ratio of 1:2. Egg yolk granules possessed similar emulsifying properties to whole egg yolk.	Motta-Romero et al. 2017

2.2. Egg yolk granules as a source of folates

Recently, studies have been performed into novel ways to extract compounds from egg yolk granules in order to obtain folate. Folate is a vitamin found in egg yolk and low serum levels are linked to gestational problems in women of child-bearing age and to cardiovascular diseases (House, Braun, Ballance, O'connor, & Guenter, 2002). In this context, Naderi, Doyen, House & Poliot (2017) extracted 5-methyltetrahydrofolate (5-MTHF), a highly bioavailable folate derivative, from egg yolk granules by high hydrostatic pressure (HHP). In this case, the best extraction conditions were 400 MPa pressure and 5 min of treatment. In such conditions, 1264.6 ± 61.4 μg of 5-MTHF per 100 g granule were extracted. If the same treatment was performed on whole egg yolk, the amount of 5-MTHF obtained decreased to 230 ± 2.7 μg/100 g. In a further study, the same authors treated egg yolk granules with high hydrostatic pressure at 600 MPa for 5 min, which caused the disintegration of the granules and the release of folate and phosvitin into the plasma fraction (Naderi, Pouliot, House & Doyen, 2017).

2.3. Preparation of films

Proteins are excellent biopolymers for preparing edible food packaging. They have the advantage of being easily improved by adding antioxidant or antimicrobial compounds and they also possess excellent mechanical properties (Mihalca et al., 2021). Therefore, bearing in mind that egg yolk granules have a large proportion of proteins in comparison with the whole egg yolk or the plasma fraction, these proteins could be revalorised by the preparation of edible films and coatings. These proteins are usually prepared by the casting method (Pirsa & Aghbolagh Sharifi, 2020), so the granular proteins have to be solubilised, mixed in the aqueous medium with a plasticiser and finally cast on an even surface in order to evaporate the water. However, egg yolk granular proteins are only significantly solubilised in solutions with a pH higher than 9.0 or with a NaCl concentration higher than 0.3 M (Anton & Gandemer, 1997), and the solubilisation of egg yolk granules in such a high concentration of NaCl is incompatible with the preparation of protein-based films.

In the light of this obstacle, Marcet, Álvarez, Paredes, Rendueles & Díaz (2018) prepared edible films with egg yolk granules treated with ultrasounds. In this respect, the granules were solubilised at alkaline pH and enhanced by ultrasound treatment. Furthermore, the main variables responsible for their solubilisation were optimized and modelled using a Box-Behnken design. The resulting films were transparent, slightly yellowish in colour and had mechanical properties suitable for wrapping pieces of food. In a further study,

Marcet, Sáez, Rendueles & Díaz (2017) prepared edible films using egg yolk granules previously delipidated with ethanol. Delipidated granules were solubilised at alkaline pH at 65 ºC and using ultrasound. Afterwards, the pH of the solution was adjusted to pH 8.6 and the proteins were crosslinked with transglutaminase. When these films were compared to others prepared using caseinate or gelatine, they showed better barrier properties and improved water resistance, but poorer mechanical properties. In addition, nisin and thymol were added to give the films antimicrobial properties. Subsequently, Sáez-Orviz, Marcet, Rendueles & Díaz (2021) prepared edible films using delipidated egg yolk granules as the film matrix with added *Lactobacillus plantarum* CECT 9567 and lactobionic acid. In this way, an egg yolk protein-based synbiotic food packaging film was developed. The film-forming solution for these films, which contained a probiotic and a prebiotic, was used to coat pieces of gelatine, employed as a food model. The concentration of *L. plantarum* inside these films remained constant for 15 days and its population was slightly increased when lactobionic acid was present during the storage period. In addition, the lactobionic acid enhanced the survival of the bacteria when the coatings were subjected to gastrointestinal digestion conditions. Finally, Fuertes et al. (2017) prepared edible films using whole egg yolk, the egg yolk subfractions and the resultant proteins obtained after the extraction of lipids from the subfractions and the whole egg yolk. However, in this case neither the granular proteins in the whole egg yolk nor those in the granular fraction were previously solubilised, so it was necessary to introduce gelatine to the mixture to prepare the films. In addition, the excess of lipids in the plasma fraction required the addition of gelatine to the film-forming solution as well. The gelatine-granules films were mechanically the strongest films, but when the granules were delipidated, the gelatine-delipidated granules films obtained were found to be among the weakest tested.

The main characteristics of all these films are summarised in Table 4.5. According to this table, the presence of lipids in the granular fraction produced the weakest but most flexible and water-resistant films tested, which could be exploited in future preparations. In addition, the puncture strength and puncture deformation properties of the films prepared by adding gelatine were provided in grams and mm, so they cannot be compared with the other films shown in this table. In addition, most films prepared using egg yolk granules were produced by solubilising the granular egg yolk fraction at alkaline pH, producing protein-based films with relatively high water-resistance at neutral or acidic pH, which are the pHs most commonly found in food. However, this

alkaline pH may affect the appearance of more delicate foodstuffs, so the long-term visual impact of these films on a large range of foods remains to be studied.

Finally, as mentioned in section 2.1, there are in the recent scientific literature several alternative ways to enhance, at least partially, the disaggregation of the granules without resorting to their solubilisation at high pH. In this line, the preparation of films using granules previously treated with ultrasound at neutral pH (Geng et al., 2021), subtilisin (Li et al., 2020), or partially disassociated by introducing lecithin (Shen et al., 2020) may generate valuable egg yolk protein-based materials at non-alkaline pHs.

Table 4.5. Main characteristics of films prepared using egg yolk granules.

Main biopolymer	Protein solubilisation	Major modifications tested	Puncture strength[a] (N/mm)	Puncture deformation[a] (%)	Water solubility[a] (%)	Reference
Whole granular fraction	Alkaline pH and ultrasounds	NA	31.6± 2.5	113 ± 7.0	14±0.5	Marcet et al. 2017
Delipidated granular protein	Alkaline pH and ultrasounds	Protein crosslinking by transglutaminase; addition of natamycin and thymol	122.0 ± 9.1	75.7 ± 5.6	24.5±2.0	Marcet et al. 2018
Delipidated granular protein	Alkaline pH	Introduction in the film matrix of a probiotic (*Lactobacillus plantarum*) and a prebiotic (lactobionic acid).	50.7± 4.9[b]	39.0 ± 0.1[b]	38.5± 2.6[b]	Sáez-Orviz et al. 2021
Delipidated granular protein and whole granular fraction	NA	Addition of gelatine	Breaking force (g): 412 ± 91	Deformation (mm): 5.4 ± 0.7	NA	Fuertes et al. 2017

NA: Not available. [a]: Results for the best preparations. [b]: Results for synbiotic films.

2.4. Encapsulation agent

Zhou, Hu, Wang, Xue & Luo (2018) prepared novel nanoscale delivery systems for the development of functional foods using HDL nanoparticles loaded with curcumin. Curcumin was specifically selected as a model lipophilic bioactive compound. The nanoparticles were alternatively coated with either chitosan or stearic acid conjugated chitosan (SACS) and had a diameter of 75 nm and 97 nm, respectively. According to the authors, SACS-HDL showed better encapsulating properties and a slower release of the curcumin in a simulated gastrointestinal tract.

Sassi, Marcet, Rendueles, Díaz & Fattouch (2020) prepared microparticles using granules from egg yolk and several concentrations of gum Arabic for encapsulating polyphenols from date pits. In this respect, when the concentration of the granular protein was increased in relation to the amount of gum Arabic, an improvement in the antioxidant effect and thermal stability of the microparticles was measured. In addition, the microparticles showed higher resistance to gastrointestinal fluids owing to the presence of the granular proteins in their wall material composition.

3. Phosvitin

Phosvitin has received considerable attention in the recent literature (Table 4.6). Three articles have been published describing novel procedures to extract and isolate phosvitin from egg yolk granules. There is also research centred on the improvement of its functional properties by structural modification or hydrolysis. In addition, studies have also been performed to increase phosvitin's antimicrobial capacity and assess its nutritional properties.

Table 4.6. Summary of recent studies about phosvitin related to food applications.

Treatment	Main objective	Best preparation	Highlighted results	Reference
Ball-mill treatment	Improvement in the emulsifying properties	20 min of treatment at 25 ºC	Dispersions of treated phosvitin showed higher viscosity. The emulsifying activity index of the phosvitin was increased 3.0-fold.	Zhang et al. 2019
Cycles of freeze-thaw	Improvement in the emulsifying properties	3 cycles of freeze-thaw	The emulsifying ability of the phosvitin was increased from 1.87 m^2/g to 3.70 m^2/g.	Li et al. 2018
Conjugated with gallic acid	Improvement in the emulsifying properties	10 mg/mL of phosvitin and 1.5 mg/mL of gallic acid at pH 9.0	A slight improvement in the emulsifying properties of the emulsion tested. The conjugate showed antioxidant properties and an increase in the viscosity of the prepared emulsions.	Jiang et al. 2020
Conjugated with pectin	Improvement in the emulsifying properties	Phosvitin and pectin at a mass ratio of 1:8 and at a protein concentration of 10 mg/mL	Emulsifying activity index increased from 19.81 m^2/g to 25.72 m^2/g and the stability index increased from 27.5% to 76.3%.	Cui et al. 2019
Conjugated with gluten	Improvement in the emulsifying properties	0.25 g of gluten, 0.25 g of phosvitin and 30 U/g of transglutaminase in 50 mL of Na_2SO_3 at 600 mg/mL	The emulsifying activity increased from 17.42 to 20.63 m^2/g.	Yang et al. 2021
Partial dephosphorylation and hydrolysis with trypsin	Identification of the peptide that binds most strongly to calcium	Fraction P3 after anion exchange, P3-1 after SEC, P3-1-1 after RP-HPLC	The sequence DEEENDQVK was detected as the best calcium-chelating peptide with a binding capacity of 151.10 ± 3.57 mg/g	Zhang et al. 2021
Subjected to high hydrostatic pressure and to enzymatic hydrolysis	Assessment of the radical scavenging and anti-inflammatory properties of the peptides	Peptides obtained at 100 MPa and using alcalase or trypsin	Peptides obtained at 100 MPa showed greater antioxidant properties. Peptides obtained with alcalase and trypsin showed higher iron chelation capacity and anti-inflammatory properties.	Yoo et al. 2017

Unmodified phosvitin	Phosvitin effect on gut health	Phosvitin was administered dissolved in water at 5 mg/mL for 14 days to several groups of mice	The *Bifidobacterium* population increased in the young group and the number of pathogenic bacteria decreased in the adult group	Li et al. 2019a
Hydrolysed with alcalase at high pressure and combined with IgY	Antimicrobial properties against *E. coli* K88 and K99 strains	IGY 100 µg/mL combined with 1 mg/mL of phosvitin peptides hydrolysed with alcalase at high hydrostatic pressure	*E. coli* growth inhibition of 2.8 and 2.67 log CFU/mL for the strains K88 and K99 respectively	Gujral et al. 2017
Granules subjected to high hydrostatic pressure and ultrafiltration membrane	Phosvitin extraction	High hydrostatic pressure of 400 MPa for 22 ºC and 5 min, centrifugation and ultrafiltration of the supernatant with a 10 kDa membrane	A phosvitin content in the retentate of 26.00 ± 4.12% w/w	Giarratano et al. 2020
Granules subjected to high hydrostatic pressure	Phosvitin extraction	High hydrostatic pressure of 600 MPa for 5 min	The transmission from the granules to the plasma of an amount of phosvitin of 33.3 ± 4.39 mg/100 g of dry plasma	Duffuler et al. 2020
Granules subjected to heat treatment and ultrasounds	Phosvitin extraction	Egg yolk granules diluted with $(NH_4)_2SO_4$, heated at 80 ºC for 15 min and then treated for 10 min with ultrasound	An extract with a phosvitin purity of 80.0 ± 2.0, with an N/P ratio of 3.1 and a phosvitin activity of 98.0 ± 1.0	Jiang et al., 2019

3.1. Novel procedures to extract phosvitin from egg yolk granules

The usual phosvitin extraction procedures involve the use of sodium chloride to dissolve the association of the granules and a further purification step using organic solvents and chromatography. This procedure and the new ones found in the literature are depicted in Figure 4.8. In a recent paper, Giarratano et al. (2020) extracted phosvitin from egg yolk granules by high hydrostatic pressure at 400 MPa for 5 min. After applying such pressure, the supernatant was concentrated by ultrafiltration with a 10 kDa membrane, obtaining a concentrate with a phosvitin content of 26.00 ± 4.12% w/w. The emulsion prepared with this concentrate showed a creaming index of 6.25% compared to a value of 93.75% for this parameter for the control emulsion. Duffuler et al. (2020), using high hydrostatic pressure, extracted phosvitin from egg yolk granules, the phosvitin accumulating in the plasma fraction. The best conditions tested were 600 MPa for 10 min of treatment, resulting in a concentration of phosvitin in the plasma fraction of 33.3 ± 4.39 mg/100 g of plasma. Jiang et al. (2019) isolated phosvitin from egg yolk granules by dissolving them in $(NH_4)_2SO_4$ solution, applying heat at 80 °C for several and different reaction times and treating the solution with ultrasound at 600 W for 10 min. After dialysis and centrifugation, the phosvitin was isolated in the supernatant. Under the best set of conditions assessed, the purity of the phosvitin was 80% and no significant differences between the properties of the extracted phosvitin and those of the phosvitin standard were detected.

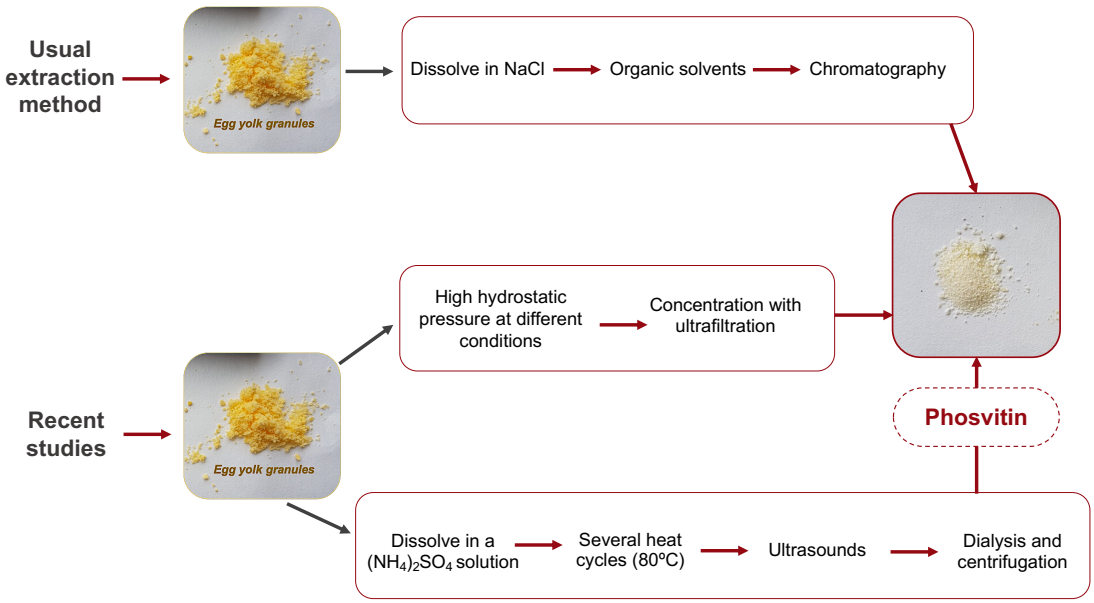


Figure 4.8. Usual extraction method and recently reported procedures for extracting phosvitin from egg yolk granules.

Although all these papers focused on the phosvitin and its functional properties, not many practical applications have been found in the literature for the leftover high-density lipoproteins without the phosvitin fraction. Investigating this issue, Chalamaiah, Esparza, Hong, Temelli & Wu (2018) studied the physicochemical and functional properties of the processed egg yolk granules and found that they showed better foam capacity, foam stability, emulsifying activity and stability indexes than the whole egg yolk granules. Bearing in mind that the different phosvitin extraction procedures may exert different effects on the structure and functionality of the remaining granular proteins, these proteins may show a wide variety of functional properties. In addition, they could be a valuable source of bioactive peptides.

3.2. Phosvitin modifications to improve its emulsifying properties

The emulsifying properties of phosvitin are based on the electrostatic repulsive force of phosphate and have been described as better than those of BSA at pH 7.0 (Chung & Ferrier, 1991) and then those of other food proteins (Sattar Khan, Babiker, Azakami, Kato, & chemistry, 1998). In recent publications, these emulsifying properties have been improved by modifying the native folded conformation of the phosvitin by physical treatments or by conjugation with other compounds.

Zhang, Yang, Hu, Liu & Duan (2019) subjected lyophilized phosvitin to a ball-milling treatment and assessed the rheological and emulsifying properties of the modified protein. After 20 min of treatment, the surface hydrophobicity of the phosvitin was doubled, and after 40 min of treatment this parameter reached its highest value, which enhanced its emulsifying properties. According to the authors, the milling treatment induces structural changes in the phosvitin, exposing its hydrophobic groups and improving its emulsifying activity index, thermal stability and increasing the viscosity of the dispersions in which it is included. In order to modify the functional properties of phosvitin by other physical treatments, Li et al. (2018) subjected this protein to several freeze-thaw cycles. The treated phosvitin showed structural changes, resulting in a decrease in the proportion of beta-sheets from 32.19% to 25.10% after 9 freeze-thaw cycles, but on this occasion the surface hydrophobicity of the phosvitin decreased, suggesting a rearrangement of the protein and a burial of the hydrophobic groups inside the protein structure. Another highlighted result was an improvement in the phosvitin emulsifying activity index value, which was noticeably increased after 3 cycles, although the emulsion stability was not significantly affected. It must be considered that, unlike the other articles reviewed, this study linked a decrease in the surface hydrophobicity of the phosvitin with an improvement in its emulsifying properties, which suggests that

although an increase in phosvitin hydrophobicity could predict an improvement in this functional property, there are other factors that must be considered to predict the performance of this protein as an emulsifier.

Regarding the conjugation of phosvitin with other compounds, Cui, Li, Lu, Liu & Duan (2019) prepared phosvitin-pectin copolymers by Maillard reaction with the objective of modifying the phosvitin structure and improving its emulsifying properties. This conjugation produced an increase in the surface hydrophobicity (H_0) from 60.3 to 183.4 and hence an increase in the emulsifying properties of the conjugated phosvitin. Taking advantage of the structural modification of the phosvitin, the conjugated phosvitin was hydrolysed with trypsin and the resultant peptides showed improved calcium binding properties. In the case of Yang et al. (2021), they increased the solubility of wheat gluten with Na_2SO_3 and crosslinked this protein with phosvitin. The surface hydrophobicity of the crosslinked proteins was also increased, enhancing their emulsifying properties. In fact, for the best preparation tested, the emulsifying activity was noticeably increased. In addition, the thermal stability of these proteins was also improved. Finally, Jiang et al. (2020) conjugated phosvitin with gallic acid in order to prepare a new emulsifier with both emulsifying and antioxidant properties. The emulsifying properties of the complexes prepared with different concentrations of gallic acid were slightly improved in comparison with the phosvitin alone, but the ABTS and DPPH free radical scavenging of these aggregates were clearly higher than those found for the isolated phosvitin.

According to all these authors, it is proven that the emulsifying properties of phosvitin can be enhanced by the procedures described above; however, except for the study performed by Cui et al. (2019) with gallic acid, it is noticeable that the antioxidant capacity of the modified phosvitins to protect the emulsified lipids against oxidation was not investigated experimentally. Considering that these phosvitin-related antioxidant properties may remain unaffected, this preservative effect in the emulsions in which the modified phosvitin is involved could noticeably increase the value of the phosvitin and hence of the whole egg yolk granular fraction.

3.3. Production of novel phosphopeptides

Phosphoproteins are considered to be resistant to protease activity, and phosvitin, the most phosphorylated protein found in nature, has been reported as highly resistant to the action of digestive enzymes such as trypsin. Therefore, in order to increase its sensitivity to the action of proteases, a well-known strategy consists in partially dephosphorylating the phosvitin before subjecting it to enzymatic hydrolysis (Jiang & Mine, 2000). In this line, and with the objective of increasing calcium bioavailability,

Zhang et al. (2021) partially dephosphorylated phosvitin and then performed a tryptic digestion, identifying and purifying a peptide with a high calcium binding capacity. The purification of this peptide was performed by anion-exchange chromatography, size exclusion chromatography and reversed-phase high-performance liquid chromatography. In another recent paper, Yoo, Bamdad, Gujral, Suh & Sunwoo (2017) avoided the partial phosvitin dephosphorylation step and directly carried out the enzymatic hydrolysis of this phosphoprotein at various high hydrostatic pressure levels and using several enzymes. According to the results, the hydrolysates obtained at 100 MPa showed superior radical scavenging properties and the alcalase and trypsin hydrolysates showed the highest anti-inflammatory properties.

3.4. Nutritional and antimicrobial properties

The combination of both high metal-chelating properties and high resistance to hydrolysis by digestive enzymes leads to phosvitin being considered as a nutritional negative protein (Ishikawa, Tamaki, Arihara, & Itoh, 2007). In this context, Li et al. (2019a) studied the effect of phosvitin on the luminal microbiota composition of mice. According to these authors, phosvitin exhibited potentially beneficial effects on gut health by decreasing the amounts of pathogenic microbes in adult mice and increasing the proportion of beneficial bacteria in young mice. In addition, Gujral et al. (2017) studied the synergistic effect of IgY and phosvitin from egg yolk on the growth of enterotoxigenic *Escherichia coli* K88 and K99. IgY was obtained from egg yolks of hens previously immunized with K88 and K99. The combination of IgY and peptides from phosvitin previously hydrolysed with alcalase at high hydrostatic pressure showed the best bactericidal effect, decreasing *E. coli* growth by 2.8 and 2.67 log CFU/mL for the K88 and K99 strains, respectively. In fact, very few papers have been published in the last five years about the nutritional effect of phosvitin on gut health or about its antimicrobial properties, but in the light of the results obtained by these authors, it could be a worthwhile topic for future research.

4. Conclusions

In this review the latest studies regarding the food-related applications of egg yolk granules have been highlighted. The emulsifying properties of egg yolk granules can be enhanced by increasing their degree of solubilisation or decreasing their size, and for that purpose, enzymatic hydrolysis and high hydrostatic pressure, partial depolymerisation with ultrasound, and mixing with lecithin were assessed. It is important that these treatments are mild, and they are not mutually exclusive, so they

may be combined in order to further depolymerise the egg yolk granules and increase their emulsifying capacity. Moreover, egg yolk granules solubilised in the ways proposed by these researchers could be exploited to prepare edible films, avoiding the use of basic pHs to cause disruption of the granules and increasing their range of applications.

In addition, the emulsifying activity of granules at different pHs or acting as a Pickering emulsifier were also tested. In this case, the good performance of native egg yolk granules in mayonnaise deserves attention and may be explained, in the light of the other papers reviewed, by the granules acting as a Pickering emulsifying agent at the typical pH of mayonnaise. If this is so, these good emulsifying properties of native granules would not be extrapolated to other emulsions prepared at non-acidic pHs. This raises the question of whether mayonnaise is a good emulsion model for assessing the emulsifying properties of this egg yolk fraction. Furthermore, phosvitin, one of the main granular proteins, has received relatively close attention in recent years, and many of these studies are about improving its emulsifying properties. Although in these articles the emulsifying ability of the phosvitin, modified in a variety of ways, was intensively studied, the antioxidant effect of the phosvitin on the emulsions, keeping lipids from being oxidised, was not generally tested. This could provide a suitable subject for future studies in order to increase the phosvitin value. In addition, bearing in mind that when the phosvitin is extracted, the remaining HDLs may be considered as a by-product, a lack of studies into the improvement of their functional properties and their possible food-related applications was also detected.

Acknowledgements

This work was financially supported by the Spanish Ministry of Science and Innovation, through the project MCIU-19-RTI-2018-094218-B-100 and by the grant *Programa Severo Ochoa de Ayudas Predoctorales para la investigación y docencia* (grant number BP19-127 to Sáez-Orviz, S.).

5. References

Anton, M. (2007). Composition and structure of hen egg yolk. *Bioactive egg compounds* (pp. 1-6): Springer.

Anton, M. (2013). Egg yolk: structures, functionalities and processes. *Journal of the Science of Food and Agriculture, 93*(12), 2871-2880.

Anton, M., & Gandemer, G. (1997). Composition, solubility and emulsifying properties of granules and plasma of egg yolk. *Journal of Food Science,62*(3), 484-487.

Chalamaiah, M., Esparza, Y., Hong, H., Temelli, F., & Wu (2018). Physicochemical and functional properties of leftover egg yolk granules after phosvitin extraction. *Food Chemistry, 268*, 369-377.

Chung, S. L., & Ferrier, L. K. (1991). Conditions affecting emulsifying properties of egg yolk phosvitin. *Journal of Food Science, 56*(5), 1259-1262.

Cui, Y., Li, X., Lu, M., Liu, X. & Duan, X. J. (2019). Role of polysaccharide conjugation in physicochemical and emulsifying properties of egg phosvitin and the calcium binding capacity of its phosphopeptides. *Food and function, 10*(4), 1808-1815.

Duffuler, P., Giarratano, M., Naderi, N., Suwal, S., Marciniak, A., Perreault, V., Offret C., Brisson G., House J.D. & Pouliot, Y. J. (2020). High hydrostatic pressure induced extraction and selective transfer of β-phosvitin from the egg yolk granule to plasma fractions. *Food Chemistry, 321*, 126696.

Fuertes, S., Laca, A., Oulego, P., Paredes, B., Rendueles, M., & Díaz, (2017). Development and characterization of egg yolk and egg yolk fractions edible films. *Food Hydrocolloids, 70*, 229-239.

Geng, F., Xie, Y., Wang, Y., & Wang, (2021). Depolymerization of chicken egg yolk granules induced by high-intensity ultrasound. *Food Chemistry, 354*, 129580.

Giarratano, M., Duffuler, P., Chamberland, J., Brisson, G., House, J. D., Pouliot, Y., & Doyen (2020). Combination of high hydrostatic pressure and ultrafiltration to generate a new emulsifying ingredient from egg yolk. *Molecules, 25*(5), 1184.

Gmach, O., Bertsch, A., Bilke-Krause, C. & Kulozik, U. J. C. (2019). Impact of oil type and pH value on oil-in-water emulsions stabilized by egg yolk granules. *Colloids and Surfaces A: Physicochemical and Engineering Aspects, 581*, 123788.

Gujral, N., Yoo, H., Bamdad, F., Y Lee, K., Suh, J.-W., & Sunwoo (2017). A combination of egg yolk IgY and phosvitin inhibits the growth of enterotoxigenic escherichia coli K88 and K99. *Current Pharmaceutical Biotechnology 18*(5), 400-409.

House, J., Braun, K., Ballance, D., O'connor, C., & Guenter. (2002). The enrichment of eggs with folic acid through supplementation of the laying hen diet. *Poultry Science, 81*(9), 1332-1337.

Ishikawa, S. I., Tamaki, S., Arihara, K., & Itoh. (2007). Egg yolk protein and egg yolk phosvitin inhibit calcium, magnesium, and iron absorptions in rats. *Journal of Food Science, 72*(6), S412-S419.

Jiang, B., Mine, Y. J. J. (2000). Preparation of novel functional oligophosphopeptides from hen egg yolk phosvitin. *Journal of Agricultural and Food Chemistry, 48*(4), 990-994.

Jiang, B., Wang, L., Wang, X., Wu, S., Li, D., Liu, C., & Feng (2019). Ultrasonic thermal-assisted extraction of phosvitin from egg yolk and evaluation of its properties. *Polymers, 11*(8), 1353.

Jiang, B., Wang, X., Wang, L., Wu, S., Li, D., Liu, C. & Feng Z. (2020). Fabrication and Characterization of a Microemulsion Stabilized by Integrated Phosvitin and Gallic Acid. *Journal of Agricultural and Food Chemistry, 68*(19), 5437-5447.

Jin, Y. G., Huang, D., Ding, T., Ma M. H., & Oh D.H. (2011). Effect of phospholipase A1 on the physicochemical and functional properties of hen's egg yolk, plasma and granules. *Journal of Food Biochemistry, 37*(1), 70-79.

Kiosseoglou, V., & Paraskevopoulou, A. (2005). Molecular interactions in gels prepared with egg yolk and its fractions. *Food Hydrocolloids, 19*(3), 527-532.

Ko, K., Nam, K., Jo, C., Lee, E., & Ahn, D. (2011). A simple and efficient method for preparing partially purified phosvitin from egg yolk using ethanol and salts. *Pultry Science, 90*(5), 1096-1104.

Laca, A., Sáenz, M., Paredes, B., & Díaz, M. (2010). Rheological properties, stability and sensory evaluation of low-cholesterol mayonnaises prepared using egg yolk granules as emulsifying agent. *Journal of Food Engineering, 97*(2), 243-252.

Le Denmat, M., Anton, M., & Beaumal, V. (2000). Characterisation of emulsion properties and of interface composition in O/W emulsions prepared with hen egg yolk, plasma and granules. *Food hydrocolloids, 14*(6), 539-549.

Lesnierowski, G., & Stangierski, J. (2018). What's new in chicken egg research and technology for human health promotion?-A review. *Trends in Food Science & Technology, 71*, 46-51.

Li, J., Shen, Y., Zhai, J., Su, Y., Gu, L., Chang, C., & Yang, Y. (2021). Enhancing the oxidative stability of algal oil powders stabilized by egg yolk granules/lecithin composites. *Food Chemistry, 345*, 128782.

Li, M., Li, X., Li, J., Lu, M., Liu, X., Duan, X. J. (2018). Effects of multiple freeze–thaw treatments on physicochemical and biological activities of egg phosvitin and its phosphopeptides. *Food and Function, 9*(9), 4602-4610.

Li, M., Zhang, Q., Wang, R., Lu, M., Xu, X., Liu, X. & Duan X. (2019a). Effects of egg phosvitin on mucosal transcriptional profiles and luminal microbiota composition in murine colon. *Food and Function, 10*(5), 2805-2816.

Li, Q., Tang, S., Mourad, F. K., Zou, W., Lu, L., & Cai, Z. (2020). Emulsifying stability of enzymatically hydrolyzed egg yolk granules and structural analysis. *Food Hydrocolloids, 101*, 105521.

Li, X., Li, J., Chang, C., Wang, C., Zhang, M., Su, Y., & Yang, Y. J. (2019b). Foaming characterization of fresh egg white proteins as a function of different proportions of egg yolk fractions. *Food Hydrocolloids, 90*, 118-125.

Marcet, I., Álvarez, C., Paredes, B., Rendueles, M., Díaz, M. (2018). Transparent and edible films from ultrasound-treated egg yolk granules. *Food and Bioprocess Technology, 11*(4), 735-747.

Marcet, I., Sáez, S., Rendueles, M., Díaz, M. (2017). Edible films from residual delipidated egg yolk proteins. *Journal of Food Science Technology, 54*(12), 3969-3978.

McWhorter, A. R., Khan, S., Sexton, M., Moyle, T. S., & Chousalkar, K. K. J. (2021). Acidification and extended storage at room temperature of mayonnaise reduce Salmonella Typhimurium virulence and viability. *Food Research International, 141*, 110117.

Mihalca, V., Kerezsi, A. D., Weber, A., Gruber-Traub, C., Schmucker, J., Vodnar, D. C., Dulf, F. V., Socaci S. A., Farcaç A., Muresan C. I., Suharoschi R. & Pop O. L. (2021). Protein-based films and coatings for food industry applications. *Polymers, 13*(5), 769.

Motta-Romero, H., Zhang, Z., Tien N., A., Schlegel, V., & Zhang, Y. J. (2017). Isolation of egg yolk granules as low-cholesterol emulsifying agent in mayonnaise. *Journal of Food Science, 82*(7), 1588-1593.

Naderi, N., Doyen, A., House, J. D., & Pouliot, Y. J. (2017). The use of high hydrostatic pressure to generate folate-enriched extracts from the granule fraction of hen's egg yolk. *Food Chemistry, 232*, 253-262.

Naderi, N., House, J. D., Pouliot, Y. J. (2016). Effect of selected pre-treatments on folate recovery of granule suspensions prepared from hen egg yolk. *LWT-Food Science and Technology, 68*, 341-348.

Naderi, N., Pouliot, Y., House, J. D., Doyen, A. J. (2017). High hydrostatic pressure effect in extraction of 5-methyltetrahydrofolate (5-MTHF) from egg yolk and granule fractions. *Innovative Food Science & Emerging Technologies, 43*, 191-200.

Orcajo, J., Marcet, I., Paredes, B., Díaz, M. J. (2013). Egg yolk hydrolysed granules: Characteristics, rheological properties and applications. *Food and Bioproducts Processing, 91*(4), 457-463.

Pirsa, S., & Aghbolagh Sharifi, K. J. (2020). A review of the applications of bioproteins in the preparation of biodegradable films and polymers. *Journal of Chemistry Letters, 1*(2), 47-58.

Sáez-Orviz, S., Marcet, I., Rendueles, M., & Díaz, M. (2021). Bioactive packaging based on delipidated egg yolk protein edible films with lactobionic acid and Lactobacillus plantarum CECT 9567: characterization and use as coating in a food model. *Food Hydrocolloids, 119.*

Sassi, C. B., Marcet, I., Rendueles, M., Díaz, M., & Fattouch, S. J. (2020). Egg yolk protein as a novel wall material used together with gum Arabic to encapsulate polyphenols extracted from Phoenix dactylifera L pits. *LWT, 131*, 109778.

Sattar Khan, M., Babiker, E. E., Azakami, H. & Kato, A. J. (1998). Effect of protease digestion and dephosphorylation on high emulsifying properties of hen egg yolk phosvitin. *Journal of Agricultural and Food Chemistry, 46*(12), 4977-4981.

Shen, Y., Chang, C., Shi, M., Su, Y., Gu, L., Li, J., & Yang, Y. (2020). Interactions between lecithin and yolk granule and their influence on the emulsifying properties. *Food Hydrocolloids, 101*.

Sirvente, H., Beaumal, V., Gaillard, C., Bialek, L., Hamm, D. & Anton, M. J. (2007). Structuring and functionalization of dispersions containing egg yolk, plasma and granules induced by mechanical treatments. *Journal of Agricultural and Food Chemistry, 55*(23), 9537-9544.

Wang, A., Xiao, Z., Wang, J., Li, G. & Wang, L. J. (2020). Fabrication and characterization of emulsion stabilized by table egg-yolk granules at different pH levels. *Journal of the Science of Food and Agriculture, 100*(4), 1470-1478.

Yang, L., Jia, J., Zhou, X., Liu, M., Zhang, Q., Tian, L., Tan W., Yang Y., Liu X. & Duan, X. (2021). Phosvitin-wheat gluten complex catalyzed by transglutaminase in the presence of Na2SO3: Formation, cross-link behavior and emulsifying properties. *Food Chemistry, 346*, 128903.

Yoo, H., Bamdad, F., Gujral, N., Suh, J.-W., & Sunwoo, H. (2017). High hydrostatic pressure-assisted enzymatic treatment improves antioxidant and anti-inflammatory properties of phosvitin. *Current Pharmaceutical Biotechnology, 18*(2), 158-167.

Zhang, Q., Yang, L., Hu, S., Liu, X., & Duan, X. (2019). Consequences of ball-milling treatment on the physicochemical, rheological and emulsifying properties of egg phosvitin. *Food Hydrocolloids, 95*, 418-425.

Zhang, X., Jia, Q., Li, M., Liu, H., Wang, Q., Wu, Y., Niu, L. & Liu Z. (2021). Isolation of a novel calcium-binding peptide from phosvitin hydrolysates and the study of its calcium chelation mechanism. *Food Research International, 141*, 110169.

Zhou, M., Hu, Q., Wang, T., Xue, J., & Luo, Y. (2018). Characterization of high density lipoprotein from egg yolk and its ability to form nanocomplexes with chitosan as natural delivery vehicles. *Food Hydrocolloids, 77*, 204-211.

4.2.2. *Films* comestibles a partir de proteína de yema de huevo delipidada

En este subapartado se recogen los resultados referentes al desarrollo de *films* comestibles empleando como matriz proteína de yema de huevo delipidada.

Como se ha recogido en el apartado anterior, la fracción proteica de la yema de huevo se puede emplear en numerosas aplicaciones alimentarias. Una de estas aplicaciones es el desarrollo de biomateriales con fines alimentarios, debido a su baja solubilidad en agua (Anton, 2013), lo que la diferencia del resto de proteínas que se emplean de forma habitual como matriz.

En este trabajo, se han extraído los gránulos de yema de huevo y se han empleado para el desarrollo novedoso de *films* alimentarios. Además, los gránulos se trataron con transglutaminasa, con el objetivo de modificar las interacciones moleculares dentro de la matriz y observar cambios a nivel mecánico en los *films* elaborados. Los *films* elaborados se caracterizaron teniendo en cuenta sus propiedades físicas, químicas y mecánicas, así como su morfología. En esta primera aproximación, se añadieron dos compuestos antimicrobianos (timol y natamicina) para analizar si la matriz era adecuada para la incorporación de compuestos activos en su formulación.

Los *films* desarrollados mostraron muy buenas propiedades, especialmente debido a su baja solubilidad. Esta característica es destacable ya que uno de los principales inconvenientes que presentan los recubrimientos alimentarios desarrollados con proteínas es su alta solubilidad en agua. En este caso, los *films* elaborados con proteína de yema de huevo delipidada serían idóneos para recubrir los productos alimentarios, que generalmente, tienen un alto contenido en agua.

- **Publicación:** Marcet, I., Sáez, S., Rendueles, M., Díaz, M. 2017. Edible films from residual delipidated egg yolk proteins. *Journal of Food Science and Technology*, 54, 3969-3978, (https://doi.org/10.1007/s13197-017-2861-8).
- **Estado:** publicada
- **Índice de impacto:** 1,701 (2017)

Edible films from residual delipidated egg yolk proteins

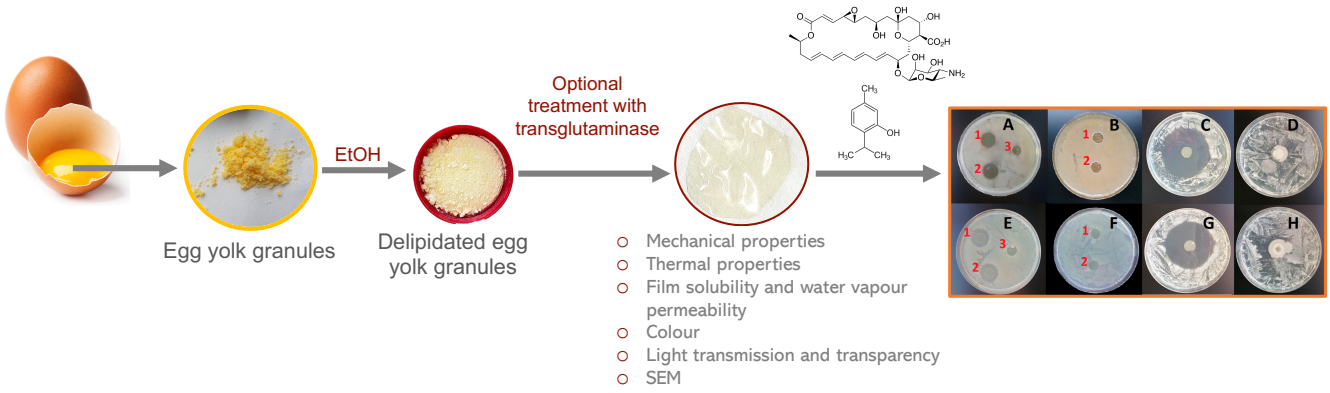
I. Marcet[a], S. Sáez-Orviz[a], M. Rendueles[a*], M. Díaz[a]

[a]Department of Chemical and Environmental Engineering, University of Oviedo, C/Julián Clavería 8, 33006, Oviedo, Spain.

[*]Corresponding author

E-mail address: mrenduel@uniovi.es (M. Rendueles)

Graphical abstract



Abstract

Commercial extraction with organic solvents of valuable lipids from egg yolk produces a highly denatured protein waste that should be valorized. In this work, the delipidated protein waste remaining after ethanol extraction was used to prepare edible films. This material was also treated with transglutaminase, obtaining films that have also been characterized. When compared with gelatin and caseinate edible films, the films made with egg yolk delipidated protein showed poorer mechanical properties, but improved light barrier properties, low water solubility and a high degree of transparency. It is particularly interesting that the presence of phosvitin in the egg yolk gives the films important ferrous chelating properties. When the egg yolk delipidated protein was treated with transglutaminase, the strength of the film was improved in comparison with films made with untreated protein. Finally, addition of thymol and natamycin in the preparation of these films is shown to be an interesting alternative, providing them with antibacterial and antifungal capacities.

Keywords: egg yolk, edible film, protein, mechanical properties, solubility

1. Introduction

Egg yolk is a source of highly valuable lipids, such as egg yolk lecithin which is composed mainly of phosphatidylcholine and phosphatidylethanolamine. These lipids have desirable functional properties widely utilized in the food industry and their emulsifying properties in particular have an important role but, on the other hand, the extraction of these compounds usually requires the use of organic solvents. Among the organic solvents which have been previously used for the extraction of these polar lipids is ethanol, a GRAS-grade organic solvent (Aro et al. 2009). However, the utilization of this type of lipid extraction method produces conformational changes in the protein structure and drastically reduces their solubility. Therefore, the delipidated protein obtained can be considered as a co-product of the lipid separation, with greatly decreased functional properties in comparison with its native state.

Egg yolk can be easily separated into two sub-fractions: egg yolk granules and plasma. The plasma fraction has a high lipid content (78%), and it has been described as an emulsifier (Le Denmat et al. 2000) and gelling agent (Kiosseoglou and Paraskevopoulou 2005). The granular fraction has a higher amount of protein (58%) than lipids (40%) (Laca et al. 2010) but this protein is only solubilized in high ionic strength mediums (>0.3 M NaCl), and therefore, its functional properties and the number of possible applications for this fraction are low. Since the revaluation of the egg yolk is associated with an increase in the number of applications developed for each of its fractions, the lipid and protein content of the egg yolk granules could be separated and used for different purposes in the food industry. In this sense, the highly denatured protein co-product obtained after egg yolk granules lipid extraction could be used to prepare edible films.

Research into edible films which can protect and increase the shelf life of food products has received increasing interest from the food industry and the scientific community in the last few years. Several sources of proteins have been used to produce protein-based edible films, such as casein (Rezvani et al. 2013) and whey (Galus and Kadzińska 2016) which are contained in milk, gelatin obtained from bovine hide (Sobral et al. 2001) or soy protein (Pan et al. 2014). These edible films, in addition to extending the shelf life of the food products, come from a renewable source, are highly biodegradable and could be an alternative to the use of environmentally harmful petroleum-derived plastics.

Additionally, protein-based edible films can be easily combined with antimicrobial compounds, and their mechanical or water vapour capacities can be modulated by treating the proteins with crosslinkers (De Carvalho and Grosso 2004). Amongst the

crosslinkers that can be used, one of the most intensively studied in food science is transglutaminase (EC 2.3.2.13), which is generally recognized as safe (GRAS) by the U.S Food and Drug Administration and by the U.S. Department of Agriculture, as well as being considered a processing aid in the European Union legislation (EU regulation 1332/2008), and therefore it is commonly used in the preparation of processed foods. Transglutaminase catalyses the formation of ε-(γ-glutamyl)-lysine crosslinks in proteins, so it is capable of introducing covalent bonds between chains of proteins, thus improving the mechanical properties of these films (Porta et al. 2015).

In this study, always bearing in mind that the granular fraction is poorly valorised by the food industry, this fraction has been delipidated with ethanol and the protein obtained treated with transglutaminase and then used to prepare films in order to characterize the modifications induced by this crosslinking agent. To understand the strengths and weaknesses of these films, their properties have been compared with other highly-studied protein-based edible films created in a similar way but using gelatine from porcine skin and sodium caseinate.

2. Materials and methods

2.1. Obtaining delipidated egg yolk granules

Egg yolk granules were obtained and delipidated according to Marcet et al. (2014). Briefly, egg yolk was separated from the egg white and dried carefully using blotting paper. The vitelline membrane was broken using tweezers and the liquid yolk mixed with water in a proportion 1:1.5 (v/v). The pH of this diluted egg yolk solution was adjusted to 7.0 with 0.1 M NaOH, and the solution was centrifuged at 10,000×g for 45 min. The sediment (granules) was collected and lyophilized. The lyophilized egg yolk granules were delipidated using ethanol (96%) and gentle agitation for 2 h. Granules were recovered by filtration using Whatman no 1 paper and a vacuum pump. Delipidated granules were dried for 2 days at 40 °C in a heater and stored at –20 °C until use. The dry matter of the delipidated granules was 96% (w/w).

2.2. Film preparation

The composition of the film-forming solution was chosen according to the results of previous tests (data not shown). A 3% suspension of delipidated egg yolk granules in water was prepared by mixing 3 g of delipidated granules, 95.5 mL of water and 1.5 mL of 1 M NaOH. The granules were totally solubilised with sonication at 65 °C for 20 min using a MS 73 probe in a Sonopuls HD 2070 system (Bandelin, Germany). In this case the amplitude of sonication selected was 70% (100% amplitude equivalent to 212 μm).

The amount of glycerine added was 50% of the total protein contained in the solution. Then, the film-forming solution was filtered using a vacuum pump and Whatman no 1 paper, degassed using a vacuum pump, and 20 mL cast in a Petri dish. This amount of film forming solution produces a dry film by evaporation of the water in an oven at 45 °C overnight (granules films). This temperature was selected to avoid the growth of microorganisms. With these parameters, the films can be completely removed from the mould, without their being sticky or brittle.

In the case of the granules treated with transglutaminase (GTT), the delipidated granules were solubilised as described above, and then the pH of the granules solution was adjusted to 8.6 with 1 M HCl. For each gram of protein contained in the sample, 10 U of transglutaminase (Probind TX, BDF ingredients, Spain) were added. In accordance with the manufacturer's instructions, this transglutaminase is mixed with maltodextrin and it has 100 U per gram of product, so, in 100 mL of film-forming solution with 3 g of delipidated egg yolk granules, 0.3 g of transglutaminase powder was added. According to the previous tests (data not shown), higher amounts of transglutaminase (0.4 g) did not produce any significant increase in the crosslinking of the granular proteins and further decreased the amount of protein per gram of film. The reaction was conducted in an oven at 45 °C for 90 min, after which the pH of the sample was adjusted to 10.0 with 1 M NaOH and the glycerin added in a similar proportion to that of the non-treated films. Finally, the film-forming solution was filtered, degassed and dried as described above.

The gelatine and sodium caseinate films were prepared in a similar way, mixing 3 g of powder of gelatine from porcine skin (G2500, Sigma-Aldrich) or sodium caseinate (C8654, Sigma-Aldrich) with 97 mL of water. The protein solutions were heated in a water bath at 65 °C for 25 min. Once the protein was solubilised, the pH of the solutions was not adjusted. Then, an amount of glycerine equivalent to 50% of the protein contained in each solution was added, and the solutions were gently stirred for 5 min. The film-forming solutions showed a pH of 5.5 for the gelatin and of 6.7 for the preparation with caseinate. Finally, the film-forming solutions were filtered, degassed and dried as described above. The amount of protein in the delipidated egg yolk granules, the gelatine and the sodium caseinate powders was determined using an Elementar Vario EL analyzer (Elementar, Germany). Prior to testing, all films were conditioned for 2 days at 21 °C and 54 ± 2% RH in a closed chamber which contained a saturated $Mg(NO_3)_2$ solution.

2.3. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The transglutaminase activity on the delipidated granular proteins was evaluated using SDS-PAGE as described by Laemmli (1970). The stacking gel was formed by polymerising a 4% (w/v) acrylamide solution, whilst a 10% (w/v) acrylamide gel was used for the running phase. Proteins were stained with two different solutions. The first was normally used to specifically stain phosphoproteins (Coomassie blue 0.05%, acetic acid 10%, triton 1%, ethanol 25%, aluminium nitrate 0.1 M). The second was a standard Coomassie stain, which stains total protein (0.1% Coomassie blue, 50% methanol, 10% acetic acid and 40% water). SDS-PAGE Molecular Weight Standards, Broad Range (Bio-Rad) were used as protein standards.

2.4. Mechanical properties of the film

To test the strength and the elongation range of the films, a TA.XT.plus Texture Analyser (Stable Microsystems, UK) with a 5 kg load cell was used. For that purpose, the films were cut into squares with a geometry of 30 × 20 mm and placed on the test platform. The film was firmly attached by means of a plate which was screwed in position above the test platform, the film lying between them. The 5 mm diameter probe (P/5S) descends perpendicularly to the surface of the film, making contact with it through the orifice formed by two holes, one in each plate, thus stretching the film and measuring its puncture strength (PS) and puncture deformation (PD) values. These parameters have been used previously to characterize the mechanical properties of other protein-based films (Otero-Pazos et al. 2016). The PS and PD were calculated according to the following equations:

$$PS = Fm/Th$$

$$PD = (\sqrt{D^2 + R^2} - R)/R$$

where *Fm* is the maximum force applied before film rupture and *Th* is the film thickness; *D* is the distance covered by the probe while it is in contact with the film until the film is broken; *R* is the radius of the orifice in the plates.

2.5. Scanning electron microscopy (SEM)

The microstructure of the film cross-section was analysed using a scanning electron microscope (SEM) (JSM-6610LV, JEOL, USA) according to Galus and Kadzińska (2016) with slight modifications. Briefly, film samples were freeze-dried and immediately cut

into squares of 1 × 1 cm using a surgical blade. The film squares were mounted around stubs perpendicularly coated with gold.

2.6. Thermal properties

Thermo-gravimetric analyses (TGA) were carried out using an SDTA851e TGA analyser (Mettler-Toledo, Switzerland) from 25 to 650 °C under a nitrogen atmosphere. The heating rate was 10 °C/min. The first derivatives of the weight loss curve thermograms were calculated (DTG curves).

2.7. Film solubility and water vapour permeability (WVP)

The water solubility of the films was measured according to Blanco-Pascual et al. (2014). Briefly, the films were cut into circumferences of 40 mm in diameter and immersed in 50 mL of distilled water for 24 h under gentle stirring in an orbital shaker. To recover the insolubilized films, the water was filtered using a vacuum pump and Whatman no 1 paper. The dry mater of the recovered films was determined using a halogen moisture analyzer (HR80, Mettler-Toledo, Switzerland) at 105 °C for 12 h. The moisture content of each type of film before water immersion was previously determined using the same moisture analyser, and the values compared to calculate the amount of film solubilized during the experiment.

The WVP test was conducted according to Rezvani et al. (2013). Briefly, polyvinyl chloride cups were filled with water and the films were cut into circles matching the size of the cup mouth and sealed to it, leaving a gap of 1 cm between the water surface and the under surface of the films. The thickness of the film samples was measured at nine points and furthermore, they were previously examined to avoid the use of film specimens with pinholes or any defects. The mounted cups were placed inside an environmental chamber which contained a saturated $Mg(NO_3)_2$ solution at 20 °C and 54 ± 2% RH. The weight loss was recorded hourly during the first 10 h and finally after 24 h. Three replicates of each film were evaluated. The weight loss was plotted against time and the water vapour transmission rate (WVTR: g m^{-2} h^{-1}) was estimated by dividing the slope in the linear region ($R^2 > 0.990$) by the film surface. The WVP was calculated using the following equation:

$$\mathrm{WVP} = (\mathrm{WVTR} \times \mathrm{Th}) / \Delta \mathrm{P}$$

where *Th* is the thickness of the film (mm) and *ΔP* is the water vapour partial pressure difference across the film (Pa).

2.8. Light transmission and transparency

The ultraviolet and visible light barrier properties of the films were tested at several wavelengths according to Hamaguchi et al. (2007), from 200 to 800 nm, using a spectrophotometer (Helios gamma, Thermo Fisher Scientific, USA). Briefly, a rectangular piece was cut from the film samples and placed directly into a spectrophotometer test cell. The measurements were performed using an empty test cell as the reference. The transparency of the films was calculated according to the following equation:

$$\text{Transparency} = A_{600}/x$$

where A_{600} is the absorbance of the film sample at 600 nm and x is film thickness (mm).

2.9. Film colour

The film colour properties were measured in the L^*, a^*, b^* system using an UltraScan VIS spectrophotometer (HunterLab, USA). Films were measured on the surface of the white standard plate, which has L*, a*, b* values of 97.12, –0.14 and 0.13 respectively.

2.10. Ferrous ion chelating assay

The ferrous ion chelating ability of the films was measured according to the method of Decker and Welch (1990) with slight modifications. Briefly, pieces of film of several weights were placed in 4 mL of de-ionized water and immediately 0.1 mL of 2 mM $FeCl_2$ was added. The solution containing the film was stirred gently in an orbital shaker for 5 min, and then the pieces of film were removed and 0.2 mL of 5 mM ferrozine was added. After incubation at room temperature for 10 min, the absorbance was measured at 562 nm. The $Fe^{2+/}$ferrozine complex has a high absorbance at this wavelength, so high chelating ability is shown as a low absorbance. The chelating ability in percentage was calculated as follows:

$$\text{Ferrous chelating ability } (\%) = (A_{\text{blank}} - A_t/A_t) \text{ x } 100$$

where A_t is the absorbance of the test sample.

2.11. Thymol and natamycin incorporation into the films and microbiological assays

Thymol (16254, Sigma-Aldrich) and natamycin (PHR1703, Sigma-Aldrich) were incorporated in the film-forming solution of the granules films and GTT films after the addition of glycerol. The thymol concentrations tested were 15 and 30% (w/w of

protein), while the natamycin concentrations tested were 2.5 and 5% (w/w of protein). These film-forming solutions were processed in a similar way to those previously described in "Film preparation" section and then the antimicrobial performance of the films obtained was assayed.

Staphylococcus aureus CECT 240, *Pseudomonas taetrolens* LMG 2336 and *Penicillium roqueforti* for cheese cultures (Choozit, Dupont, USA) were selected and the inhibition assays were carried out using the agar diffusion test as follows: Staphylococcus aureus was cultured in a Tryptic Soy Broth medium for 16 h at 37 °C under agitation at 250 rpm in an orbital shaker. *P. taetrolens* was cultured in a Nutrient Broth medium for 16 h at 30 °C and under agitation at 250 rpm. A solution which contained 300×10^6 spores/mL of *P. roqueforti* was diluted in peptone water to 1×10^6 spores/mL. In each case, an inoculum of 200 µL was spread on the respective agar mediums and immediately the 10 mm diameter pieces of film were placed on the surface of the agar plates. In the case of *P. roqueforti*, the agar medium selected was Plate Count Agar medium. *S. aureus* plates were incubated for 10 h at 37 °C, *P. taetrolens* for 16 h at 30 °C and *P. roqueforti* for 4 days at 30 °C. At the end of the experiment, the plates were photographed.

2.12. Statistical analysis

Analysis of variance (ANOVA) was applied. Least significant differences (LSD) were calculated by Fisher's test to determine significant differences between the tested samples. These analyses were performed using the statistical software Statgraphics® V.15.2.06.

3. Results and discussion

To get some idea about the physical parameters obtained for the new granules and GTT films in relation with other protein-based edible films, these values were compared with those obtained for gelatin and caseinate films. Since the relative humidity and the type and amount of plasticizer have relevance for the film properties, these films were prepared in a similar way to those made using granules protein. The amount of glycerine selected (50% w/w of protein) was the optimal quantity for the egg yolk protein films. In the case of gelatin and caseinate films, similar levels of glycerol were successfully used by other authors (Audic and Chaufer 2005; De Carvalho and Grosso 2004).

3.1. SDS-PAGE

In the SDS-PAGE shown in Figure 4.9 1a, at each time tested, it is possible to detect a decrease in the intensity of the apo-HDL bands of 110 and 100 kDa, and at the same

time, an appreciable increase in the amount of protein which cannot enter or can hardly enter the separation gel. In fact, after 30 min of reaction, these high molecular weight proteins almost disappear from the reaction medium, not being detectable after 90 min of reaction. This experiment suggests a high capacity of the transglutaminase to catalyse the crosslinking reaction for these proteins. However, the action of the transglutaminase on the other proteins of lower size was limited. It should be taken into account that the proteins involved in the crosslinking reaction must have a glutamine and a lysine residue, and they must be accessible to the enzyme. Furthermore, the amino acid sequence and the secondary structure in which the glutamine and lysine are included appear to be important for recognition by the enzyme (Rachel and Pelletier 2013).

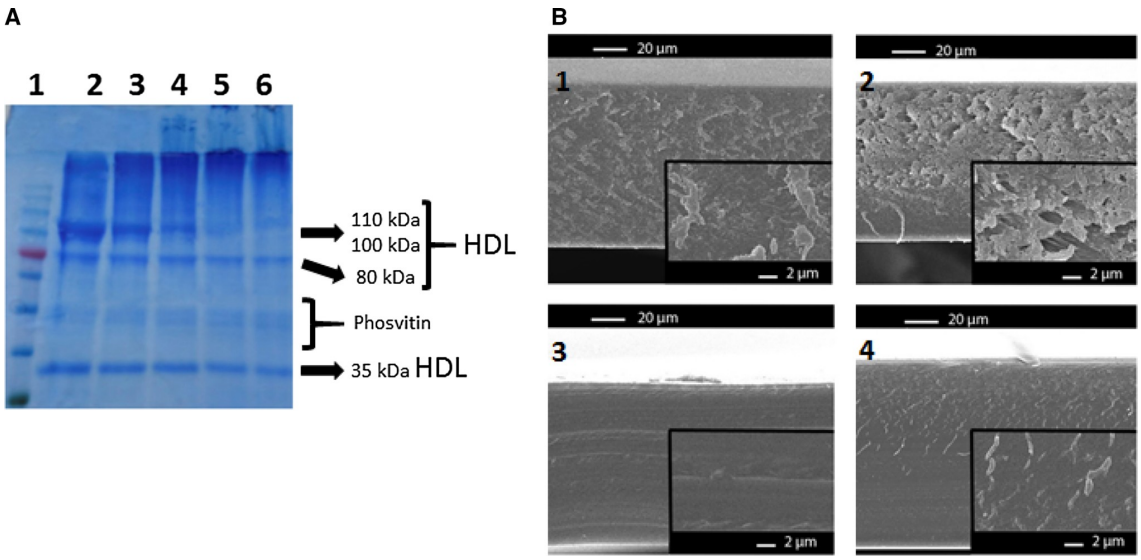


Figure 4.9. Effect of transglutaminase on the delipidated granules proteins and on the microstructure of the films obtained. a Electrophoresis. 1: Molecular weight markers. 2: Transglutaminase-treated granules. 3: 15 min of reaction. 4: 30 min of reaction. 5: 60 min of reaction. 6: 90 min of reaction. b Micrographs of the film cross-Section. 1: Untreated granules. 2: Transglutaminase-treated granules. 3: Gelatin. 4: Caseinate.

3.2. Microstructure

The surface microstructure of all tested films was completely smooth and with no difference between them (data not shown). However, the micrographs of the film cross-section showed particularities in the way the proteins form the film in each case (Figure. 4.9 b). In the granules films the cross-sectional area was observed to be slightly heterogeneous, with protein agglomerations appearing throughout the film matrix. The GTT film cross-sectional area showed a flaky, fibrous appearance, which produced stronger egg yolk protein packaging. The gelatin film showed a smooth and structured cross-sectional area, with a high degree of homogeneity, and this resulted in the film

with the best mechanical properties, as reported below. Finally, in the caseinate film, as in the case of the gelatin film, the cross-sectional area looked uniform except for the presence of some protein aggregates in the upper half.

3.3. Mechanical properties

To study the mechanical properties of the granules-based films with and without the transglutaminase treatment, the PS and PD values were obtained (Table 4.7). These values were compared with those obtained for the gelatin and caseinate films.

The gelatin films obtained the highest PS and PD values. This is possibly because the mechanical properties of the protein-based films are modulated by the chain-to-chain interactions produced between the proteins (hydrogen, electrostatic and hydrophobic bonds). So, the primary structure of the proteins as well as the degree of extension of the protein chains are factors that have an influence on the strength and flexibility of the film formed (Bourtoom 2008). In this case, the high PS and PD values of the gelatin film are due to the gelatin being obtained from collagen, which is a fibrous protein with structural functions that can form intermolecular interactions easily.

The PS value of the caseinate films was similar to that of the gelatin films. The casein structure has a high degree of flexibility and confers on this protein a good chance of establishing interactions with other proteins (Kinsella and Morr 1984). It could explain the formation of a highly interconnected net of proteins, forming strong films.

The PS and PD values of the granules films were found to be the lowest of those obtained by the evaluated films, this being in accordance with the micrographs shown previously. The granular fraction is composed mainly of high density lipoproteins, which are globular-type proteins, and since the amount of protein and glycerol was similar to that used for the gelatin and caseinate films, it is possible that the number and/or quality of protein interactions produced in the granules film matrix is lower than those in the other films tested.

Furthermore, when this egg yolk protein was treated with transglutaminase, the PS value of the films obtained was increased by 54%. Increments in the strength of the protein-based films after treatment with transglutaminase were also reported by other authors. Weng and Zheng (2015) made films using gelatin and transglutaminase (10 U/g of protein) and obtained an increase in the tensile strength of 29%. However, the effect of the transglutaminase on the flexibility of the films was slight. This marginal effect corresponds with other reported films in which the transglutaminase had no effect or had a negative effect on this film property (Jiang et al. 2007; Porta et al. 2015).

Table 4.7. Puncture strength (PS), puncture deformation (PD), water solubility (WS), water vapour permeability (WVP), thickness, moisture content (MC), colour, light transmission and transparency of the tested films.

	PS (N/mm)	PD (%)	WS (%)	WVP (g mm/m^2 h kPa)	Thickness (µm)	MC (%)	L*	a*	b*	200	280	350	400	500	600	Transparency
Granules	79.0 ± 8.4^c	50.0 ± 2.1^c	22.1 ± 2.0^c	3.38 ± 0.10^a	122 ± 5^a	29.5 ± 5^a	95.10 ± 0.30^c	-0.53 ± 0.07^c	12.80 ± 1.00^a	0.1	0.1	52.0	78.4	96.0	96.0	0.18
GTT	122.0 ± 9.1^b	58.0 ± 3.0^b	24.5 ± 2.0^c	3.05 ± 0.05^b	119 ± 4^a	30.5 ± 0.5^a	94.50 ± 0.40^c	-0.58 ± 0.01^c	13.70 ± 0.19^a	0.1	0.1	53.5	78.3	95.0	96.2	0.18
Gelatin	190.6 ± 21.5^a	75.7 ± 5.6^a	48.9 ± 2.5^b	2.90 ± 0.05^c	100 ± 10^b	23.2 ± 1.0^b	98.48 ± 0.20^a	-0.40 ± 0.12^b	3.50 ± 0.70^b	0.1	25.6	83.5	92.4	95.1	97.3	0.21
Caseinate	188.6 ± 13.8^a	30.5 ± 5.2^d	99.0 ± 1.0^a	2.93 ± 0.30^c	115 ± 3^a	21.0 ± 0.3^c	97.60 ± 0.25^b	-0.10 ± 0.01^a	4.40 ± 0.50^b	0.1	0.1	42.1	66.9	84.0	84.5	0.65

3.4. Thermal properties

The TGA curves of the films are shown in Figure 4.10a. The tested films showed behaviour typical of this type of material, with a loss of weight with increasing temperature that can be divided into three different stages. In the first stage, from 40 to 150–160 °C, the adsorbed water is evaporated from the film (Guo et al. 2014). In the second phase, from around 150–160 °C to 270–280 °C, the glycerol is volatilized from the sample (Hoque et al. 2011). Finally, the third stage starts with the thermal degradation of the protein, and it begins at 270–280 °C (Ge et al. 2015). For a better understanding of the thermal degradation of the films, the derivatives of the TGA thermograms were calculated and they are shown in the form of DTG curves (Figure 4.10b, c). Furthermore, some parameters were calculated from TGA and DTG curves and they are shown in Table 4.8. In the DTG curve of the granules film the three stages described above can be clearly observed in the form of three peaks. However, when the DTG curve of the GTT films was analysed, the peak corresponding to the glycerol evaporation could not be observed and appeared to be combined with the protein degradation stage.

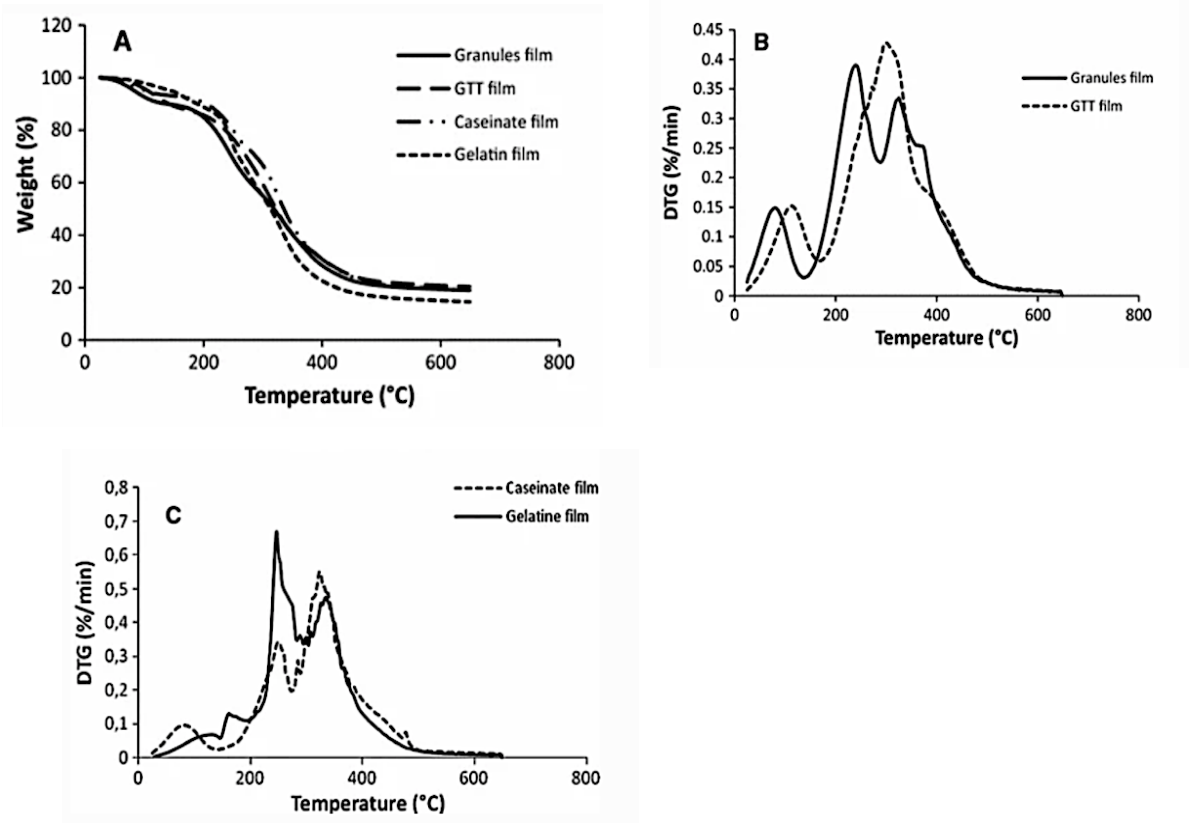


Figure 4.10. Films TGA (a) and DTG (b, c) curves.

Table 4.8. Thermal property values obtained from Figure 4.10.

Films	Decomposition stage	Temperature range (ºC)	DTG peak	Weight loss (%)	Residue at 650ºC	T_{50} (ºC) [a]
Granules	1	25-140	85	11	18.9	313
	2	140-291	243	32.6		
	3	291-500	335	35.8		
GTT	1	25-170	120	12.5	20.3	319
	2 and 3	170-500	303	65.4		
Caseinate	1	25-167	85	7.5	18.9	332
	2	167-260	259	15.7		
	3	260-500	325	55.6		
Gelatin	1	24-145	139	5	14.5	313
	2	145-278	249	32.5		
	3	278-500	335	46.2		

[a]Temperature at which 50% of the film weight was lost.

This could be due to an improvement in the compatibility between the glycerol and the egg yolk protein (Ramos et al. 2013), probably produced by a better interaction between the glycerol molecules and the protein chains in the film matrix. Furthermore, the GTT film showed an increase in the amount of residue at 650 °C and a higher T50 value with respect to the granules film, evidencing an improvement in the thermal properties of the transglutaminase-treated films. In agreement with this, several authors have reported similar effects on the thermal properties of this type of packaging material when using different crosslinking agents (Ge et al. 2015). In comparison with the other films tested, the caseinate film showed the highest T50 value and a low level of thermal degradation in the glycerol evaporation stage. This suggests a good compatibility between the glycerol and the caseinate and the best thermal resistance among the films tested. Finally, the gelatin film had the lowest values of T50 and amount of residue at 650 °C and it also showed higher thermal degradation in the glycerol and in the protein degradation stages than did the granules and GTT films.

3.5. Film solubility, water vapour permeability (WVP)

Films made using delipidated egg yolk granules showed low water solubility in comparison with the gelatin and caseinate films (Table 4.7). The egg yolk granules proteins are soluble in water at high ionic strength (> 0.3 M), so the films obtained using

these proteins are also expected to be insoluble in distilled water. In fact, the conditions for solubilizing the egg yolk granules cannot be easily found in the food industry, and therefore, these films could be suitable for covering food. The caseinate films dissolved almost immediately in distilled water, and the gelatin films showed a water solubility value that was intermediate between that of egg yolk and caseinate films. The WVP value of the films tested varied within narrower limits, although the differences were statistically significant. This could be explained by the fact that this film property is strongly modulated by the type of biopolymer used and by the amount and type of plasticizer (Wihodo and Moraru 2013). In this sense, all films have a similar protein-based composition, with a similar amount of protein and glycerol in the film forming solution. Among them, the gelatin and caseinate films showed a slightly lower WVP value than that of the granules. This could be due to better packaging of the proteins in the film matrix, leaving less free space to facilitate the migration of water through the film. This granules film property was improved by the transglutaminase treatment, which increased the degree of packaging of the proteins and decreased the WVP of the film. This effect caused by the transglutaminase was also observed by other authors using mixtures of gelatin and soy protein (Weng and Zheng 2015).

3.6. Colour, light transmission and transparency

The colour values of the films tested are shown in Table 4.8. For all films tested the L* (lightness) and a* (reddish/greenish) parameters were found to be only slightly different and have values similar to those for the white standard. However, the b* parameter showed high values in the egg yolk films with respect to the gelatin and caseinate films. This yellowish colour of the films was produced by the fat-soluble carotenoids (Li-Chan and Kim 2008) which were not totally removed during the ethanol treatment. Although after the delipidation process a dry powder with a high content in proteins (87 ± 1%) was obtained, it must be expected that part of the non-protein content is composed of these coloured compounds. Finally, it is noteworthy that the transglutaminase does not produce any change in the colour of these films.

The light transmission of the granules, GTT and caseinate films in the UV range was very low, this being a desirable property in order to protect food items high in lipid, since UV radiation is an important starter for their oxidative degradation (Coupland and McClements 1996). In the case of the gelatin film, it showed a decreased light barrier capacity at 280 nm in comparison with the other tested films. In broad terms, the protein-based edible films have a high capacity to prevent UV damage due to the natural presence of aromatic amino acids in their composition. However, the gelatin does not

have tryptophan in its composition, and it is low in phenylalanine and tyrosine (Nhari et al. 2011). This resulted in lower light barrier properties than in the other films tested. Regarding the wavelengths in the visible region, the tested films showed good properties for food packaging, since they allow easy visual examination of the product covered. This was confirmed by the transparency index, which was very low for all of them.

3.7. Ferrous ion chelating assay

Phosvitin is the most phosphorylated protein found in nature, and its chelating properties have been broadly studied. In the granular fraction, the phosvitin content corresponds to 16% of the whole protein content, and as indicated by the electrophoresis results presented previously, phosvitin can be found as part of the protein obtained after the delipidation process. In Figure 4.11 the ferrous chelating property of the egg yolk protein-based films is shown. In this case, the caseinate film could not be tested due to its solubilisation in the aqueous medium during the assay, and the gelatin film, as was expected, does not show any chelating property.

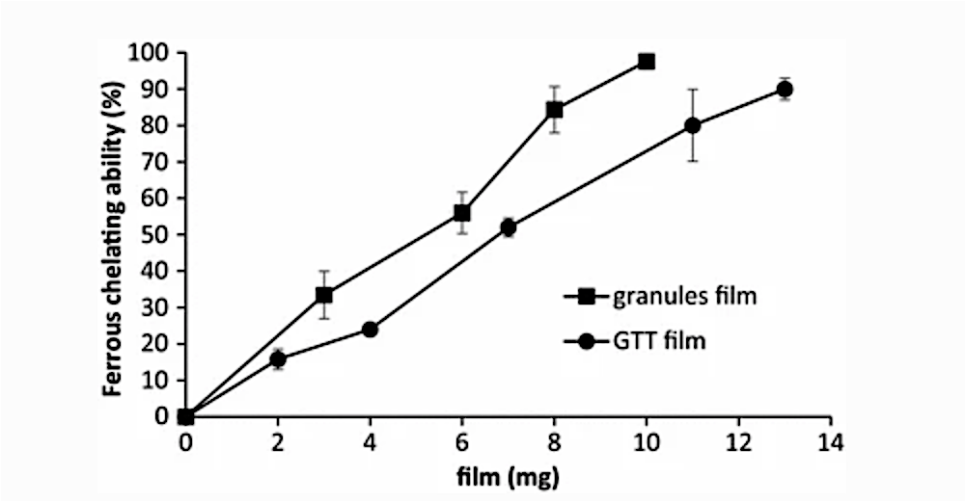


Figure 4.11. Ferrous chelating ability of the granules and GTT films.

The ferrous chelating ability of both the granules and GTT films showed dose-dependent behaviour with a strong capacity to chelate ferrous ion. The higher chelating capacity of the granules film could be due in part to the dilution of the egg yolk protein in the case of the GTT film. In the GTT films, the addition of transglutaminase decreases by 10% the amount of phosvitin and other egg yolk proteins per gram of film, in agreement with the preparation method reported in the materials and methods section. Furthermore, the fibrous film matrix produced by the action of transglutaminase could have developed a tighter net of fibres which might hinder the interactions between the

aqueous solvent and the phosvitin. In any case, this antioxidant activity could be of interest to protect foods containing iron which are rich in lipids, as for example meat-based products, since ferrous iron is involved in the oxidation of lipids (Love and Pearson 1971).

3.8. Films with thymol and natamycin added

The antimicrobial and antifungal properties of the egg yolk protein-based films, with and without adding thymol (30% w/w of protein) and natamycin (5% w/w of protein), were tested (Figure 4.12). In the case of the control films, bacterial growth in the area under the granules and GTT films was impeded both in the case of *P. taetrolens* and *S. aureus*. This could be due to the presence in the films of phosvitin, which can remove the iron in the medium, showing bactericidal properties (Zhou et al. 2014). In the case of *P. roqueforti,* the fungi were able to grow under the film without any problem, not being affected by the presence of the egg yolk proteins.

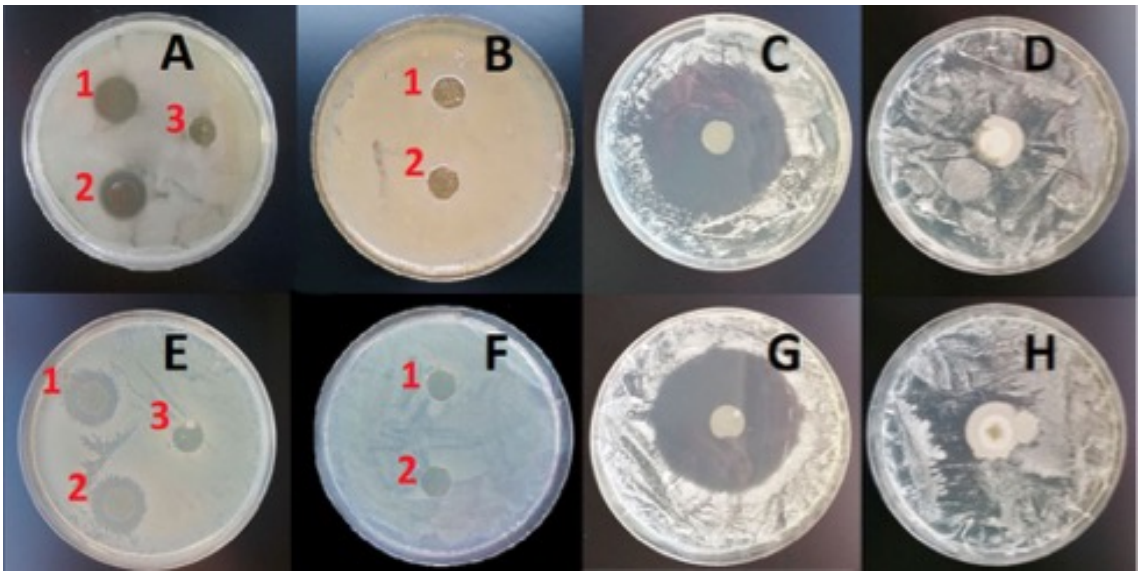


Figure 4.12. a (*S. aureus*) and e (*P. taetrolens*): GTT film with thymol 30% (1), granules film with thymol 30% (2) and granules film control (3). b (*S. aureus*) and f (*P. taetrolens*): GTT film control (1) and granules film control (2). c (*P. roqueforti*): GTT film with natamycin 5%. g (*P. roqueforti*): granules film with natamycin 5%. d and h (*P. roqueforti*): GTT film and granules film control respectively.

In the case of the films with thymol, an inhibition area greater than the film surface was observed in both *S. aureus* and *P. taetrolens* agar medium. To produce this inhibition zone, the thymol contained in the films must diffuse from the film to the culture medium, confirming in this way that the addition of an antibacterial agent to improve the functionality of these films is possible. Thymol has been used previously by other authors with a similar purpose (González and Igarzabal 2013). As in the case of thymol,

the films with natamycin produced a large inhibition area in the *P. roqueforti* culture medium. In these experiments, the large inhibition area shown in Figure 4.12 is explained by the fact that the films' formulation was modified with a high concentration of natamycin (5% w/w egg yolk protein), but lower concentrations of this active agent produced a smaller inhibition zone (data not shown).

4. Conclusions

The delipidated egg yolk protein-based edible films prepared in this study showed optical properties which are desirable for the food industry, but their low solubility and their ferrous ion chelating capacity could be considered the main characteristics of these packaging materials. Their low solubility probably derives from the fact that the egg yolk granules are highly insoluble in distilled water but could also be due to the structural protein modifications which occur during lipid extraction. Furthermore, ferrous ion chelating capacity is seen due to the presence of phosvitin in the granular fraction. So, the addition of phosvitin to other edible films, thus allowing them to acquire this property, is a possibility, although it would increase the cost of the packaging material. Finally, the treatment with transglutaminase produced a noticeable increase in the strength of the film obtained, but the other film parameters were barely affected, so in this case, the transglutaminase treatment would be useful only if the mechanical properties of the non-treated film were considered to be insufficient.

5. References

Aro H, Järvenpää EP, Könkö K, Sihvonen M, Hietaniemi V, Huopalahti R (2009) Isolation and purification of egg yolk phospholipids using liquid extraction and pilot-scale supercrit- ical fluid techniques. *Eur Food Res Techn*ol 228:857–863

Audic J-L, Chaufer B (2005) Influence of plasticizers and crosslink- ing on the properties of biodegradable films made from sodium caseinate. *Eur Polym J* 41:1934–1942

Blanco-Pascual N, Montero MP, Go´mez-Guille´n MC (2014) Antioxidant film development from unrefined extracts of brown seaweeds Laminaria digitata and Ascophyllum nodosum. *Food Hydrocoll* 37:100–110. doi:10.1016/j.foodhyd.2013.10.021

Bourtoom T (2008) Edible films and coatings: characteristics and properties. *Int Food Res J* 15:237–248

Coupland JN, McClements DJ (1996) Lipid oxidation in food emulsions. *Trends Food Sci Technol* 7:83–91

De Carvalho R, Grosso C (2004) Characterization of gelatin based films modified with transglutaminase, glyoxal and formaldehyde. *Food Hydrocoll* 18:717–726

Decker EA, Welch B (1990) Role of ferritin as a lipid oxidation catalyst in muscle food. *J Agric Food Chem* 38:674–677

Galus S, Kadzin´ska J (2016) Whey protein edible films modified with almond and walnut oils. *Food Hydrocoll* 52:78–86

Ge L, Li X, Zhang R, Yang T, Ye X, Li D, Mu C (2015) Development and characterization of dialdehyde xanthan gum crosslinked gelatin based edible films incorporated with amino-functional- ized montmorillonite. *Food Hydrocoll* 51:129–135. doi:10.1016/j.foodhyd.2015.04.029

González A, Igarzabal CIA (2013) Soy protein–Poly (lactic acid) bilayer films as biodegradable material for active food packaging. *Food Hydrocoll* 33:289–296

Guo J, Ge L, Li X, Mu C, Li D (2014) Periodate oxidation of xanthan gum and its crosslinking effects on gelatin-based edible films. *Food Hydrocoll* 39:243–250. doi:10.1016/j.foodhyd.2014.01.026

Hamaguchi PY, WuYin W, Tanaka M (2007) Effect of pH on the formation of edible films made from the muscle proteins of Blue marlin (Makaira mazara). *Food Chem* 100:914–920. doi:10.1016/j.foodchem.2005.10.045

Hoque MS, Benjakul S, Prodpran T (2011) Properties of film from cuttlefish (Sepia pharaonis) skin gelatin incorporated with cinnamon, clove and star anise extracts. *Food Hydrocoll* 25:1085–1097. doi:10.1016/j.foodhyd.2010.10.005.

Jiang Y, Tang C-H, Wen Q-B, Li L, Yang X-Q (2007) Effect of processing parameters on the properties of transglutaminase- treated soy protein isolate films. *Innov Food Sci Emerg Technol* 8:218–225

Kinsella JE, Morr CV (1984) Milk proteins: physicochemical and functional properties. *Crit Rev Food Sci Nutr* 21:197–262

Kiosseoglou V, Paraskevopoulou A (2005) Molecular interactions in gels prepared with egg yolk and its fractions. *Food Hydrocoll* 19:527–532

Laca A, Paredes B, D´ıaz M (2010) A method of egg yolk fractionation. Characterization of fractions. *Food Hydrocoll* 24:434–443. doi:10.1016/j.foodhyd.2009.11.010

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685

Le Denmat M, Anton M, Beaumal V (2000) Characterisation of emulsion properties and of interface composition in O/W emulsions prepared with hen egg yolk, plasma and granules. *Food Hydrocoll* 14:539–549

Li-Chan EC, Kim H-O (2008) Structure and chemical composition of eggs. In: Mine Y (ed) Egg bioscience and biotechnology, 1st edn. Wiley, New York, pp 1–96

Love JD, Pearson AM (1971) Lipid oxidation in meat and meat products—a review. J Am Oil Chem Soc 48:547–549. doi:10. 1007/BF02544559

Marcet I, Á lvarez C, Paredes B, Díaz M (2014) Inert and oxidative subcritical water hydrolysis of insoluble egg yolk granular protein, functional properties, and comparison to enzymatic hydrolysis. *J Agric Food Chem* 62:8179–8186

Nhari R, Hafidz RM, Che Man Y, Ismail A, Anuar N (2011) Chemical and functional properties of bovine and porcine skin gelatin. *Int Food Res J* 18:813–817

Otero-Pazos P et al (2016) Preparation of antioxidant active films based on chitosan: diffusivity study of a-tocopherol into food simulants. *J Food Sci Technol* 53:2817–2826

Pan H, Jiang B, Chen J, Jin Z (2014) Blend-modification of soy protein/lauric acid edible films using polysaccharides. *Food Chem* 151:1–6. doi:10.1016/j.foodchem.2013.11.075

Porta R, Di Pierro P, Rossi-Marquez G, Mariniello L, Kadivar M, Arabestani A (2015) Microstructure and properties of bitter vetch (Vicia ervilia) protein films reinforced by microbial transglutaminase. *Food Hydrocoll* 50:102–107

Rachel NM, Pelletier JN (2013) Biotechnological applications of transglutaminases. *Biomolecules* 3:870–888

Ramos O´ L et al (2013) Effect of whey protein purity and glycerol content upon physical properties of edible films manufactured therefrom. *Food Hydrocoll* 30:110–122. doi:10.1016/j.foodhyd. 2012.05.001

Rezvani E, Schleining G, Su¨men G, Taherian AR (2013) Assessment of physical and mechanical properties of sodium caseinate and stearic acid based film-forming emulsions and edible films. *J Food Eng* 116:598–605

Sobral PJA, Menegalli FC, Hubinger MD, Roques MA (2001) Mechanical, water vapor barrier and thermal properties of gelatin based edible films. *Food Hydrocoll* 15:423–432. doi:10.1016/S0268-005X(01)00061-3

Weng W, Zheng H (2015) Effect of transglutaminase on properties of tilapia scale gelatin films incorporated with soy protein isolate. *Food Chem* 169:255–260

Wihodo M, Moraru CI (2013) Physical and chemical methods used to enhance the structure and mechanical properties of protein films: a review. *J Food Eng* 114:292–302

Zhou B, Hu Y, Li J, Li B (2014) Chitosan/phosvitin antibacterial films fabricated via layer-by-layer deposition. *Int J Biol Macromol* 64:402–408. doi:10.1016/j.ijbiomac.2013.12.016

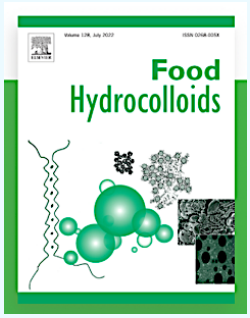
4.2.3. Packaging bioactivo basado en *films* comestibles de proteína de yema de huevo delipidada con ácido lactobiónico y *Lactobacillus plantarum* CECT 9567: caracterización y uso como *coating* en un modelo alimentario

Tras estudiar y caracterizar los *films* con proteína de yema de huevo delipidada como matriz expuestos en el subapartado anterior, se procedió a la incorporación, de forma independiente y conjunta, de ácido lactobiónico como compuesto prebiótico y *L. plantarum* CECT 9567 como probiótico en su formulación. De esta forma se elaboraron *films* prebióticos, probióticos y sinbióticos. En este caso se empleó ácido lactobiónico comercial, tras comprobar que no había diferencia entre éste y el obtenido por síntesis biológica.

Estos *films* bioactivos fueron estudiados en cuanto a sus propiedades físicas, químicas y mecánicas, así como en relación a su morfología. Además, se analizó la viabilidad del probiótico durante el periodo de almacenamiento.

Por otro lado, para comprobar su aplicabilidad en alimentos, se desarrollaron *coatings* con la misma composición que los *films* y se utilizaron para recubrir gelatina comercial como modelo alimentario real a través del método de inmersión. En este caso, además de la viabilidad del probiótico durante el almacenamiento y el consumo de ácido lactobiónico por el mismo, se realizó una prueba de digestión *in vitro* simulada para comprobar la capacidad protectora, tanto del *coating* como del ácido lactobiónico, sobre la bacteria probiótica.

- **Publicación:** Sáez-Orviz, S., Marcet, I., Rendueles, M., Díaz, M. 2021. Bioactive packaging based on delipidated egg yolk protein edible films with lactobionic acid and *Lactobacillus plantarum* CECT 9567: Characterization and use as coating in a food model. *Food Hydrocolloids*, 119, 106849, ISSN 0268-005X, (https://doi.org/10.1016/j.foodhyd.2021.106849).
- **Estado:** publicada
- **Índice de impacto:** 9,147 (2020)



Bioactive packaging based on delipidated egg yolk protein edible films with lactobionic acid and *Lactobacillus plantarum* CECT 9567: characterization and use as coating in a food model

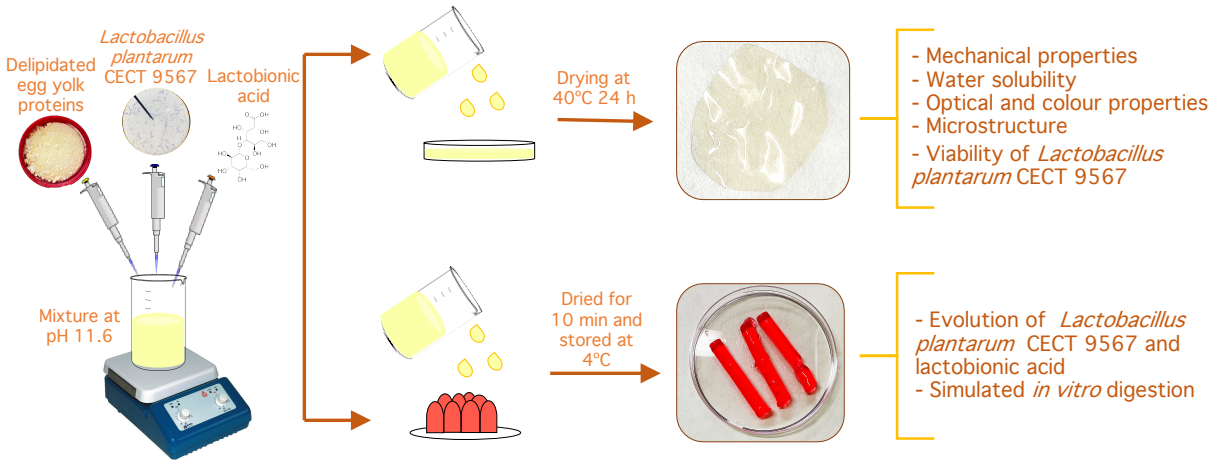
S. Sáez-Orviz[a], I. Marcet[a], M. Rendueles[a*], M. Díaz[a]

[a]Department of Chemical and Environmental Engineering, University of Oviedo, C/Julián Clavería 8, 33006, Oviedo, Spain.

*Corresponding author

E-mail address: mrenduel@uniovi.es (M. Rendueles)

Graphical abstract



Abstract

Egg yolk is a source of proteins that can be used to develop novel food packaging material. In this study, three different bioactive films were developed using delipidated egg yolk proteins, the first was loaded with a probiotic (PRO, *Lactobacillus plantarum* CECT 9567), the second one with a prebiotic (PRE, lactobionic acid (LBA), 10 g/L) and the third with both (SYN, containing *L. plantarum* and LBA). The mechanical properties, water solubility, light transmittance, colour and microstructure of the films were fully characterised. Furthermore, LBA and probiotic concentrations inside the films were monitored under storage conditions. Finally, in order to test the performance of these packaging materials on a piece of real food, gelatine portions were coated by immersion in PRO and SYN film-forming solutions and the microorganism survival after a simulated

in vitro digestion was assessed. The strength and water solubility of the films were slightly affected by the presence of LBA at the concentration tested, while the light transmittance decreased in the SYN film in comparison with the other films. Before simulated digestion, PRO and SYN coatings met the minimal requirements to attain the "probiotic" category (6 $\log_{10}$ CFU/g) but after the simulated digestion, only SYN maintained acceptable values. It was noted that the addition of LBA increased the viability of the probiotic, in both the edible films and coatings.

Keywords: egg yolk protein, edible film, lactobionic acid, prebiotic, probiotic, *in vitro* digestion

1. Introduction

Currently, conventional packaging materials made using non-renewable sources are being replaced by other more sustainable alternatives. In this sense, proteins are suitable biopolymers for the development of biodegradable films and coatings, as they usually result in materials with adequate mechanical and physical properties (Fernandes et al., 2020a). Proteins from egg yolk can be used to develop packaging materials, as egg is broadly recognised as a valuable source of proteins. In this respect, egg yolk can be easily separated into the plasma and the granular fraction by dilution with water and centrifugation (Froning, 2007). The plasma fraction is mainly comprised of lipids (78%) and is the most valuable fraction, due to its emulsifying and foaming capacities (Laca, Paredes, & Díaz, 2010). However, the granular fraction is mainly composed of water-insoluble proteins (58%) (Laca et al., 2010) and consequently, its direct use as a food ingredient with functional properties is limited. Therefore, the exploitation of the granular fraction requires the development of a large range of new applications, specifically designed to take advantage of its relatively high protein content, in order to raise the interest of the industry in egg yolk fractionation. In this sense, one way to revalorise egg yolk granules is by developing new protein-based food packaging materials (Marcet, Sáez, Rendueles, & Díaz, 2017), which can also undergo enhancement by the addition of different types of compounds to prepare bioactive packaging.

The inclusion of bioactive compounds is a completely new trend in the development of packaging. The main objective is to add value to the packaging market, and this can be achieved by using protein-based edible films and coatings as carriers. This kind of packaging has the capacity to hold and maintain the bioactive principles in optimum condition until their release, which could be in the food product itself or during its consumption (Lopez-Rubio, Gavara, & Lagaron, 2006). In this regard, the food industry

makes widespread use of probiotics and prebiotics, and these two types of compounds could be also combined and introduced into protein-based edible films or coatings to enhance their properties. Furthermore, these packaging materials could well be an excellent alternative to petroleum-based plastics in the food industry, whilst at the same time converting the food products into healthier ones for consumers.

Regarding probiotics, there are many bacterial strains that can be used, but the most commonly employed belong to the genera *Lactobacillus* and *Bifidobacterium* (Espitia, Batista, Azeredo, & Otoni, 2016). Traditionally, probiotics are mostly used in dairy food matrices, but their inclusion in other products, such as fruits, meat, gelatine, cereals and chocolate have also been studied (Fernandes et al., 2020a). In addition, it is important to bear in mind that bioactive coatings are very versatile and tend to maintain the viability of the probiotics over time (Espitia et al., 2016). As for the prebiotics, there are a wide range that could be considered for producing edible packaging systems with valuable features, but lactobionic acid (LBA) has generated growing interest in the food industry in recent times. It can be used as an antioxidant, moisturizer and chelating agent (Cardoso, Marques, Dagostin, & Masson, 2019), but also as a prebiotic (Alonso, Rendueles, & Díaz, 2013), as it is resistant to digestive enzymes and can be metabolised by gastrointestinal microflora (Saarela, Hallamaa, Mattila-Sandholm, & Mättö, 2003) (Schaafsma, 2008). At the moment, LBA has been approved by the Food and Drug Administration (FDA) (Cardoso et al., 2019) (Alonso et al., 2013) for use only as the salt form (*FDA. Code of Federal Regulations, Title 21, 21 CFR 172.720. US Food and Drug Administration*, 2017), but it is expected that its human consumption will be approved by other food authorities in the short term. Therefore, LBA can be considered as a very promising compound in the food industry, and its inclusion in films and coatings to cover foodstuffs, such as prepared slices or pieces of fruit, cheese or sausages, will help to protect them from dehydration and oxidation while enhancing their nutritional value by including a prebiotic compound that can be combined with a probiotic microorganism. Thus, its inclusion in a sustainable and bioactive packaging material should be very attractive for both the food industry and consumers.

Therefore, the aim of this research is to develop delipidated egg yolk protein-based films and coatings by introducing probiotics and prebiotics simultaneously in their formulation. For that purpose, the packaging materials will contain *Lactobacillus plantarum* CECT 9567 or LBA as additive(s), with the two combined to obtain a synbiotic food packaging. The effect of the probiotic and prebiotic, separately and combined, on the physical properties of the films will be tested. Furthermore, changes in the LBA

concentrations and the probiotic viability inside the films under storage conditions will also be assessed. Finally, in order to evaluate the performance of the materials prepared in a real commercial scenario, pieces of gelatine, as a real food model, will be coated, and the variations in the probiotic and prebiotic concentrations will be analysed over time and after a simulated *in vitro* digestion test.

2. Materials and methods

2.1. Obtaining delipidated egg yolk granules

Delipidated egg yolk granules were obtained as described by Marcet *et al.* (2014) (Marcet, Álvarez, Paredes, & Díaz, 2014). Briefly, after separating the egg yolk from the egg white, the egg yolk was dried using blotting paper. The vitelline membrane was broken to obtain only the liquid yolk, which was mixed with distilled water to a proportion of 1:1.5 (v/v). The pH of this diluted egg yolk solution was adjusted to 7.0 with 0.1 M NaOH (Sigma-Aldrich, Steinheim, Germany). The solution was centrifuged at 10 000 rpm for 45 min. The pellet, mainly composed of granules, was collected and lyophilized (Tesltar Cryodos, 0.1 mBar, -70ºC for 24 h). The lyophilized egg yolk granules were delipidated using absolute ethanol (50:1 v/w) (99%, VWR Chemicals, PA, USA) under gentle agitation for 2 h. Granules were recovered by filtration using Whatman no 1 paper (Sigma-Aldrich) and a vacuum pump. Delipidated granules were dried at 40 ºC for 24 h in an oven and stored in a freezer at -20 ºC until use. The dry matter content of the delipidated granules was 96% (w/w).

2.2. Film preparation

Film-forming solution was prepared according Marcet et al. (2014) with some modifications. Briefly, 3 g of delipidated granules of egg yolk protein were mixed with 75.5 mL of distilled water and 1.5 mL of 1 M NaOH. The mixture was kept under agitation for 20 minutes at 65 ºC until a homogeneous appearance was observed. After that, the mixture was centrifuged at 10 000 rpm for 10 min and the supernatant was recovered. Glycerine (Panreac S.A., Barcelona, Spain) was added at a concentration of 50% (w/w) with respect to the total protein content and four different types of film-forming solution were prepared (Table 4.9). LBA was previously dissolved in ultrapure water at a concentration of 40 g/L (pH adjusted to 11.6 with 5 M NaOH) and then added to the prebiotic (PRE) and synbiotic (SYN) film-forming solutions until a concentration of 10 g/L was reached in the final volume. This concentration was selected on the basis of the results of previous studies (García, Bautista, Rendueles, & Díaz, 2018) (Sáez-Orviz, Puertas, Marcet, Rendueles, & Díaz, 2020). *Lactobacillus plantarum* CECT 9567

(from the Spanish Type Culture Collection, Valencia, Spain) was added to the probiotic (PRO) and SYN film-forming solutions as a probiotic microorganism. *L. plantarum* was previously grown in 100 mL of MRS Broth (de Man, Rogosa and Sharpe, Sigma-Aldrich) in an orbital shaker at 200 rpm and 30 ºC for 24 h. The *L. plantarum* culture was centrifuged at 13200 rpm for 10 min and the pellet was dispersed in the film-forming solution until a concentration of 8 $\log_{10}$ CFU/mL was reached in the final volume. A control film without prebiotic or probiotic was also made. In all cases, the final pH of the film-forming solutions was 11.6, with the aim of completely dissolving the delipidated egg yolk protein.

In order to obtain the dried films, 20 mL of the film-forming solution was cast in a Petri dish and dried in an oven at 40 ºC for 24 h. Finally, the films could be removed from the Petri dishes without being sticky or brittle.

Table 4.9. Composition of the four different types of film-forming solution.

Film sample	Lactobionic acid (LBA)	*Lactobacillus plantarum* CECT 9567
Control	-	-
Prebiotic (PRE)	10 g/L	-
Probiotic (PRO)	-	8 $\log_{10}$ CFU/mL
Synbiotic (SYN)	10 g/L	8 $\log_{10}$ CFU/mL

2.3. Film characterization

2.3.1. Thickness and mechanical properties

The thickness of the samples was determined with a digital micrometer (Mitutoyo, Japan). Measurements were performed at ten different points, both on the outside and inside of the films. The average of these values was reported as the film thickness.

A TA.XT plus Texture Analyser (Stable Microsystems, UK) was employed to assess the puncture strength (PS) and puncture deformation (PD) of the films. For that purpose, films were cut into squares, placed on the test platform, and subjected to a penetration test at room temperature using a P/5S spherical probe with a test speed of 2.0 mm/s and a load cell of 5 kg. PS and PD parameters were calculated according to the following equations (Otero-Pazos et al., 2016):

$$PS = Fm/Th$$

$$PD = (\sqrt{D^2 + R^2} - R)/R$$

where *Fm* is the maximum force applied before the breakage of the film (N) and *Th* is the film thickness (mm). *D* is the distance covered by the probe while it is in contact with the film until the film is broken (mm) and *R* is the radius of the orifice in the plates (mm). Experiments were carried out in triplicate and reported results correspond to the mean value.

2.3.2. Water solubility

To test the water solubility of the film (WS), pieces of film were immersed in 20 mL of distilled water with 2% (w/w) Tris-HCl pH 7.0 (Sigma-Aldrich) and were kept for 24 h at room temperature. To recover the undissolved films, the solution was filtered using a vacuum pump and Whatman no 1 paper (Sigma-Aldrich) and they were dried in an oven at 105 ºC for 24 h. In addition, other pieces of film were dried under the same conditions without being previously dissolved. The solubility was calculated as follows (Blanco-Pascual, Montero, & Gómez-Guillén, 2014):

$$WS\ (\%) = (m1 - m2)/m1\ x\ 100$$

where $m1$ is the weight (g) of the film pieces dried in an oven at 105 ºC for 24 h and $m2$ is the weight (g) of the fragments of undissolved film once they have been dried. Experiments were carried out in triplicate and reported results correspond to the mean value.

2.3.3. Optical transmittance and transparency

Ultraviolet (UV) and visible light barrier properties of the films were tested at various wavelengths, from 200 to 800 nm (Marcet et al., 2014), employing a spectrophotometer (Helios gamma, Thermo Fischer Scientific, USA). For this purpose, a rectangular piece of film was cut from the film samples and placed directly into a spectrophotometer test cell. Measurements were performed employing an empty test cell as reference. The transparency of the films was calculated as follows:

$$Transparency = A_{600}/x$$

where A_{600} is the absorbance of the film at 600 nm and x is film thickness (mm). Experiments were carried out in triplicate and reported results correspond to the mean value.

2.3.4. Colour properties

The colour of the film samples was assessed on the surface of a previously measured white standard plate using a Lovibond® LC 100 Spectrocolorimeter (Tintometer® Group,

Lovibond house, UK). Three colour parameters were measured: *L** (lightness/brightness), *a** (redness/greenness) and *b** (yellow/blueness). The total colour difference (Δ*E*) was calculated as follows (Zabihollahi, Alizadeh, Almasi, Hanifian, & Hamishekar, 2020):

$$\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

where Δ*L**, Δ*a** and Δ*b** are the difference between the colour of the white standard plate (*L**= 97.12, *a**= -0.14 and *b**=0.13) and film samples. The *b** and *L** parameters are linked to yellowness index (*YI*) according to the following equation (Francis F. J. & Clydesdale F. M., 1977):

$$YI = (142.86\ x\ b^*)/L^*$$

Experiments were carried out in triplicate and reported results correspond to the mean value.

2.3.5. Scanning electron microscopy (SEM)

The morphology of the films was observed by employing scanning electron microscopy (SEM) (JSM-6610LV, JEOL, USA). For this purpose, the pieces of film were cut with a surgical blade, mounted on stubs and coated with gold. The cross-sectional morphology of the films was observed at a magnification of 500x.

2.4. Viability of *Lactobacillus plantarum* CECT 9567 inside the films under storage conditions

The viability of the probiotic within the films was analysed with a confocal laser microscope (confocal laser microscope SP8-Leica TCS-SP2-AOBS; Leica Microsystems, Germany) equipped with a camera lens of 63x/1.400iL. To check the cellular state of the bacteria, seven-day films stored at 4 ºC were dyed with two different fluorochromes. SYBRGreen (Sigma-Aldrich) was diluted (1: 10 000) in TE buffer (10 mM Tris-HCl and 1 mM EDTA, all from Sigma-Aldrich, at pH 8.0). Propidium iodide (Sigma-Aldrich) was dissolved in distilled water at a concentration of 5 µg/mL. Films were first dyed with SYBRGreen and then with propidium iodide, both for 20 minutes at room temperature in the dark. For SYBRGreen a white laser ($\lambda_{excitation}$ of 488 nm and $\lambda_{emission}$ of 588-531 nm) was used. For propidium iodide a white laser (λ excitation of 434 nm and $\lambda_{emission}$ of 663-759 nm) was employed. In order to detect the fluorescence, a photomultiplier was used.

2.5. Preparation of probiotic and synbiotic coatings to analyse the viability of *Lactobacillus plantarum* CECT 9567 using commercial gelatine as food model

Gelatine pieces were coated by immersion in the film-forming solutions as this allows a homogeneous and uniform covering on the foodstuff. For this purpose, commercial gelatine pieces (Reina, Postres Reina S.L., Murcia, Spain) of 10 g were dipped into the PRO and SYN film-forming solutions for 2 minutes and then left to dry in a laminar flow cabin for 10 minutes. The coated pieces of gelatine were stored in closed plastic trays at 4 ºC for further analysis. The growth of the microorganisms was followed for 15 days, by sampling at 0, 1, 5, 9, 12 and 15 days. Sampling was performed as follows: 1 g of coated gelatine was placed in a Stomacher ™ bag (Seward, UK) with 1 mL NaCl 0.7% (w/v) (Sigma-Aldrich) and was homogenized with a Stomacher ™ device (Seward, UK) at maximum speed for 120 s to break the piece down. Microbial growth was analysed by preparing serial dilutions (1:10) and incubating on MRS agar plates for 48 h at 30 ºC. Each sample was carried out in triplicate. Results were expressed in $\log_{10}$ CFU/g.

2.6. Lactobionic acid analysis under storage conditions

The concentration of LBA was measured in the edible films (PRE and SYN samples) and in pieces of gelatine coated with SYN film-forming solution by High Performance Liquid Chromatography (HPLC) (Agilent 1200, Agilent Technologies Inc., CA, USA). Sampling was performed at days 0, 1, 3, 6 and 15 for LBA in SYN edible films and at days 0, 1, 5, 9, 12 and 15 for LBA in SYN coatings. Samples were prepared as follows: 1 g of film or coated gelatine was mixed with 1 mL of distilled water, and they were broken down using a Stomacher ™ device (2 min at maximum speed). Afterwards, the samples were filtered using a syringe filter with a pore size of 0.45 µm (Whatman, Fischer Scientific, UK) and analysed by HPLC. The column used was Coregel ION 300 (Teknocroma, Barcelona, Spain) coupled to a refractive index detector (at 40 ºC). The mobile phase employed was a sulphuric acid solution (0.0450 mM/L, pH 3.1) with a flow rate of 0.3 mL/min and a column temperature of 75 ºC (Sáez-Orviz et al., 2019). Data acquisition and analysis were performed with ChemStation software (Agilent).

2.7. Simulated *in vitro* digestion of probiotic and synbiotic coated gelatine

The simulated digestion test of the gelatine coated with PRO and SYN was performed to test only the survival of the probiotic, as LBA is a non-digestible fibre resistant to human digestive enzymes (Cardoso et al., 2019). For that purpose, the concentration of microorganisms in the coatings was assessed after every digestive fluid tested and calculated per gram of gelatine. In addition, to test the survival of the free bacteria in

the digestive fluids, an MRS broth culture with a concentration of *L. plantarum* of 7 log_{10} CFU/mL was also considered (control sample).

Salivary, gastric and intestinal conditions were simulated according to Minekus et al., (2014) with some modifications. Simulated salivary fluid (SSF) was prepared by adding 1.12 g/L KCl, 0.50 g/L KH_2PO_4, 1.14 g/L $NaHCO_3$, 0.031 g/L $MgCl_2(H_2O)_6$ and 0.0058 g/L $(NH_4)_2CO_3$. The composition of the simulated gastric fluid (SGF) employed was 0.51 g/L KCl, 0.12 g/L KH_2PO_4, 2.10 g/L $NaHCO_3$, 2.76 g/L NaCl, 0.020 g/L $MgCl_2(H_2O)_6$ and 0.048 g/L $(NH_4)_2CO_3$. Simulated intestinal fluid (SIF) was prepared by mixing 0.51 g/L KCl, 0.11 g/L KH_2PO_4, 7.14 g/L $NaHCO_3$, 2.24 g/L NaCl and 0.067 g/L $MgCl_2(H_2O)_6$ (all reagents from Sigma-Aldrich).

Pieces of gelatine coated with PRO and SYN film-forming solutions, prepared as explained in Section 2.5, were tested after 5 days stored at 4ºC. For the oral simulation, $CaCl_2$ (Sigma-Aldrich) and α-amylase (1333 U/mg protein, CAS 900-90-2, Sigma-Aldrich) were mixed with 25 mL of SSF to achieve a final concentration of 0.083 g/L and 75 U/mL, respectively. 10 g of coated gelatine were added to this mixture and incubated for 3 minutes at 37°C. Then, 25 mL of SGF were added to the mixture. Porcine pepsin (2500 U/mg protein, CAS 9001-75-6, Sigma-Aldrich) and $CaCl_2$ were also added to this solution until respective concentrations of 2000 U/mL and 0.0083 g/L in the final volume were reached. Afterwards, the mixture was well homogenized, the pH was adjusted to 2.0 with HCl 5 M (Sigma-Aldrich), and incubated at 37 ºC for 2 h. Finally, 50 mL of SIF was added with previously solubilised porcine pancreatin (0.1% (w/v), CAS 9049-47-6, Sigma-Aldrich), bovine chymotrypsin (3% (w/v), CAS 9004-07-3, Sigma-Aldrich) and $CaCl_2$ (0.033 g/L). The mixture was well homogenized, the pH adjusted to 6.5 with NaOH 5 M (Sigma-Aldrich), and incubated at 37 ºC for 2 h.

The bacterial survival was assessed by taking samples after the following times: one after the simulated salivary phase and one after each hour of the simulated gastric and intestinal phases (5 samples in total for the entire digestive simulation). For the sampling, 1 g of gelatine was collected and homogenized with 1 mL NaCl (0.7%) using a Stomacher™ device (2 min at maximum speed). Serial dilutions (1:10) were incubated on MRS agar plates (30 ºC for 48 h). Regarding the control, 1 mL of liquid was collected after the simulated salivary, gastric and intestinal digestion and was centrifugated (10000 rpm, 10 min). Afterwards, the pellet was resuspended in 1 mL of NaCl 0.7% (w/v) and incubated in MRS agar plates for 48 h at 30 ºC. Each sample was carried out in triplicate. Results were expressed in log_{10} CFU/g of gelatine (CFU/mL in the case of control).

2.8. Statistical analysis

Analysis of variance (ANOVA) was carried out. Fischer's Least Significant Difference (LSD) was used to determine significant differences between the data. A level of $p < 0.05$ was considered significant. Analysis was performed using Statgraphics 18® Centurion statistical software.

3. Results and discussion

3.1. Film characterization

3.1.1. Thickness and mechanical properties

The thickness of edible films ranged from 127.8 to 155.3 µm (Table 4.10), and significant differences were detected between the PRE and SYN samples on the one hand and the Control and PRO samples. Therefore, the addition of LBA affected the thickness of the films, but the presence of the probiotic did not.

Table 4.10. Puncture strength (PS), puncture deformation (PD), thickness, water solubility (WS) and transparency index of the four edible film samples (Control, PRE, PRO and SYN). Different letters in the same column indicate significant differences ($p < 0.05$).

	PS (N/mm)	PD (%)	Thickness (µm)	WS (%)	Transparency index
Control	88.2 ± 9.9 ^{a}	34.5 ± 3.1 ^{a}	133.2 ± 8.7 ^{a}	19.0 ± 1.8 ^{a}	0.32 ± 0.04 ^{a}
PRE	62.8 ± 4.0 ^{b}	30.9 ± 5.8 ^{a}	155.3 ± 8.1 ^{b}	43.1 ± 4.9 ^{b}	0.62 ± 0.13 ^{a}
PRO	78.5 ± 5.7 ^{a}	25.2 ± 3.9 ^{b}	127.8 ± 7.6 ^{a}	16.1 ± 5.8 ^{a}	0.42 ± 0.05 ^{a}
SYN	50.7 ± 4.9 ^{b}	39.0 ± 0.1 ^{a}	151.2 ± 9.2 ^{b}	38.5 ± 2.6 ^{b}	1.90 ± 0.08 ^{b}

Regarding mechanical properties, the presence of LBA affected PS values, as PRE and SYN edible films showed values lower than did Control and PRO films (Table 4.10), with significant differences ($p < 0.05$). Therefore, the presence of LBA in the edible films made them less resistant. LBA molecules are very small, have a low molecular weight and are hygroscopic, so the inclusion of LBA in the film matrix produced thicker films, with more water retained inside, and with fewer protein-protein interactions, resulting in mechanically weaker materials compared with those prepared without LBA. Other authors have observed similar changes in the strength values of protein films when prebiotics were added, compared with the non-prebiotic films. Fernandes et al. (2020b) observed a decrease in strength values in protein whey films when galacto-oligosaccharides and xylo-oligosaccharides were added as prebiotics at a concentration of 20 g of prebiotic/g film or higher. In this case, the decrease in strength values was due to the branched structure of the oligosaccharides and the presence of

hydroxyl molecules that caused complex interactions between the prebiotics and film proteins, which made these structures more flexible.

In the case of PD, the only significantly different results were found in the PRO samples, which had the lowest value for this parameter. This may be because the PRE and SYN films contained LBA, which is a very hygroscopic molecule that may hinder a possible desiccant effect of the bacteria. In addition, bearing in mind that plasticizers have the capacity to extensively modify the mechanical properties of the protein-based films (Vieira, Da Silva, Dos Santos, & Beppu, 2011), LBA has properties that might lead to it being considered as a plasticizer in its own right, since it has a similar molecular weight to, and a higher polarity than sucrose, which has also shown some plasticizer activity in other protein-based films (Wan, Moon, & Lee, 2005). In fact, PRE films only differ from control films in the presence of LBA in their composition, the former having lower PS values than the latter, which may be caused by the plasticizer effect of the LBA augmenting that already produced by the glycerol.

3.1.2. Water solubility

Delipidated egg yolk protein edible films showed low WS (Table 4.10), the value being less than 50% in all samples. This is because egg yolk granular proteins are only soluble in water at high ionic strength (> 0.3 M) or at high pH, so, once the protein network is already established in the dried film, they would be expected to be quite insoluble in distilled water (Marcet et al., 2017). Even with the addition of LBA, the films are less soluble than common films such as gelatine (Pellá et al., 2020), sodium caseinate (Marcet et al., 2017) and whey protein films (Ozdemir & Floros, 2008). High ionic strength or pH conditions are uncommon in the food industry, so these edible films could be excellent candidates for covering a variety of foodstuffs for long periods of time. The addition of LBA to the PRE and SYN samples produced an increase in the film solubility values, likely due to the high solubility and hygroscopicity of LBA (Alonso et al., 2013).

3.1.3. Light transmittance and transparency

The light transmittance of the delipidated egg yolk edible films was measured over the wavelength range between 200 and 800 nm (Figure 4.13).

For UVC (100-280 nm) and UVB (280-320 nm) regions, the transmittance of the four samples was very low. In the UVA (320-400 nm) range, there was an increase in the optical transmittance in Control, PRE and PRO samples but SYN edible film showed the lowest values at all wavelengths tested. A good property for this kind of packaging materials is to permit only low light transmission in the UV range, since 98% of the UV

radiation that reaches the earth falls within the 315 to 400 nm range (Criado, Fraschini, Salmieri, & Lacroix, 2020), this radiation being an important starter for the oxidative degradation of foodstuffs with high lipid content (Marcet et al., 2017). In this sense, protein-based films have a high capacity to prevent UV damage due to the natural presence of aromatic amino acids in their composition, which are able to absorb the UV light. Regarding the wavelengths in the visible region, Control, PRE and PRO films showed good properties for food packaging, since they are transparent enough to facilitate the inspection of the product covered. This was confirmed by the transparency index (Table 2), which was very low for all of them. In the case of the SYN edible films, the light transmission values at these wavelengths were low compared with those values for the other samples, reaching a maximum of 63.32% at 800 nm. The relatively high opacity of the SYN samples at every wavelength tested may be due to an increase in the microbial load contained inside these films, which could be produced by the prebiotic effect of the LBA on *L. plantarum*. Therefore, this higher microbial presence in the SYN films resulted in a higher light dispersion capacity than in the samples loaded only with probiotics (PRO).

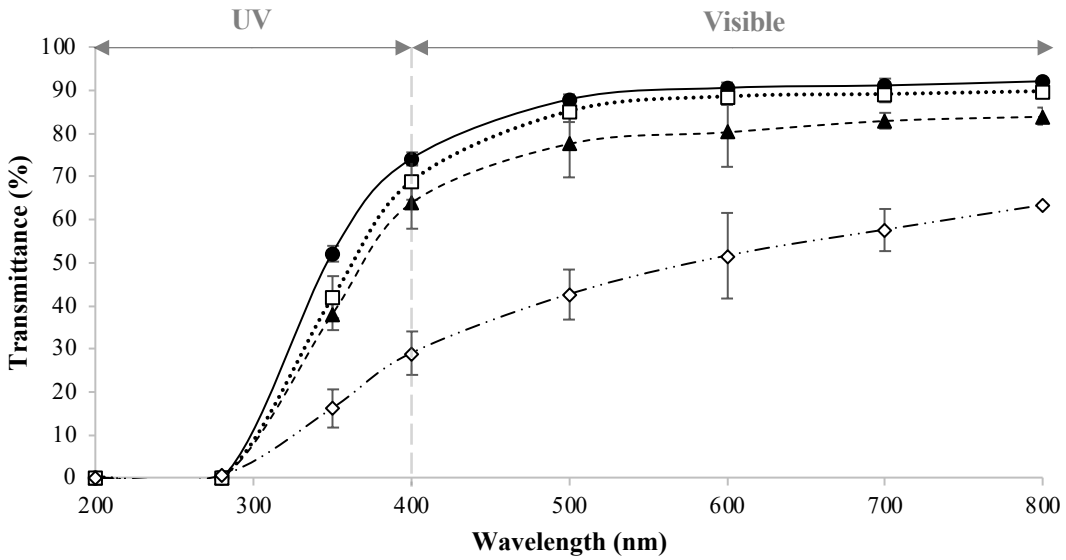


Figure 4.13. Light transmittance (%) of the edible films at different wavelengths (200-800 nm). (●) Control, (▲) PRE, (□) PRO and (◊) SYN. Significant differences were only observed in SYN films ($p < 0.05$).

3.1.4. Colour properties

Together with the transparency of the edible film, the packaging colour properties are quite important because they affect the appearance of the food products, and they exert an influence on the consumers' acceptance of the products. The colour properties of the delipidated egg yolk protein edible films are shown in Table 4.11. There were no

significant differences ($p > 0.05$) between Control, PRE, PRO and SYN edible films in any of the parameters measured (L^*, a^*, b^*, ΔE and *YI*). The visual appearance of the films was similar in the four cases (Figure 4.14). Films were transparent, homogeneous and slightly yellowish. This yellowish appearance of the films was quantified by calculating the YI parameter, and it could be produced by the presence of fat-soluble carotenoids, such as β-carotene, which are soluble in solvents like hexane (Kovalcuks & Duma, 2016) and are not completely removed during the lipid extraction with ethanol (Li-Chan, E.C.Y & Kim, 2008).

Table 4.11. Colour of the four film samples (Control, PRE, PRO and SYN). L^* represented lightness/brightness, a^* redness/greenness and b^* yellow/blueness. ΔE represented the total colour difference. *YI* represented the yellowness index. Different letters in the same column indicate significant differences ($p < 0.05$).

	L*	a*	b*	ΔE	YI
Control	94.3 ± 0.1 a	-1.05 ± 0.2 a	11.5 ± 0.5 a	11.8 a	17.4 a
PRE	93.5 ± 0.6 a	-0.9 ± 0.5 a	11.1 ± 0.6 a	11.6 a	16.9 a
PRO	94.2 ± 0.5 a	-1.4 ± 0.3 a	11.7 ± 0.3 a	12.0 a	17.7 a
SYN	93.6 ± 0.5 a	-1.24 ± 0.3 a	11.6 ± 0.9 a	12.1 a	17.7 a

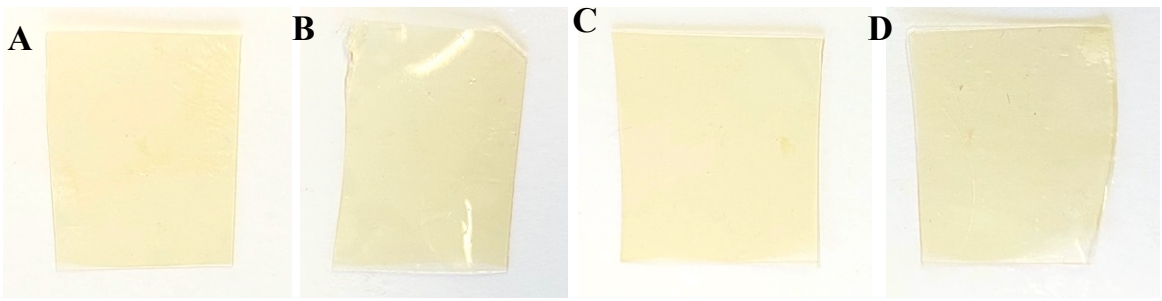


Figure 4.14. Visual appearance of the edible films. (A) Control, (B) PRE, (C) PRO and (D) SYN edible films.

3.1.5. Scanning electron microscopy (SEM)

The surface microstructure of all tested films was completely smooth, homogeneous, without pores and with no difference between them (data not shown). Micrographs of the film cross-section are shown in Figure 4.15. The cross-sections of the films showed a scaly and fibrous appearance, with a lot of protein agglomeration throughout the film matrix for every film tested. Therefore, the incorporation of LBA and *L. plantarum* did not produce any noticeable change in the film microstructure.

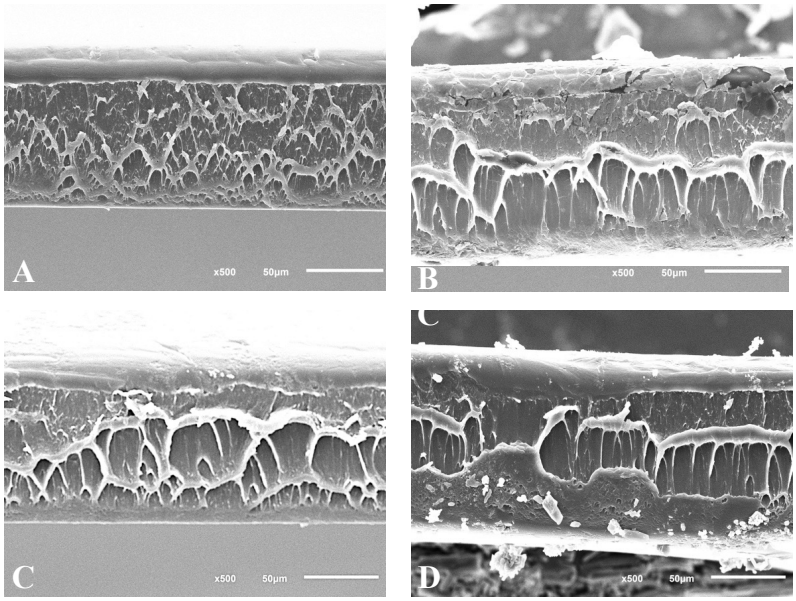


Figure 4.15. SEM images of the edible film cross-sections (500x). (A) Control, (B) PRE, (C) PRO and (D) SYN samples. Scale bars correspond to 50 µm.

3.2. Viability of *Lactobacillus plantarum* CECT 9567 inside films during storage

In order to identify the viability of *L. plantarum* at different depths, pieces of the films were stained with two different fluorochromes and a confocal microscope was employed to determine the metabolic state of the probiotic inside PRO and SYN edible films after 7 days of storage at 4 ºC.

The information provided by the confocal microscope about the PRO and SYN edible films (Figure 4.16 A and B) showed that the concentration of probiotic was higher for the SYN film than for the PRO film, but microorganisms were mostly dead, since red was the predominant colour in both cases. However, a microscopic analysis of the same film fragment was carried out to see how the depth within the film affects microbial growth. Images numbered from 0 to 7 were taken at different depths from the surface (0) to the innermost point of the film (7). As could be seen, in the most superficial layers the microbial load was higher, but the probiotic microorganisms were almost all dead (Figure 4.16-0A to 3A and 0B to 3B). As deeper layers of the film were analysed, it was observed that the microbial concentration decreased but the vast majority were alive, as they were seen to have been dyed green (Figure 4.16-4A to 7A and 4.16B to 7B). Therefore, it was observed that the deeper inside the film, the greater was the survival of the probiotic. *L. plantarum* is an aerobic microorganism but oxygen transmission is likely to be good, as the films were very thin (between 127.8 and 155.3 µm) and, as Figure 4.16 shows, it did not affect the growth at the deepest levels. Thus, this greater viability at the deepest levels may be due to the fact that inside the film the microorganisms are better protected during the film drying step. In addition, another

difference observed was that the presence of LBA had a protective effect, since more viable bacteria were observed in the deepest layers of the SYN film (Figure 4.16-5A to 7A) than were observed in the same layers of the PRO film. Furthermore, it was observed that the microorganisms in SYN films were in a state of aggregation, while in PRO films they were more dispersed (Figure 4.16-6A and 6B). This phenomenon may be due to the presence of LBA, since the influence of prebiotics on the aggregation of probiotic microorganisms has been evaluated by some authors. Ouldchikh et al. (2020) showed that the presence of prebiotics increased the aggregation capacity of the probiotic *Lactobacillus salivarius* significantly ($p < 0.05$). Another study (Saran, Bisht, Singh, & Teotia, 2012) showed that the presence of inulin increased both the ability to autoaggregate and the cell surface hydrophobicity of *Lactobacillus acidophilus* NCDC 13 and *L. acidophilus* NCD 291. Therefore, the presence of LBA in the edible films could improve the aggregation ability of the probiotic which is related to adhesion and is essential for colonisation of the low gastrointestinal tract (GIT).

The variation in the LBA concentration over 15 days in SYN edible films is seen in Figure 4.17, which shows that there was a noticeable decrease in the concentration of LBA from the beginning of the film storage, which implies that *L. plantarum* had been consuming the prebiotic. Therefore, LBA promoted the viability of the probiotic over time, reinforcing the results observed in Figure 4.17. The concentration of LBA decreased from 187.44 mg LBA/g at day 0 to 136.17 mg LBA/g in SYN edible film. Despite the fact that the existing regulations do not establish a concentration range for consumption (*FDA. Code of Federal Regulations, Title 21, 21 CFR 172.720. US Food and Drug Administration*, 2017), the concentrations used in this work are far lower than the values that would cause lactose intolerance-like effects (24 g LBA/day) (Cardoso et al., 2019).

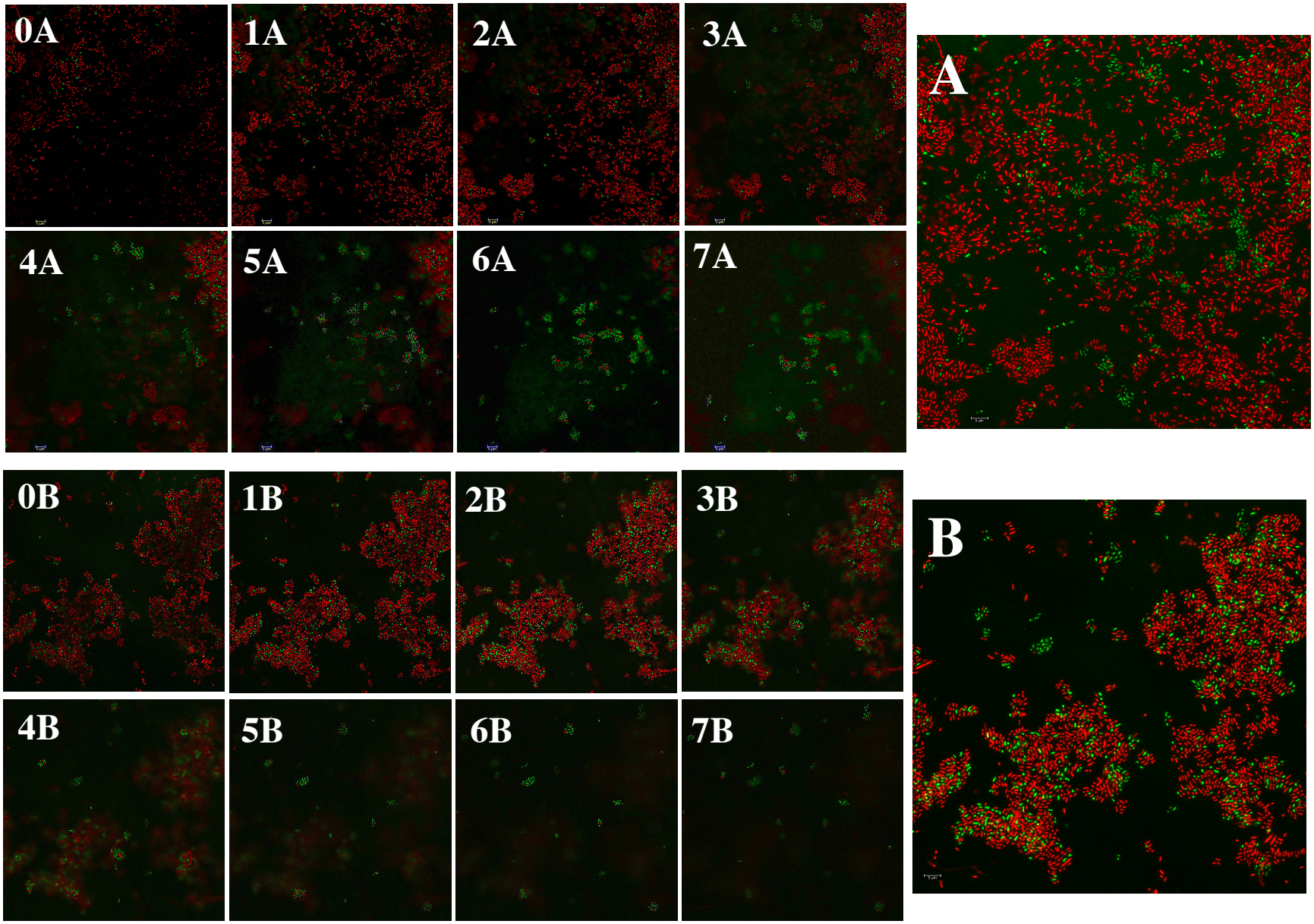


Figure 4.16. Confocal laser microscopy images of the edible films after 7 days of storage at 4ºC (100x). Green-stained cells are alive, red ones are dead and orange ones are alive but damaged. (A) PRO edible film and (B) SYN edible film. Numbers from 0 to 7 refer to the layers of the film. 0 corresponds to the surface and 7 is the innermost point of depth. (A) and (B) photos are the collection of the previous eight.

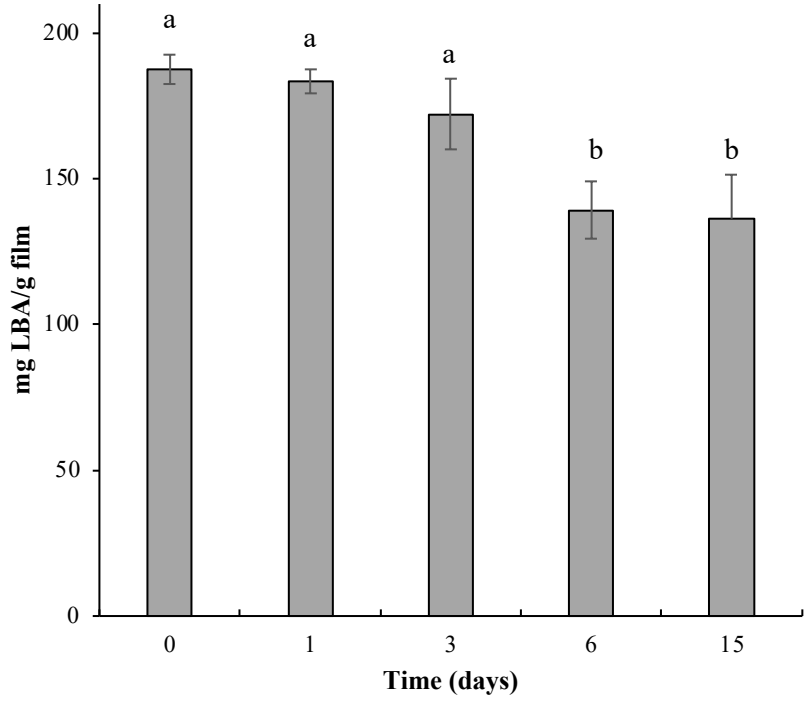


Figure 4.17. Variation of LBA in SYN edible film over 15 days of storage. LBA concentration is represented as mg of LBA per g of film. Experiments were carried out in triplicate and reported results correspond to the mean value. Different letters indicate significant differences ($p < 0.05$).

3.3. Growth and viability of *Lactobacillus plantarum* CECT 9567 in the bioactive coating during storage

The above experiments were performed by drying the film-forming solution and characterising the resulting sheet of protein film. In this way, it is possible to assess the physical properties of any novel edible film and to compare the results obtained with others found in the literature. However, the usual way to apply these kinds of films on foodstuff is by immersion of the piece of food in the film-forming solution and subsequently drying the coating at room temperature. In general, the products to be covered are fresh, such as cheese, cottage cheese, vegetables or fruit (Suhag, Kumar, Petkoska, & Upadhyay, 2020) and, in these cases, one of the best methods to achieve a homogeneous and uniform covering is the use of coatings (Lu, Ding, Ye, & Liu, 2010) (Zhong, Cavender, & Zhao, 2014), dipping being the process most commonly used on the laboratory scale (Suhag et al., 2020). With the aim of obtaining a good degree of covering and a regular surface, and in order to assess the growth and viability of *L. plantarum* in a real application scenario, pieces of commercial gelatine were dipped into

the PRO and SYN film-forming solutions. The probiotic growth inside the coatings is shown in Figure 4.18.

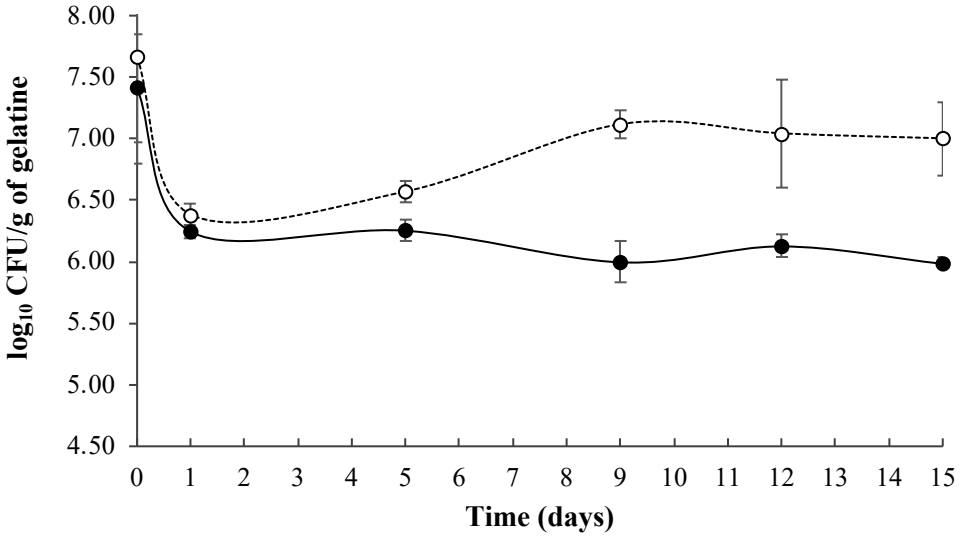


Figure 4.18. Growth of *L. plantarum* CECT 9567 in gelatine coated with (●) PRO and (○) SYN film-forming solutions over 15 days of storage. The amount of microorganism is represented as log10 CFU per g of gelatine. There are only significant differences between PRO and SYN coatings from day 9 to 15. Experiments were carried out in triplicate and reported results correspond to the mean value.

For PRO and SYN coatings, there was a very rapid decrease in the probiotic concentration between the initial time and the first day, with the reduction of one logarithmic unit in both cases. This may be due to the abrupt change in the conditions when the coated gelatine pieces were transferred from the aeration process in a laminar flow hood at 30 ºC to a fridge for storage in at 4ºC. After this initial decrease in the microbial load, the concentration remained constant for 15 days in both cases. In order to develop a functional product, the number of viable bacteria is crucial, as the product can only obtain the category of probiotic if the bacterial concentration is adequate. Although there is no scientific consensus about the probiotic concentration necessary to exert beneficial effects, many authors estimate that the minimum quantity is 6 $\log_{10}$ CFU/g or CFU/mL of food product (Angiolillo, Conte, Faccia, Zambrini, & Nobile, 2014) (Aureli et al., 2011) (Kechagia et al., 2013) (Pavli et al., 2017). On the last day of storage, the concentration was 6 $\log_{10}$ CFU/g and 7 $\log_{10}$ CFU/g for PRO and SYN coated gelatine, respectively. Therefore, the concentration reached during the storage period analysed was adequate, and both coatings would obtain the category of "probiotic". In addition, the probiotic concentration was higher in SYN than in PRO coated gelatine,

with significant differences ($p < 0.05$). As was observed in the case of edible films (Section 3.2), the presence of LBA had a positive effect on the viability of *L. plantarum*. The observed increase in the concentration of *L. plantarum* in SYN coatings can be explained by assessing the evolution of the LBA content inside the coatings during storage time (Figure 4.19). There was a noticeable decrease in the concentration of LBA from day 5 which implies that the probiotic had been consuming the prebiotic and its growth has been enhanced. As described in Section 3.2, the values of LBA are adequate and far lower than the levels that might lead to lactose intolerance-like effects (Cardoso et al., 2019). Recent studies by other authors have observed similar results when probiotics are mixed with prebiotics in different kinds of coatings. In the case of Orozco-Parra *et al.* (Orozco-Parra, Mejía, & Villa, 2020), inulin helped *Lactobacillus casei* strain R4603008 preservation and viability during storage (10ºC). Bambace, Alvarez, & Moreira (2019) also found that the viability of *Lactobacillus rhamnosus* CECT 8361 in their coating was improved during cold storage by the use of fructo-oligosaccharides. No studies have been found in which egg yolk protein is used as food packaging by itself or when mixed with prebiotic or probiotic in order to develop bioactive coatings or films, which highlights the novelty of this work.

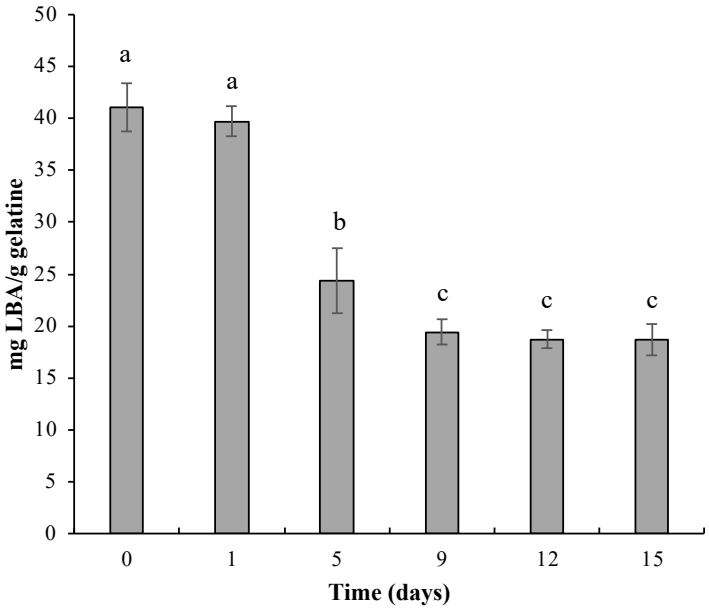


Figure 4.19. Variation of LBA concentration in the SYN coated gelatine over 15 days of storage. Experiments were carried out in triplicate and reported results correspond to the mean value. Different letters indicate significant differences ($p < 0.05$).

3.4. Simulated *in vitro* digestion of PRO and SYN coatings

In order to be effective, probiotics must reach the large intestine, after first withstanding acidic conditions and stomach gastric juices. With the purpose of testing the protective

capacity of the delipidated egg yolk coatings and the effect of the LBA on *L. plantarum* survival, gelatine pieces coated with the PRO and SYN film-forming solutions were analysed in a simulated digestion assay. As control, *L. plantarum* without coating (7 log_{10} CFU/mL) was employed. Results are shown in Figure 4.20.

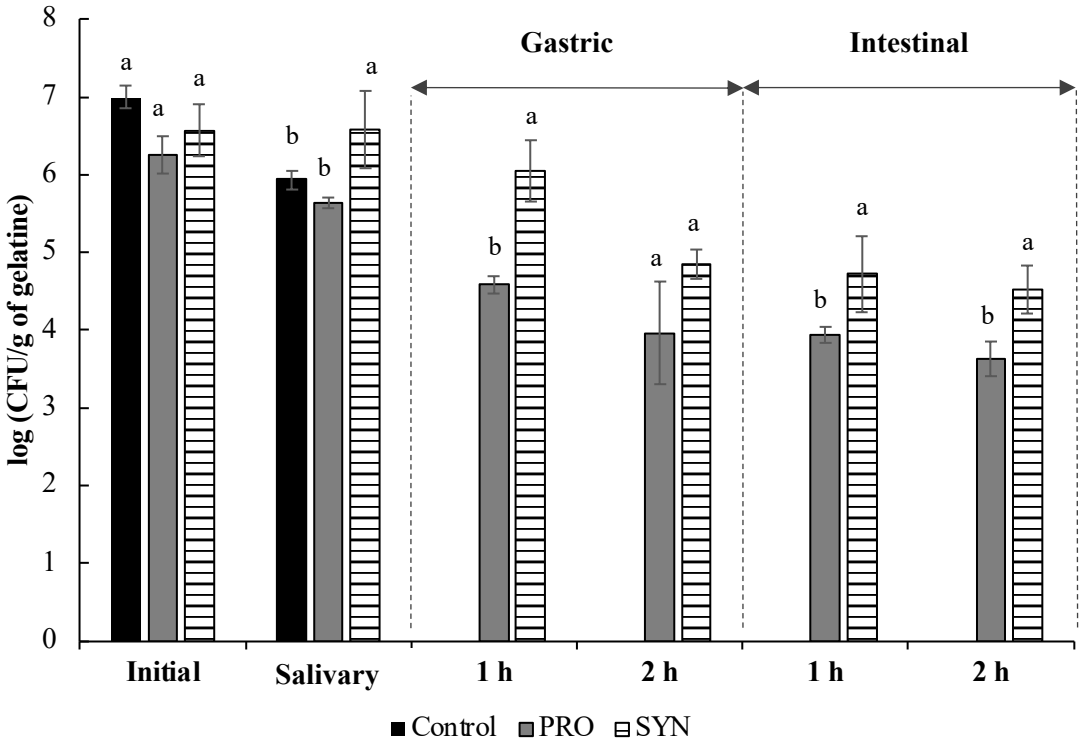


Figure 4.20. Variation of the concentration of probiotic (log_{10} CFU/g of gelatine in PRO and SYN samples and log_{10} CFU/mL in the case of control sample) throughout the in vitro digestion test. "Initial" refers the concentration of microorganisms before the digestion trial. Experiments were carried out in triplicate and reported results correspond to the mean value. Different letters indicate significant differences ($p < 0.05$).

At initial time, the probiotic load was similar in the three cases. After the simulated salivary phase, there was a reduction in the number of bacteria in all samples except in the SYN sample (Figure 4.20-Salivary). This reduction may be due to the change in environmental conditions, the presence of different salts and α-amylase activity. After the first hour of the simulated gastric step, no survival of the microorganism without coating was observed (Figure 4.20- Gastric-1h). It is known that the presence of pepsin as well as the long exposure to an acid pH can cause the death of the bacteria (Chan & Zhang, 2005) (Melchior, Marino, Innocente, Calligaris, & Nicoli, 2020). However, bacteria inside PRO and SYN coatings did survive. After the simulated gastric phase, the microbial reduction was similar for both samples (1.67 ± 0.4 and 1.73 ± 0.2 log_{10} CFU/g of gelatine for PRO and SYN samples, respectively). Therefore, the delipidated egg yolk

protein coatings exerted a protective effect against pepsin activity and the acidic gastric environment.

After the simulated gastric phase, there was barely any microbial reduction in the simulated intestinal phase (0.34 ± 0.1 and 0.32 ± 0.1 log_{10} CFU/g of gelatine for PRO and SYN samples, respectively). However, the final concentrations in the two samples were different, with mean values of 3.63 ± 0.22 and 4.52 ± 0.3 log_{10} CFU/g of PRO and SYN coated gelatine, respectively. Thus, viability was higher in SYN coated gelatine. Although the initial concentrations of the PRO and SYN samples were different, the analysis of the microbial reduction showed that the logarithmic microbial decrease was similar throughout all the simulated *in vitro* digestion, except in the simulated salivary phase. In this first phase, there was no microbial reduction for SYN samples while for PRO samples there was a reduction of 0.63 ± 0.1 log_{10} CFU/g of gelatine. Thus, the simulated salivary phase would be the only one in which the LBA showed a protective effect, although in other studies, prebiotics provided protection to the probiotic microorganism during all phases of simulated *in vitro* digestion (Lopez-Rubio et al., 2006) (Orozco-Parra et al., 2020) (Langa et al., 2019).

Coatings can be used to cover different types of food, both dairy (such as cheese or cottage cheese) and non-dairy (such as vegetables or fruit) products (Parreidt, Müller, & Schmid, 2018). In this case and with the aim of simplifying the experimentation, commercial gelatine pieces were chosen for coating, but any of the food products mentioned above could also have been used. For probiotics to be effective, they must resist the conditions in the digestive tract in order to reach the lower GIT. Although there is no scientific consensus about the concentration required for health benefits, several authors agree that the minimum dose would be between 6 to 9 log_{10} CFU per day (Espitia et al., 2016) (Saad, Delattre, Urdaci, Schmitter, & Bressollier, 2013). In the case of gelatine, a commercial individual portion has a weight of 100 g. Taking into account the results of the simulated digestion and a 100 g portion, the PRO coatings would have a final value of 5.6 ± 0.2 log_{10} CFU/portion and the SYN coatings of 6.5 ± 0.3 log_{10} CFU/portion. Therefore, although the values are very close, only SYN coatings would be considered as probiotics after the simulated *in vitro* digestion, as they are within the established range. Regarding LBA, it was not measured in the digestion test, as it is a prebiotic, it is a non-digestible fibre, so is resistant to human digestive enzymes and, in consequence, it reaches the large intestine intact (Sáez-Orviz et al., 2019) (Cardoso et al., 2019). As can be seen from the results obtained in sections 3.3 and 3.4, PRO and SYN coatings would meet the minimum legal requirements to attain the “probiotic”

category but only SYN coatings would also have the capacity to deliver the adequate probiotic dose to the lower GIT.

4. Conclusions

Bioactive delipidated egg yolk food packaging materials with LBA as prebiotic and *L. plantarum* CECT 9567 as probiotic were developed and characterised. Control, PRE, PRO and SYN edible films showed adequate mechanical properties, although PS was influenced by the presence of LBA and PD by the presence of the probiotic microorganism. These films showed low water solubility values, so they are optimal to cover food products, which are usually high in moisture content. The films were transparent, homogenous and only slightly yellowish, as the b^* parameter showed. With respect to the bioactive properties, the SYN formulation was found to be the best option, mainly due to the influence of the LBA on *L. plantarum* growth. In this case, the results obtained suggest that the LBA enhances the growth of the bacteria inside the film and coating during the storage period, which ensures the microorganism is present in numbers high enough for it to be considered "probiotic" when it is used to coat a piece of gelatine. However, the LBA only exerted a positive effect on microorganism survival when the coated pieces of gelatine were subjected under *in vitro* GIT conditions to the simulated salivary phase. Nevertheless, as LBA had no influence in the other phases of the simulated *in vitro* digestion, the effect of the LBA might be also achieved through adapting the formula of the PRO film-forming solution by simply increasing the initial concentration of *L. plantarum*, although this assumption has to be corroborated and, in this case, the beneficial properties of the LBA on the intestinal flora would also be lost.

Acknowledgements

This work was financially supported by the Principality of Asturias, by the project GRUPIN IDI/2018/000127 and by the grant *Programa Severo Ochoa de Ayudas Predoctorales para la investigación y docencia* (grant number BP19-127 to Sáez-Orviz, S.). Marta Alonso is gratefully acknowledged for her technical assistance (Photonic Microscopy and Image Processing Area, Scientific-Technical Services, University of Oviedo).

5. References

Alonso, S., Rendueles, M., & Díaz, M. (2013). Bio-production of lactobionic acid: Current status, applications and future prospects. *Biotechnology Advances*, *31*(8), 1275–1291. https://doi.org/10.1016/j.biotechadv.2013.04.010

Angiolillo, L., Conte, A., Faccia, M., Zambrini, A. V, & Nobile, M. A. Del. (2014). A new method to produce synbiotic Fiordilatte cheese. *Innovative Food Science and Emerging Technologies*, *22*, 180–187. https://doi.org/10.1016/j.ifset.2013.09.010

Aureli, P., Capurso, L., Castellazzi, A. M., Clerici, M., Giovannini, M., Morelli, L., Poli, A., Pregliasco F., Salvini F. & Zuccotti, G. V. (2011). Probiotics and health: An evidence-based review. *Pharmacological Research*, *63*(5), 366–376. https://doi.org/10.1016/j.phrs.2011.02.006

Bambace, M. F., Alvarez, M. V., & Moreira, M. del R. (2019). Novel functional blueberries: Fructo-oligosaccharides and probiotic lactobacilli incorporated into alginate edible coatings. *Food Research International*, *122*(December 2018), 653–660. https://doi.org/10.1016/j.foodres.2019.01.040

Blanco-Pascual, N., Montero, M. P., & Gómez-Guillén, M. C. (2014). Antioxidant film development from unrefined extracts of brown seaweeds Laminaria digitata and Ascophyllum nodosum. *Food Hydrocolloids*, *37*, 100–110. https://doi.org/10.1016/j.foodhyd.2013.10.021

Cardoso, T., Marques, C., Dagostin, J. L. A., & Masson, M. L. (2019). Lactobionic Acid as a Potential Food Ingredient: Recent Studies and Applications. *Journal of Food Science*, *84*, 1672–1681. https://doi.org/10.1111/1750-3841.14686

Chan, E. S., & Zhang, Z. (2005). Bioencapsulation by compression coating of probiotic bacteria for their protection in an acidic medium. *Process Biochemistry*, *40*(10), 3346–3351. https://doi.org/10.1016/j.procbio.2005.03.001

Criado, P., Fraschini, C., Salmieri, S., & Lacroix, M. (2020). Cellulose nanocrystals (CNCs) loaded alginate films against lipid oxidation of chicken breast. *Food Research International*, *132*(October 2019), 109110. https://doi.org/10.1016/j.foodres.2020.109110

Espitia, P. J. P., Batista, R. A., Azeredo, H. M. C., & Otoni, C. G. (2016). Probiotics and their potential applications in active edible films and coatings. *Food Research International*, *90*, 42–52. https://doi.org/10.1016/j.foodres.2016.10.026

FDA. Code of Federal Regulations, Title 21, 21 CFR 172.720. US Food and Drug Administration. (2017). Retrieved from https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=172.720

Fernandes, Leonardo M. Guimaraes Jonas T., Pimentel Tatiana C., Esmerino Erick A., Freitas, M. Q., Carvalho C. W. P., Cruz A. G. and Marcia. C.S. (2020a). Chapter 7. Edible whey protein films and coatings added with prebiotic ingredients. In *Agri-Food Industry Sytrategies for Healthy Diets and sustainability* (pp. 177–193). https://doi.org/https://doi.org/10.1016/B978-0-12-817226-1.00007-2

Fernandes, L. M., Guimarães, J. T., Silva, R., Rocha, R. S., Coutinho, N. M., Balthazar, C. F., Rodrigo, N. C., Piler, C. W., Pimentel, T. C., Neto P. C. N., Tavares, M. I. B., Esmerino, E. A., Freitas, M. Q., Marcia, C. S. & Cruz, A. G. (2020b). Whey protein films added with

galactooligosaccharide and xylooligosaccharide. *Food Hydrocolloids*, *104*(February). https://doi.org/10.1016/j.foodhyd.2020.105755

Francis F. J. & Clydesdale F. M. (1977). *Food Colorimetry: Theroy and Applications*. Retrieved from https://doi.org/10.1002/food.19770210122

Froning, G. W. (2007). Egg Products Industry and Future Perspectives. *Egg Bioscience and Biotechnology*, 307–325. https://doi.org/10.1002/9780470181249.ch8

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. https://doi.org/10.1111/1471-0307.12558

Kechagia, M., Basoulis, D., Konstantopoulou, S., Dimitriadi, D., Gyftopoulou, K., Skarmoutsou, N., & Fakiri, E. M. (2013). Health Benefits of Probiotics: A Review. *ISRN Nutrition*, *2013*, 1–7. https://doi.org/10.5402/2013/481651

Kovalcuks, A., & Duma, M. (2016). Distribution of Phospholipids, Cholesterol and Carotenoids in Two-Solvent System during Egg Yolk Oil Solvent Extraction. *World Academy of Science, Engineering and Technology, International Journal of Biological, Biomolecular, Agricultural, Food and Biotechnological Engineering*, *10*(5), 323–328.

Laca, A., Paredes, B., & Díaz, M. (2010). A method of egg yolk fractionation. Characterization of fractions. *Food Hydrocolloids*, *24*(4), 434–443. https://doi.org/10.1016/j.foodhyd.2009.11.010

Langa, S., van den Bulck, E., Peirotén, A., Gaya, P., Schols, H. A., & Arqués, J. L. (2019). Application of lactobacilli and prebiotic oligosaccharides for the development of a synbiotic semi-hard cheese. *Lwt*, *114*(April), 108361. https://doi.org/10.1016/j.lwt.2019.108361

Li-Chan, E.C.Y and Kim, H. . (2008). Composition of Eggs. *In Egg Bioscience and Biotechnology*, 1–95. Retrieved from https://doi.org/10.1016/j.plefa.2018.06.003%0Ahttp://www.journals.cambridge.org/abstract_S0007114504001102%0Ahttps://doi.org/10.1016/j.plefa.2018.07.010%0Ahttps://academic.oup.com/jn/article/139/1/5/4750894%0Ahttp://www.nap.edu/catalog/4758%0Ahttps://en.e

Lopez-Rubio, A., Gavara, R., & Lagaron, J. M. (2006). Bioactive packaging: turning foods into healthier foods through biomaterials. *Trends in Food Science and Technology*, *17*(10), 567–575. https://doi.org/10.1016/j.tifs.2006.04.012

Lu, F., Ding, Y., Ye, X., & Liu, D. (2010). Cinnamon and nisin in alginate-calcium coating maintain quality of fresh northern snakehead fish fillets. *LWT - Food Science and Technology*, *43*(9), 1331–1335. https://doi.org/10.1016/j.lwt.2010.05.003

Marcet, I., Álvarez, C., Paredes, B., & Díaz, M. (2014). Inert and Oxidative Subcritical Water Hydrolysis of Insoluble Egg Yolk Granular Protein, Functional Properties, and Comparison to Enzymatic Hydrolysis. *Journal of Agricultural and Food Chemistry*, *62*(32), 8179–8186.

Marcet, I., Sáez, S., Rendueles, M., & Díaz, M. (2017). Edible films from residual delipidated egg yolk proteins. *Journal of Food Science and Technology*, *54*(12), 3969–3978. https://doi.org/10.1007/s13197-017-2861-8

Melchior, S., Marino, M., Innocente, N., Calligaris, S., & Nicoli, M. C. (2020). Effect of different biopolymer-based structured systems on the survival of probiotic strains during storage and in vitro digestion. *Journal of the Science of Food and Agriculture*, *100*(10), 3902–3909. https://doi.org/10.1002/jsfa.10432

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carrière, F., Boutrou, R., Corredig, M., Dupont, D., Dufour, C., Egger L., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A., Marze, S., McClements, D.J., Ménard, O., Recio, I., Santos, C.N., Singh, R.P., Vegarud, G. E., Wickham, M.S.J., Weiteschies, W. & Brodkorb, A. (2014). A standardised static in vitro digestion method suitable for food-an international consensus. *Food and Function*, *5*(6), 1113–1124. https://doi.org/10.1039/c3fo60702j

Orozco-Parra, J., Mejía, C. M., & Villa, C. C. (2020). Development of a bioactive synbiotic edible film based on cassava starch, inulin, and Lactobacillus casei. *Food Hydrocolloids*, *104*(January), 105754. https://doi.org/10.1016/j.foodhyd.2020.105754

Otero-Pazos, P., Sendón, R., Blanco-Fernandez, B., Blanco-Dorado, S., Alvarez-Lorenzo, C., Concheiro, A., Angulo I., Paseiro-Losada P. & Rodríguez-Bernaldo de Quirós, A. (2016). Preparation of antioxidant active films based on chitosan: diffusivity study of α-tocopherol into food simulants. *Journal of Food Science and Technology*, *53*(6), 2817–2826. https://doi.org/10.1007/s13197-016-2256-2

Ouldchikh, S., TirTouil, A., & Meddah, B. (2020). Zizyphus lotus (L.) extracts as prebiotics in the aggregation and adhesion of probiotic and inhibition of pathologic bacteria from patients with colorectal cancer. *International Journal of Food Studies*, *9*(1), 160–177. https://doi.org/10.7455/IJFS/9.1.2020.A3

Ozdemir, M., & Floros, J. D. (2008). Optimization of edible whey protein films containing preservatives for water vapor permeability, water solubility and sensory characteristics. *Journal of Food Engineering*, *86*(2), 215–224. https://doi.org/10.1016/j.jfoodeng.2007.09.028

Parreidt, T. S., Müller, K., & Schmid, M. (2018). Alginate-based edible films and coatings for food packaging applications. *Foods*, *7*(10), 1–38. https://doi.org/10.3390/foods7100170

Pavli, F., Kovaiou, I., Apostolakopoulou, G., Kapetanakou, A., Skandamis, P., Nychas, G. J. E., Tassou C. & Chorianopoulos, N. (2017). Alginate-based edible films delivering probiotic bacteria to sliced ham pretreated with high pressure processing. *International Journal of Molecular Sciences*, *18*(9). https://doi.org/10.3390/ijms18091867

Pellá, M. C. G., Silva, O. A., Pellá, M. G., Beneton, A. G., Caetano, J., Simões, M. R., & Dragunski, D. C. (2020). Effect of gelatin and casein additions on starch edible biodegradable films for

fruit surface coating. *Food Chemistry*, *309*(March 2019), 125764. https://doi.org/10.1016/j.foodchem.2019.125764

Saad, N., Delattre, C., Urdaci, M., Schmitter, J. M., & Bressollier, P. (2013). An overview of the last advances in probiotic and prebiotic field. *LWT - Food Science and Technology*, *50*(1), 1–16. https://doi.org/10.1016/j.lwt.2012.05.014

Saarela, M., Hallamaa, K., Mattila-Sandholm, T., & Mättö, J. (2003). The effect of lactose derivatives lactulose, lactitol and lactobionic acid on the functional and technological properties of potentially probiotic Lactobacillus strains. *International Dairy Journal*, *13*(4), 291–302. https://doi.org/10.1016/S0958-6946(02)00158-9

Sáez-Orviz, S., Camilleri, P., Marcet, I., Rendueles, M., & Díaz, M. (2019). Microencapsulation of Calcium Lactobionate for Protection From Microorganisms in a Solid Phase Food. *Biochemical Engineering Journal*, *150*(June), 107281. https://doi.org/10.1016/j.bej.2019.107281

Sáez-Orviz, S., Puertas, C., Marcet, I., Rendueles, M., & Díaz, M. (2020). Bioactive synbiotic coatings with lactobionic acid and Lactobacillus plantarum CECT 9567 in the production and characterization of a new functional dairy product. *Journal of Functional Foods*, *75*(October). https://doi.org/10.1016/j.jff.2020.104263

Saran, S., Bisht, M. S., Singh, K., & Teotia, U. S. (2012). Analyzing Probiotic Attributes to Assess Comparatively Two Isolates of Lactobacillus acidophilus in Prebiotics , Honey and Inulin. *International Journal of Scientific and Research Publications*, *2*(4), 75–81.

Schaafsma, G. (2008). Lactose and lactose derivatives as bioactive ingredients in human nutrition. *International Dairy Journal*, *18*(5), 458–465. https://doi.org/10.1016/j.idairyj.2007.11.013

Suhag, R., Kumar, N., Petkoska, A. T., & Upadhyay, A. (2020). Film formation and deposition methods of edible coating on food products: A review. *Food Research International*, *136*(July), 109582. https://doi.org/10.1016/j.foodres.2020.109582

Vieira, M. G. A., Da Silva, M. A., Dos Santos, L. O., & Beppu, M. M. (2011). Natural-based plasticizers and biopolymer films: A review. *European Polymer Journal*, *47*(3), 254–263. https://doi.org/10.1016/j.eurpolymj.2010.12.011

Wan, V. C. H., Moon, S. K., & Lee, S. Y. (2005). Water vapor permeability and mechanical properties of soy protein isolate edible films composed of different plasticizer combinations. *Journal of Food Science*, *70*(6), e387–e391. https://doi.org/10.1111/j.1365-2621.2005.tb11443.x

Zabihollahi, N., Alizadeh, A., Almasi, H., Hanifian, S., & Hamishekar, H. (2020). Development and characterization of carboxymethyl cellulose based probiotic nanocomposite film containing cellulose nanofiber and inulin for chicken fillet shelf life extension. *International Journal of Biological Macromolecules*, *160*, 409–417. https://doi.org/10.1016/j.ijbiomac.2020.05.066

Zhong, Y., Cavender, G., & Zhao, Y. (2014). Investigation of different coating application methods on the performance of edible coatings on Mozzarella cheese. *LWT - Food Science and Technology*, *56*(1), 1–8. https://doi.org/10.1016/j.lwt.2013.11.006

Capítulo 5.
Desarrollo de materiales con polisacáridos como matriz

En este capítulo se recogen las investigaciones llevadas a cabo empleando polisacáridos como matriz en la elaboración de diferentes biomateriales de aplicación alimentaria.

Los polisacáridos son otro de los biopolímeros más utilizados en el desarrollo de materiales de uso alimentario y pueden ser obtenidos a partir de fuentes animales o vegetales (Cazón, Velazquez, Ramírez, & Vázquez, 2017). Entre los que se usan de forma más habitual destacan la celulosa y sus derivados, el almidón, el chitosano, la pectina y el alginato (Amin et al., 2021). Los materiales desarrollados con estos biopolímeros muestran buenas propiedades mecánicas, debido en gran medida al buen grado de compactación entre los polímeros (Ribeiro et al., 2021). Sin embargo, al igual que sucede con las proteínas, estos materiales son bastante hidrofílicos, lo que hace ciertas propiedades, como la barrera contra el vapor de agua, no sean óptimas. Algunos de los biomateriales desarrollados con polisacáridos, como en el caso del alginato, son muy higroscópicos, lo que puede ayudar a evitar la pérdida de humedad de ciertos alimentos (Cazón et al., 2017) (Hassan, Chatha, Hussain, Zia, & Akhtar, 2018).

En este capítulo se han desarrollado otros tipos de biomateriales de uso alimentario. Además de *films* y *coatings*, también se pueden elaborar otro tipo de formulaciones como micropartículas y cápsulas, que se pueden incluir en la matriz de los alimentos. Ambos son muy versátiles, ya que también pueden elaborarse incorporando compuestos bioactivos, como prebióticos y bacterias probióticas. Incluyéndolos en la matriz de los productos alimentarios, permiten obtener alimentos funcionales.

Este capítulo está divido en dos apartados. En el primero, se recoge la investigación llevada a cabo con una mezcla de matrices para la elaboración de micropartículas prebióticas, mientras que en el segundo se exponen los dos trabajos elaborados utilizando alginato de sodio como matriz.

5.1. Mezcla de matrices

En este apartado se muestra el trabajo desarrollado empleando diferentes polisacáridos (maltodextrina y goma arábiga, entre otros biopolímeros) para el desarrollo de micropartículas como materiales alimentarios.

5.1.1. Microencapsulación de lactobionato de calcio por el método de liofilización e incorporación para el desarrollo de productos lácteos funcionales

Como otro tipo de biomateriales de uso alimentario se han desarrollado micropartículas empleando diferentes polisacáridos como matriz. Las micropartículas son frecuentes en el campo de la industria alimentaria (Dias, Ferreira, & Barreiro, 2015) (Sarao & Arora, 2017) y tienen un tamaño comprendido entre 1 y 1000 µm de tamaño (Dias et al., 2015).

En este primer trabajo empleando polisacáridos como matriz, se elaboraron únicamente micropartículas prebióticas, empleando como compuesto prebiótico lactobionato de calcio. En este caso se utilizó el lactobionato de calcio, ya que es la sal más común del ácido lactobiónico (Alonso et al., 2013), y es el compuesto que actualmente está aprobado por la FDA para su uso en alimentación (E-399) (FDA, 2017). Las micropartículas se elaboraron por medio de técnicas de liofilización. El propósito de incluir el prebiótico en las micropartículas fue la protección del mismo frente al consumo por parte de los microorganismos que se encuentran de forma natural en los alimentos, permitiendo la llegada del prebiótico de forma intacta al GIT, donde podría ser empleado como sustrato para la fermentación de la microflora gastrointestinal. En este estudio, las micropartículas se añadieron a quesos frescos de cabra como modelo alimentario real con el objetivo de desarrollar un alimento funcional. Se observó que las micropartículas protegieron al prebiótico de ser usado como sustrato por las BAL presentes de forma natural en el queso. Además, por medio de digestiones *in vitro* simuladas, se verificó que el prebiótico no es digerido por enzimas humanas y que es capaz de llegar al GIT intacto.

- **Publicación:** Sáez-Orviz, S., Camilleri, P., Marcet, I., Rendueles, M., Díaz, M. 2019. Microencapsulation of calcium lactobionate for protection from microorganisms in solid phase food. *Biochemical Engineering Journal*, 150., 107281, ISSN 1369-703X, (https://doi.org/10.1016/j.bej.2019.107281).
- **Estado:** publicada
- **Índice de impacto:** 3,475 (2019)

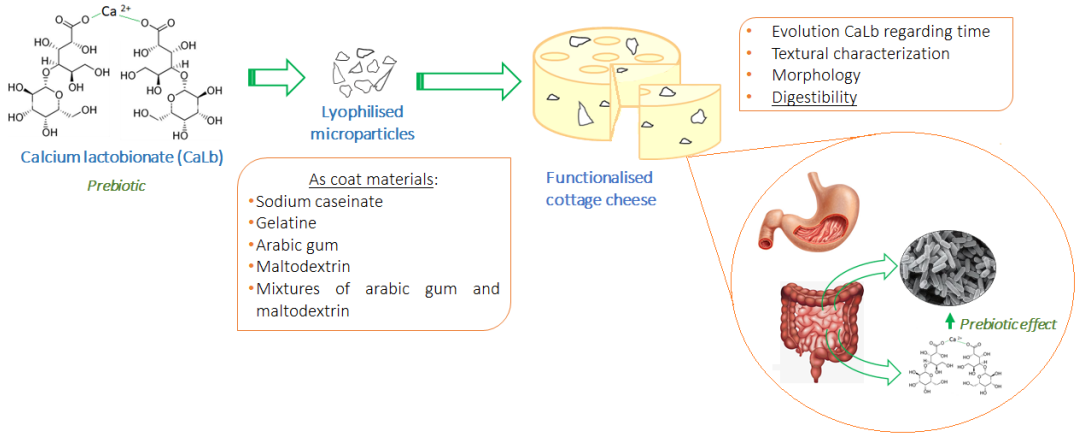
Microencapsulation of calcium lactobionate by lyophilized method and incorporation in functional dairy products

S. Sáez-Orviz[a], P. Camilleri[a], I. Marcet[a], M. Rendueles[a], M. Díaz[a]*

[a]Department of Chemical and Environmental Engineering, University of Oviedo, C/Julián Clavería s/n, 33071 Oviedo, Spain.

*Corresponding author.
E-mail address: mariodiaz@uniovi.es (M. Díaz)

Graphical abstract



Abstract

Prebiotic compounds can be consumed during storage process by the foodstuff's own microflora and are thus prevented from reaching the gastrointestinal tract to promote probiotic growth. Microencapsulation techniques can protect these bioactive compounds. In the present research, lyophilized calcium lactobionate microparticles employing different coat materials, some after treatment with transglutaminase enzyme, were developed, characterized and tested in cottage cheeses as an example of a food matrix with low water activity. These microparticles possessed good properties with respect to their encapsulation efficiency and morphology, and no great differences were observed when transglutaminase was employed with casein and gelatine. All microparticles were shown to exert an enduring protective effect against lactic acid bacteria, since after 12 days a very worthwhile amount of calcium lactobionate (about 1.165 mg g-1 of product) was still in the final product. Diffusion of the calcium

lactobionate to the outlet of the microparticles and diffusion of the lactic bacteria inside the microparticles are negligible after the period tested. Microparticles of sodium caseinate were chosen for the final digestibility analyses. In the digestibility tests calcium lactobionate was released completely in both conditions, gastric and intestinal. Thus, an innovative dairy functional product has been developed.

Keywords: prebiotic, calcium lactobionate, microencapsulation, cottage cheese, functional dairy product

1. Introduction

Nowadays, there is a great demand for high quality food products with no risk of any ill effects and which, besides satisfying the consumers' nutritional demands, have the capacity to improve their health. To fulfil this perceived need, what has been called "functional food" has acquired growing attention lately. According to the International Life Sciences Institute (ILSI), functional food can be defined as "that which beneficially affects one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease" (Ashwell, 2002).

The functional food market is currently growing, and Japan is one of the main producers and consumers. About 45% of Japanese functional foodstuffs aim to help the maintenance of good gastrointestinal tract (GIT) conditions (Ohama, Ikeda & Moriyama, 2014). The GIT microbiota is arranged in a complex ecosystem which directly affects human nutrition and health. Thus, it is of interest to increase the number of beneficial bacteria to the point where they represent more than half the population. There are two ways to achieve this goal: using living bacteria, known as probiotics, and/or employing prebiotic compounds (Ashwell, 2002).

Prebiotics constitute a vast variety of compounds. Within this group, lactose derivates such as lactobionic acid are of great interest. This compound has received attention from the food industry due to its properties as an antioxidant, stabilizer and gelling agent (Alonso, Rendueles & Díaz, 2013), but also for its prebiotic effect. The CaLb consumption in the colon by the human microflora is capable of improving the growth of some probiotics of the Bifidobacterium genus (Schaafsma, 2008) (Suguri, Yanagidaria & Kobayashi, 1995). Lactobionic acid has been approved for its use as calcium lactobionate (CaLb) by the FDA (FDA, 2017), although it is still being studied by European Committees (Alonso, Rendueles & Díaz, 2013).

Functional foods incorporate bioactive compounds which are usually sensitive to chemical and physical factors (Dias, Ferreira & Barreriro, 2015). Specifically, for prebiotics, introducing them directly into food products leads to the risk of their being consumed by the foodstuff's own microflora. If that happens, the prebiotics will not be able to reach the consumers' GIT and so promote the growth of beneficial probiotics. To deal with the problems of the degradation of bioactive-compounds, the food industry has developed several tools, such as microencapsulation (Dias, Ferreira & Barreriro, 2015) (Sarao & Arora, 2017). There are many microencapsulation techniques, but that of lyophilization allows the creation of microparticles that are stable over time (Morita, Horikiri, Suzuki & Yoshino, 2001). There have been several studies on the microencapsulation of bioactive compounds, the vast majority of which do not include experimental investigation of the incorporation of microparticles into food matrices. The most usual food matrices employed to test microparticles are dairy products (Francisco et al. 2018) (Caleja et al. 2016) (Dias, Ferreira & Barreriro, 2015). The use of low water activity (aw) food matrices such as bread is also common (Ezhilarasi, Indrani, Jena & Anandharamakrishnan, 2014), as is the use of cereals.

As far as is known, there are few prebiotic microencapsulated products on the market. In this respect, the purpose of the present research was to develop and characterize CaLb microparticles, employing different coat materials and transglutaminase enzyme (TGase), with the objective of improving the microparticle properties. After analysing the microparticles, they were tested in cottage cheeses as an example of a food matrix with low aw. The aim was to determine the protective effect of microparticles against the consumption of lactobionic acid by the cottage cheese's own microflora and to generate an innovative functional dairy product that could be attractive for both consumers and the food industry.

2. Materials and methods

2.1. Materials

Several coat materials with different concentrations (w/w) were employed as described by other authors (Table 5.1). Sodium caseinate (NaCas), maltodextrin (MD) and gelatine (Gel) were purchased at Sigma-Aldrich Chemical Co. (Steinheim, Germany). Gum arabic (GA) was provided at Panreac (Panreac S.A., Barcelona Spain). CaLb (≥ 98% purity) was supplied by Sigma-Aldrich.

Table 5.1. Coating materials employed to encapsulate CaLb and the concentration of CaLb inside microparticles and cheeses.

Coating material with its concentration (% weight)	mg CaLb/ g microparticle	mg CaLb/ g cheese
Maltodextrin (MD) 12%	172.4 ± 5.3	0.479 ± 0.032
MD 16%	135.2 ± 4.2	0.716 ± 0.072
Gum arabic (GA) 4%	385.6 ± 2.9	2.803 ± 0.023
GA 8%	238.6 ± 3.4	1.845 ± 0.095
Sodium caseinate (NaCas)	143.7 ± 0.4	1.11 ± 0.066
NaCas-TGase	143.3 ± 2.0	0.809 ± 0.077
Gelatine (Gel)	455.3 ± 1.2	2.246 ±0.045
Gel-TGase	455.2 ±4.5	1.520 ± 0.034
GA:MD 75:25	200.9 ± 5.0	0.962 ± 0.007
GA:MD 50:50	238.1 ± 9.9	0.997 ± 0.178
GA:MD 25:75	294.3 ±1.1	0.763 ± 0.110
Positive control	201.4 ± 68.6	2.601± 0.551

2.2. Microparticle preparation

Mixtures of 0.05 g mL -1 CaLb and the different coat agent solutions were prepared at a ratio of 1:3 and they were frozen at -80ºC for 12 hours. Microparticles were produced using the lyophilisation method (Tesltar Cryodos, 0.1 mBar, -70ºC for 24 hours). After lyophilisation, microparticles were ground and sieved with a pore-size of 355 µm and stored in a dry atmosphere until use.

Mixtures of NaCas and Gel with the active material were also treated with transglutaminase (TGase), in an attempt to improve their properties. In this case, pH was adjusted to 7.0 with 1M NaOH (Sigma-Aldrich) and TGase was added (0.33 g L-1, 50 U g-1 protein) (Probind TX, BDF ingredients, Spain). The reaction was conducted in an oven at 45ºC for 90 min. After that time, the temperature was increased to 70ºC for 10 min to deactivate the enzyme. As in the case of the other microparticles, they were produced by the lyophilization method.

2.3. Microparticle characterization

2.3.1. Encapsulation efficiency

Encapsulation efficiency (EE) was calculated according to Cilek et al. (2012). EE is defined as the ratio of encapsulated active compound content (EAC) to total active

compound (TAC). EAC is determined by calculating the difference between TAC and the surface-active compound content (SAC):

$$EE=[(TAC\text{-}SAC)/TAC]x100 \qquad \text{(Equation 1)}$$

To determinate SAC, 1 mL of distilled water was added to 0.005 g of microparticles, and they were gently shaken for 10 minutes. Samples were centrifuged at 13,200 rpm (Centrifuge 5415D, Sigma-Aldrich) for 5 minutes. The supernatant was used to measure SAC as described in Section 2.5.

2.3.2. Solubility

This test investigates which kind of microparticle prevents further release of CaLb. 1 mL of distilled water was added to 0.005 g of each type of microparticle. Samples were incubated at 20ºC and 300 rpm for 30 min (Thermomixer, Eppendorf, Hamburg, Germany). After centrifugation (13,200 rpm for 5 min), the supernatant was collected and analysed as described in Section 2.5.

2.3.3. Microstructure characterization

The morphology of the microparticles was observed using scanning electron microscopy (SEM) (JSM-6610LV, JEOL, USA). Microparticles were mounted on stubs and coated with gold. The surface morphology of the microparticles was observed with magnifications of 100x.

2.4. Functionalization of cheese with calcium lactobionate

2.4.1. Functional cottage cheese manufacture

Milk was obtained from a herd of Murciano-Granadino goats from a local farm in San Martín del Rey Aurelio (Asturias, Spain). Cottage cheeses were made with low temperature-long time (LTLT) pasteurised milk, heated at 60ºC for 25 minutes. This gentle pasteurization process allows some lactic acid bacteria (LAB) to survive, so it was not necessary to use a starter culture. Subsequently, milk was cooled to 34ºC and rennet (Chy-Max®, CHR-Hansen, Denmark) was added (0.0225 g L^{-1}). After 40 minutes, the curd was cut several times to stimulate syneresis. 0.005 g of each type of microparticle was added with careful mixing to 40 g of curd. Cheeses were stored at 21ºC for a day and then at 4ºC for further analysis.

Analysis of microparticles inside functional cottage cheeses was carried out using 5 g samples of each cheese. 20 mL of H_2SO_4 (0.013N) (Sigma-Aldrich) (Jo, Benoist, Ameerally & Drake, 2017) was added and samples were homogenised using a

Stomacher®80 (Seward, United Kingdom) for 90 seconds at maximum speed. Afterwards, samples were heated at 64ºC for 30 minutes. Finally, samples were centrifuged for 10 minutes at 13,200 rpm and the supernatant was stored until analysis as described in Section 2.5.

2.4.2. Textural analysis

For textural characterisation, a TA.XTplus Texture Analyzer (Stable Systems, Godalming, Surrey, United Kingdom) was employed. Cottage cheese samples were submitted to a penetration test, at room temperature, using the spherical probe SMS P/0.5S with a test speed of 2.0 mm s^{-1} and a load cell of 5 kg. Results are expressed in terms of firmness and stickiness values (grams). Experiments were triplicated and reported results correspond to the mean value.

2.4.3. Digestibility test

Gastric and intestinal conditions were simulated according to Minekus et al. (2014) with some modifications. The composition of the simulated gastric fluid (SGF) employed was 0.517 g L^{-1} KCl, 0.123 g L^{-1} KH_2PO_4, 2.106 g L^{-1} $NaHCO_3$ and 2.75 g L^{-1} NaCl. Simulated intestinal fluid (SIF) was made by mixing 0.509 g L^{-1} KCl, 0.110 g L^{-1} KH_2PO_4, 11.68 g L^{-1} $NaHCO_3$ and 2.24 g L^{-1} NaCl.

For the gastric simulation, 5 grams of cottage cheese were mixed with 7.5 ml SGF, 1.6 mL of pepsin (863 U mg^{-1} protein, CAS 9001-75-6, Sigma-Aldrich), with a concentration of 15.15 g L^{-1}, using SGF as solvent, 5 µL of $CaCl_2$ 0.3M (Sigma-Aldrich) and 0.696 µL of distilled water. The pH was adjusted to 3.0 with 1M HCl (Sigma-Aldrich). The mixtures were shaken at 300 rpm and 37ºC for 90 minutes and samples were collected at 45 and 90 minutes.

After gastric simulation, intestinal simulation was carried out. 4.95 mL of SIF, bovine chymotrypsin (0.3% w/v) (60 U mg^{-1} protein, EC 232-671-2, Sigma-Aldrich), porcine pancreatin (0.1% w/v) (Sigma-Aldrich), 40 µL of 0.3M $CaCl_2$ and 1.3 mL of distilled water were added to the previous mixes. The pH was adjusted to 7.0 using 1M NaOH (Sigma-Aldrich). The mixtures were shaken at 300 rpm and 37ºC for 2 hours and samples were collected at 15, 30, 45, 60 and 120 minutes. All samples taken at different times were centrifuged (13,200 rpm for 5 minutes) and filtered with a 0.45 µL syringe filter (Whatman, Sigma-Aldrich) before analysis.

2.4.4. Morphological analysis

Structural analysis was carried out to search for any changes in the cottage cheese structure when microparticles were added. Cottage cheeses were frozen at -80ºC o/n

and then were lyophilised (0.1 mBar, -70ºC for 24 hours). Morphology was studied using SEM as described in Section 2.3.3.

2.5. Analytical methods

CaLb was measured as lactobionic acid using High Performance Liquid Chromatography (HPLC). The system of liquid chromatography employed (Agilent 1200, Agilent Technologies Inc., Santa Clara, CA, USA) was equipped with a Coregel ION 300 column (Teknokroma, Barcelona, Spain) coupled to a refractive index detector. The mobile phase was a sulphuric acid solution (0.450 mmol L^{-1}, pH 3.1) with a flow rate of 0.3 mL min^{-1} and a column temperature of 75ºC. Data acquisition and analysis were performed with ChemStation software (Agilent).

3. Results and discussion

3.1. Characterization of microparticles

3.1.1. Encapsulation efficiency (EE)

EE indicates the amount of active compound encapsulated with respect to the initial quantity used. Experiments were performed in triplicate and results are shown as the mean value with the standard deviation (Table 5.2).

The NaCas and Gel microparticles treated with TGase showed an improvement in the EE, NaCas being the better core material (Table 5.2). TGase catalyses the acetyltransferase reaction (intra or inter chain) between the γ-carboxamide groups of glutamic residues and the ε-amine groups of the lysine residues (Fatima & Khare, 2018), which creates crosslinking. It has been shown that treatment with this enzyme improves the microparticle characteristics of different kinds of core material, leading to a higher EE (Mihalcea et al. 2018). It is also capable of enhancing water retention (Tello et al. 2016) and as CaLb is a hydrophilic compound, core material treated with TGase achieved a better EE.

In the case of MD and GA, the EE values increased when more core material was employed. GA 8% showed an EE of 88.4±1.8%, while at 4% the EE decreased to 64.8±2.8%. Something similar happened with MD microparticles, where MD 12% showed a reduction of 10% in its EE value with respect to MD 16%. So, the greater the amount of core material, the higher the encapsulation efficiency. Other authors explain it as a faster precipitation of the core material (Jyothi et al. 2010).

Mixtures of MD and GA had the highest amount encapsulated and it can be seen that the EE rose when there was a higher proportion of GA. This can be explained by the properties of GA as a stabilizing and emulsifying agent (Cilek et al. 2012) and by the fact

that GA has a higher molecular weight, a characteristic that enables it to retain more active material (Kaushik & Roos, 2007).

Table 5.2. Encapsulation efficiency (EE) and solubility (% weight) of microparticles made with different materials.

Microparticle	EE (%)	Solubility (% weight)
NaCas	79.4 ± 1.1	69.4 ± 8.2
NaCas-TGase	85.3 ± 1.1	68.8 ± 8.1
Gel	51.4 ± 2.6	76.7± 1.2
Gel-TGase	86.8 ± 2.7	96.8± 2.6
GA 4%	64.8 ± 2.8	87.1 ± 1.9
GA 8%	88.4 ± 1.8	95.5 ± 3.1
MD 12%	69.2 ± 1.0	61.5 ± 1.5
MD 16%	81.0 ± 1.2	75.1 ± 3.7
GA:MD 75:25	91.7 ± 2.0	11.9 ± 1.6
GA:MD 50:50	87.6 ± 1.9	14.8 ± 1.2
GA:MD 25:75	83.8 ± 1.6	20.5 ± 2.4

3.1.2. Microparticles solubility

Solubility experiments were performed in triplicate and results are shown as the mean value with the standard deviation (Table 5.2). Excepting GA:MD mixtures, the rest of the microparticles have a CaLb release of 60%. It is a high percentage, as expected, because most coating materials employed were hydrosoluble.

GA:MD mixtures were extremely water-resistant, but GA and MD separately were not. The two polysaccharides together form water-resistant microparticles, possibly as a result of some interaction that leads to longer polysaccharide chain length, which implies lower solubility (Sarao & Arora, 2017). Gel was also soluble even after being treated with TGase and was even less water resistant. TGase activity may form some new bonds but others might be destroyed due to physical and chemical factors like pH or temperature (Fatima & Kahre, 2018) changing the gelatine structure and thus affecting parameters like solubility. NaCas and NaCas-TGase had a mean solubility value of 69.1%. Caseins had a low solubility in an acid environment (Glab & Boratynski, 2017) but the salt form increases solubility.

Excluding GA:MD mixtures, the results demonstrated that these microparticles were not appropriate for addition to food matrixes with high aw, such as milk, but that they could be effective in food products with a low aw, like cheeses or bread. This is why microparticles have been used more frequently in such foods. There are several examples of the use of microparticles in such foods; for instance, casein microparticles

were used with citric acid for chewing gums (Soleiman & Rahimi, 2009) or whey and MD microparticles with fruit extract for bread (Ezhilarasi et al. 2014).

3.1.3. Microcapsule morphology

SEM was employed to study surface morphology (Figure 5.1). The visual appearance of all the microparticles was similar: a white powder with a particle size ≤ 355 μm.

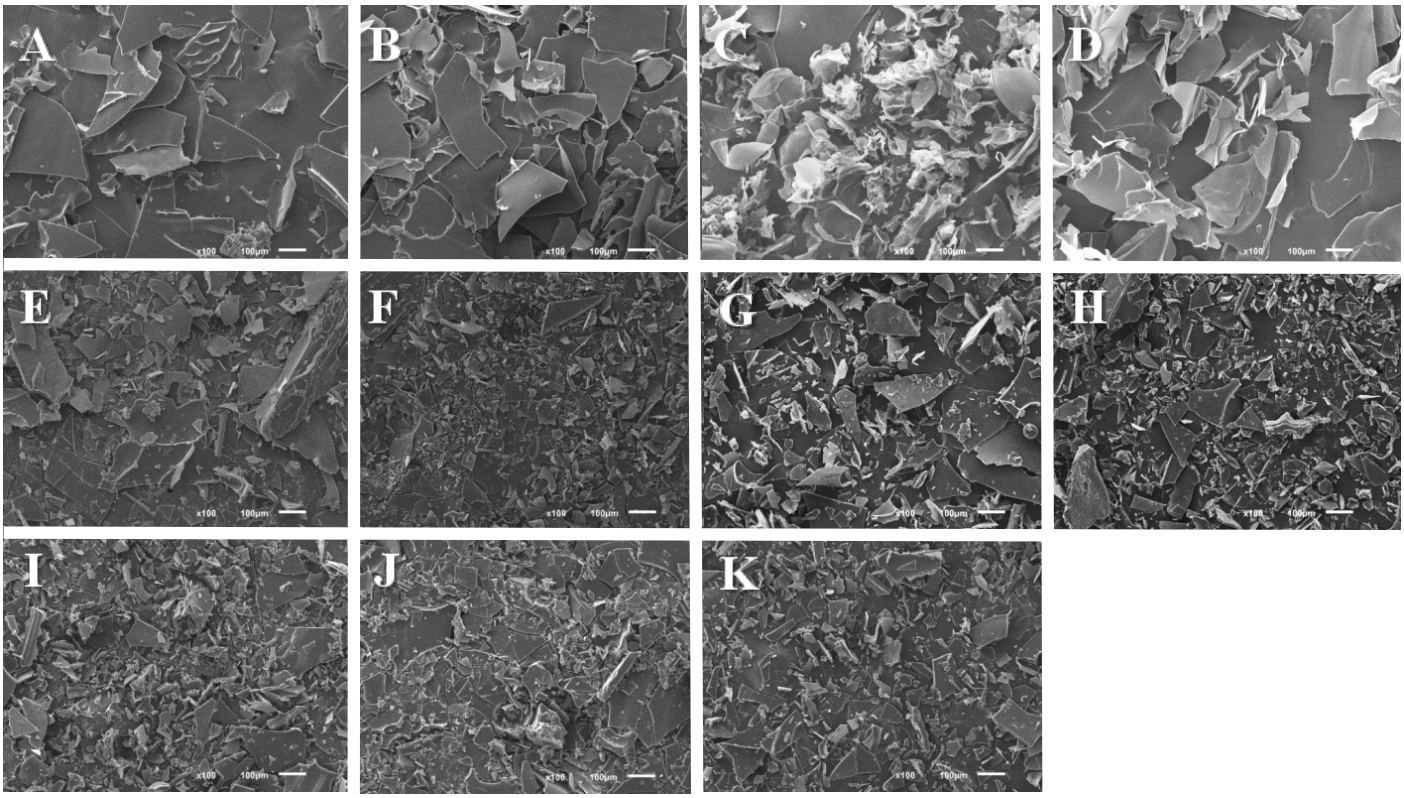


Figure 5.1. SEM images of (A) NaCas, (B) NaCas-TGase, (C) Gel, (D) Gel-TGase, (E) GA 4%, (F) GA 8%, (G) MD 12%, (H) MD 16%, (I) GA:MD 75:25, (J) GA:MD 50:50 and (K) GA:MD 25:75 microparticles; scale bars correspond to 100 μm.

There was no morphological difference between the microparticles treated with TGase and those that had not been treated. Only the Gel microparticles had a different appearance, with a less uniform surface and less compact structure (Figure 1C and D), while the others had a generally uniform appearance, despite having irregular shapes, and were smooth and with no pores (Figure 1E-K). Microparticle morphology is related to the active compound and the method employed to produce the microparticles. In this study, after lyophilisation, the microparticles were crushed and sifted and, as a result, all microparticles had a uniform and irregular shape despite the core material. Similar surface morphologies were obtained when biopolymers, such as chitosan, xanthan or β-cyclodextrin, were employed as coat material with phenolics as active

compounds in lyophilised microparticles developed by other authors (da Rosa et al. 2013) (Chandrasekar, Coupland & Anantheswaran, 2017).

3.2. Functionalization of cheese with microparticles

Milk and dairy products have been the preferred food matrices for the incorporation of microparticles (Dias, Ferreira & Barreiro, 2015). Some active compounds added to dairy products are plant-derived (Caleja et al. 2016), vitamins (Stratulat et al. 2014) and organic acids (Francisco et al. 2018). There are very few studies investigating prebiotic microencapsulation. Employing dairy products in which probiotics are present along with a prebiotic that is protected against consumption by the food probiotic allows the production of synbiotic products (García, Bautista, Rendueles & Díaz, 2018) with interesting health properties and that are appealing to consumers.

3.2.1. Evolution of CaLb regarding time

After being characterized, the microparticles were added to the cheese matrix. CaLb was measured at initial time (t 0) and after 5 and 12 days. Experiments were performed in triplicate and results are expressed as the mean percentage of CaLb with the standard deviation (Figure 5.2).

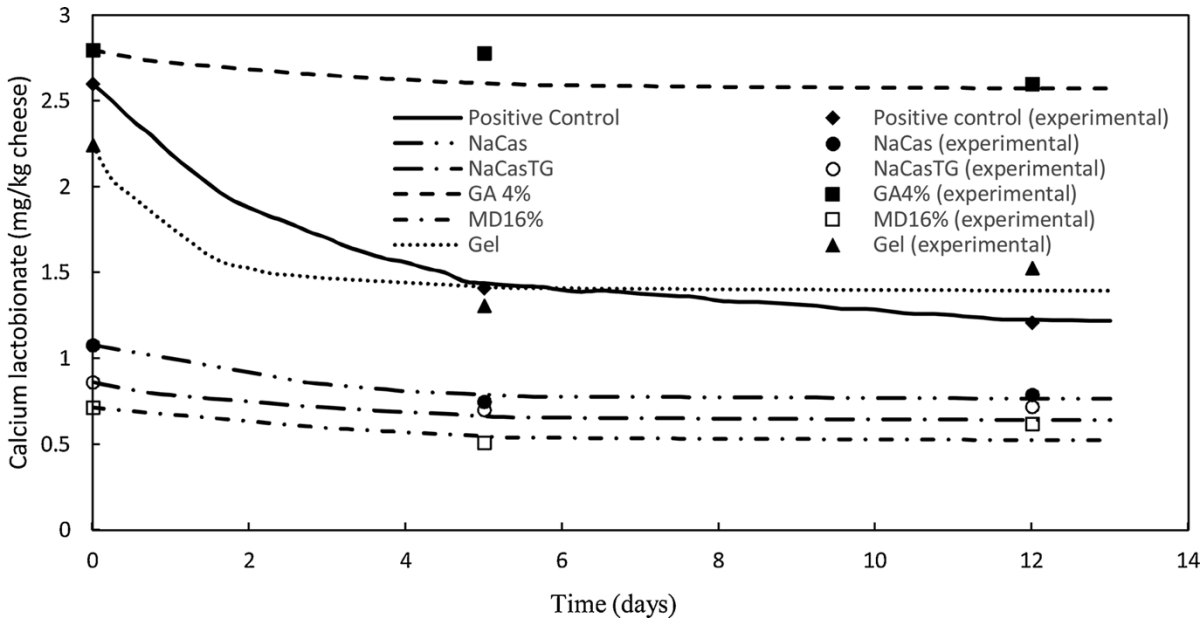


Figure 5.2. Experimental and simulated evolution of CaLb percentage in cottage cheese-micropartciles mixes with time along 12 days. Points indicate experimental results: (⧫) Positive control (free calcium lactobionate); (cirf) NaCas; (○) TGase; (squf) Gel; (cirf) GA 8%; (squf) MD 165. Lines show simulated evolution using the proposal model.

Due to the LTLT process, the concentration of CaLb decreased over time because some LAB were still alive inside the cheese matrix. CaLb is used as substrate by some LAB (Schaafsma, 2008) but microparticles have protective capacity against bacterial consumption. Figure 5.2A shows the percentage of CaLb in cottage cheeses when microparticles of NaCas, NaCas-TGase, Gel and Gel-TGase were added. Results demonstrated an increase in the degree of protection against consumption when the core material is treated with TGase. CaLb contained in GA and MD microparticles is shown in Figure 5.2B and, as can be seen, they offered protection against LAB consumption. Results for mixtures of GA and MD microparticles are shown in Figure 5.2C. The three mixtures showed a similar percentage of CaLb, although microparticles of GA:MD 50:50 showed a slightly better performance.

The real amount of active compound inside the microparticles was between 1.14±0.03 and 2.67±0.6, expressed as mg of CaLb g^{-1} of cottage cheese. This may seem a very large variation in CaLb content, but what is really significant is that even the lowest value is much greater than the amount found in the only CaLb-containing food product marketed today. This is Caucasian yogurt ("Caspian Sea yogurt"), which is sold in Japan, where FOSHU ("Food for specified health use") products have been extensively developed. This is the only yogurt that contains CaLb and its concentration is approximately 0.45 mg of CaLb g^{-1} of yogurt (Kiryu et al. 2009). Protecting CaLb against LAB consumption by employing microparticles allowed an increase of 60%. The FDA and committees are investigating suitable concentrations for consumption (FDA, 2017) but there is no specific regulation at the moment. There are several studies and some authors have concluded that amounts of up to 24 g of CaLb per day were well tolerated (Schaafsma, 2008). So, developing innovative new products with high concentrations of CaLb could be particularly interesting from a technological point of view. Specifically, NaCas and NaCas-TGase microparticles had a concentration of 1.14±0.03 and 1.19±0.04 mg of CaLb g^{-1} of cottage cheese respectively. Casein is found naturally in dairy products as the major protein. Employing NaCas microparticles for developing functional dairy products will be accepted more readily by consumers, as it does not involve the use of foreign proteins or sugars.

3.2.2. Modelling of CaLb transport and consumption in cheese

A schematic description of the experimental work considers two phases, the microparticles phase and the cheese phase. The CaLb is present initially in the microparticles phase; there will be diffusion, J_1, to the surface, where there could be some biodegradation, and then diffusion to the bulk cheese phase, J_2. The

biodegradation reactions will take place in the neighbourhood of the interphase and in the cheese bulk. The concentrations of CaLb in the microparticles, interphase and cheese bulk will be c_p, c_s, and c_b, while the concentration of the bacterial population will be assumed as uniform in the cheese phase (Figure 5.3).

A general mass balance for the cheese phase will consider the accumulation of CaLb to be equal to its mass transfer ($J_1 = k_s (c_p - c_s)$) ($Ak_s c_s Xs Y^{s}_{x/s}$) to the surface and in the cheese bulk ($Vk_x X_s Y^{s}_{x/s}$), which would give:

$$V_b \frac{dc_b}{dt} = J_1 \sum r = J_1 - [(Ak_s c_s X_s Y^s_{x/s}) + (V_b k_x X_b Y^b_{x/s})] \qquad \text{(Equation 2)}$$

A being the interphase surface, V_b the volume of the cheese bulk, K_s and K_X the kinetic constants, *Xs* the biomass in the interphase and $Y_{X/s}$ the biomass/substrate stoichiometric coefficient.

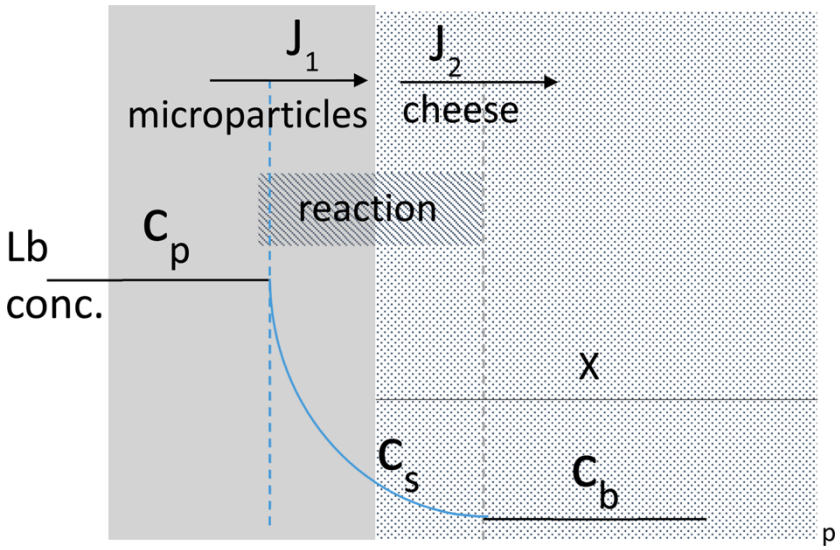


Figure 5.3. Flows/ reactions and concentration profiles assumed in the experimental system for modelization.

A global mass balance for the cheese-microparticles mix could also be of interest in order to obtain experimental data (Figure 5.3) for the global concentration c. Experiments carried out to investigate the biodegradation of CaLb homogeneously distributed in the cheese have indicated a rapid degradation process, so the reaction which could take place in the cheese and in the microparticles, and clustering the parameters, simplified equations can be obtained for the analysis of the experimental results.

A local mass balance for the microparticles phase will give

$$\frac{dC_p}{dt} = k'(c_p - c_s) \qquad \text{(Equation 3)}$$

And if $c_x \cong 0$ we will obtain $\frac{dC_p}{dt} = k' c_p$

A global mass balance over time gives rise to:

$$V_p C_{po} = V_c + \int_0^t V_p \frac{dc_p}{dt} dt = V_c + \int_0^t V_p k' c_p dt \quad \text{(Equation 4)}$$

The term c_{po} being the initial concentration of CaLb in the microparticles, and V_p the volume of the microparticles. Consequently,

$$\frac{V_p}{V} c_{po} = c + \int_0^t k' c dt \quad \text{(Equation 5)}$$

This equation can be used to obtain the values of k´ from the experimental data of c vs. t. When NaCas and NaCasTG microparticles were introduced into the cheese, the values obtained for k' were 4.43 x 10^{-5} s^{-1} and 4.40 x 10^{-5} s^{-1} respectively, indicating that the use of transglutaminase did not exert an important effect on the protection process. When gum arabic (GA) was used, k' was found to be 4.61 x 10^{-5} s^{-1}, while the value of k' was 3.03 x 10^{-5} s^{-1} with maltodextrin (MD), and if gelatine (Gel) was used for making the microparticles, the value of k' was 3.1 X 10-5 s-1. The values of the fitting of the model to the experimental results are shown in Figure 5.3, with the simulated lines.

As a comparison, the consumption in the positive control, using the same equation (5) gave k' as 4.66 x 10^{-5} s^{-1} when the lactobionate was not encapsulated in the microparticles. Taking into account the concentration of lactobionate in the cheese after 12 days, it can be seen that the microparticles exerted a protective effect, impeding the consumption of this compound by the lactic acid bacteria present in the cheese.

3.2.3. Textural characterization of functional cheeses

To produce attractive food products for consumers, it is particularly important to understand their texture and structure (Wilkinson, Dijksterhuis & Minekus, 2001) (Foegeding & Drake, 2007). Textural characterization allows the measurement of the firmness and stickiness of food products. Experiments were performed in triplicate and results are shown as the mean value with the standard deviation (Figure 5.4A and B).

In terms of firmness, the negative control (cheese without microparticles) had a mean value of 249.18±26.55 g (Figure 5.4A). All the cheeses had a similar value, except the positive control, Gel and Gel-TGase and GA cheeses. In the case of the positive control, the firmness value was 48% higher than the negative control due to the increase in free calcium in the matrix. CaCl2 is one of the salts which is added to improve the curdling process. CaLb was added free to positive control cheeses, so calcium was able to enhance the precipitation of casein and the strength of its bonds, leading to a greater value of firmness (Everett & Auty, 2008). Firmness of cheeses with GA microparticles

showed an increase of 59% with respect to the negative control. GA has useful properties in the food sector due to its stabilising capacity (Cilek et al. 2012). It may stabilize casein-casein bonds, thus increasing the firmness of the matrix. The opposite happens with Gel microparticles where cheese firmness was 32% lower. The pH of cheese was between 3.5 and 4.5. At this pH, the number of basic amino acid residues available to participate in the TGase bond with caseins decreased rapidly and therefore firmness also decreased (Pang et al. 2014).

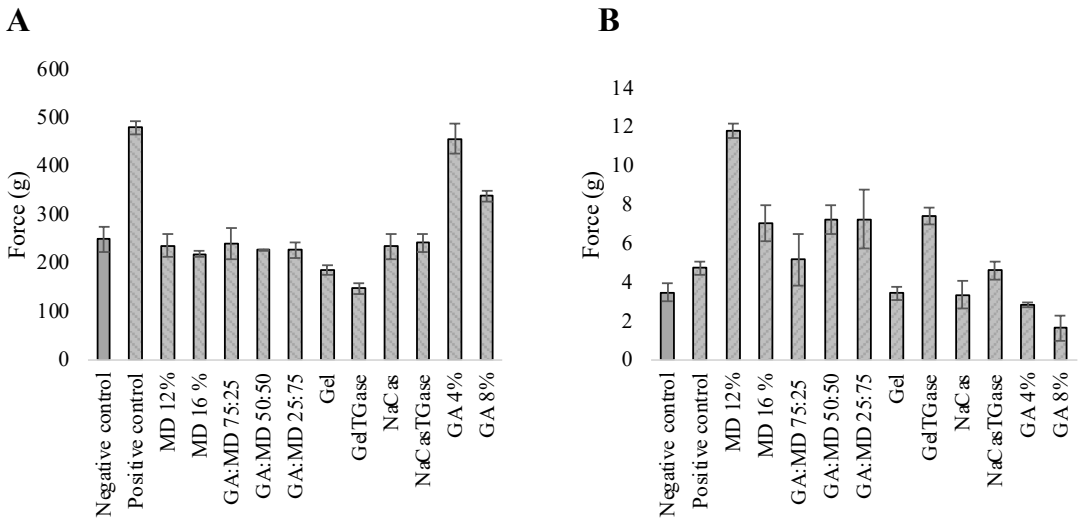


Figure 5.4. Textural analysis of functionalised cottage cheeses; (A) firmness analysis and (B) stickiness analysis. Both are expressed in force units (g).

Referring to stickiness, the values obtained differed notably from the negative control (3.498± 0.474 g) (Figure 5.4B). The highest values were obtained with MD microparticles and the mixtures of MD and GA. As there was no difference between the GA microparticle cheeses and the control, it can be assumed that MD was responsible for the increase in stickiness values. The presence of MD in the cheeses raised their moisture content (Jack, 2011) and that could affect the structure of the protein matrix, increasing stickiness. And an increase in the stickiness value was also observed with TGase microparticles, particularly with Gel-TGase. In the case of casein microparticles TGase catalyses bonds between different residues in caseins (Fatima & Khare, 2018). The increase in this kind of interaction could have as a result a higher stickiness in the final product.

As there was no great difference between the protective effect exerted by the different microparticles, and in all of them the amount of CaLb encapsulated was acceptable, as were their textural properties, NaCas and NaCas-TGase microparticles were the ones chosen for digestibility and cheese morphology analysis.

3.2.4. Functionalized cheese morphology

The visual appearance of the functional cheeses was similar to the cottage cheese without microparticles in its structure. Functionalised cottage cheeses were examined for matrix differences with SEM (Figure 5.5).

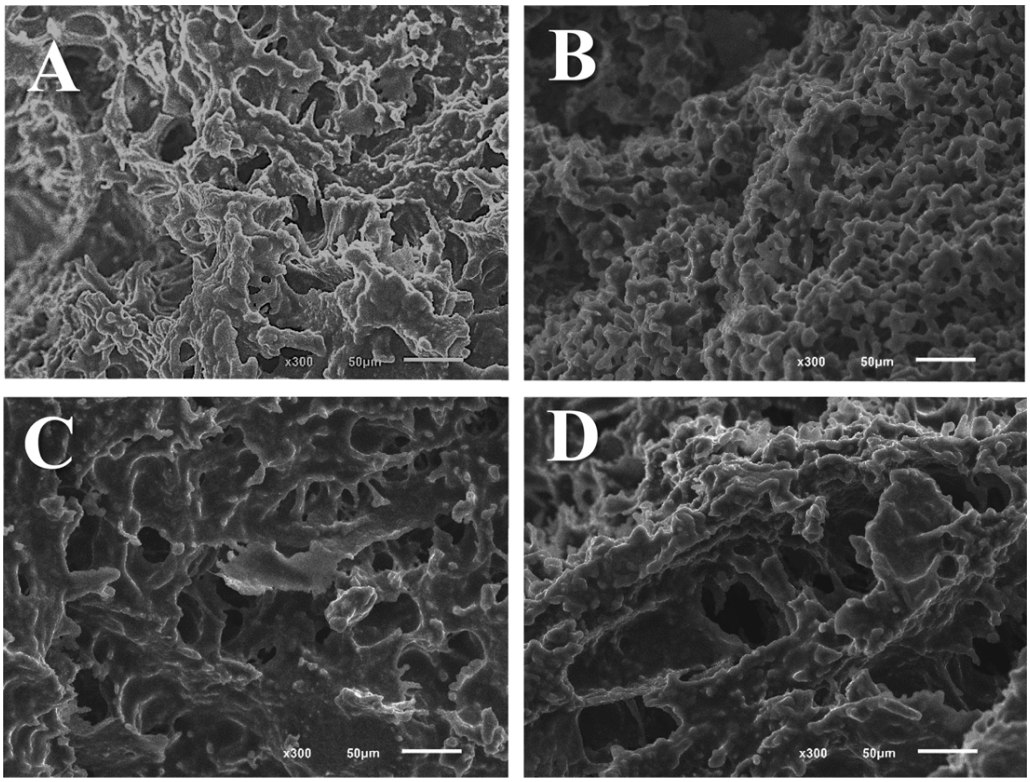


Figure 5.5. SEM images of cheese (A) without microparticles in its structure, (B) with free calcium lactobionate, (C) with CasNa and (D) with NaCas-TGase microparticles; scale bars correspond to 50 µm.

There was no difference between the negative control matrix and cheeses functionalised with NaCas and NaCas-TGase (Figure 5.5A, C and D, respectively). The morphology results corresponded with textural analysis since there was no difference in firmness and stickiness values between the negative control and cheese functionalized with NaCas and NaCas-TGase microparticles.

The matrix of the positive control showed evident changes (Figure 5.5B). It was more homogeneous, rounded and less rough. This could be due to the presence of free calcium. Calcium promotes the curdling process, helping and improving casein precipitation (Everett & Auty, 2008). Free calcium could interact with casein, and so change the matrix structure and textural properties, resulting in a firmer and more compact cheese.

3.2.5. Digestibility of microparticles and cheeses

The digestibility test showed the degree of degradation of microparticles with the aim of discovering whether CaLb can be released in the GIT to enhance the growth of its microflora. The digestibility test was carried out employing NaCas and NaCas-TGase after characterization of these microparticles and their functionalized cheeses.

NaCas and NaCas-TGase powder was firstly tested to ensure microparticle degradation. Experiments were performed in triplicate. NaCas-TGase microparticles were more resistant to the acid stomach environment with a CaLb release of 84.8±1.2% (w/w), while NaCas had a liberation of 99.2±0.8% (w/w). Casein microparticles can resist low pH but they are rapidly digested by pepsin (Glab & Boratynski, 2017). But nevertheless, when casein is treated with TGase, the microparticles are more resistant to pH and enzymes due to the cross-linking between proteins (Xing et al. 2016). In the intestinal simulation, CaLb release with NaCas microparticles was similar to that seen in the stomach, while NaCas-TGase showed a higher degree of liberation (85.7±0.3%). A modification in the pH, from acid (3.0) to neutral (7.0), might encourage a change in the protein structure by breaking bonds formed by TGase and increasing CaLb liberation.

Once it had been proved that the microparticles were degraded, functional cheeses were tested in triplicate. As occurred with microparticle powder, NaCas showed more release than NaCas-TGase (75.6±0.7% (w/w) versus 63.0±0.7% (w/w)) in the stomach. In the intestine, the liberation was similar, although NaCas-TGase functionalised cheeses had a greater degree of release (78.8±2.0% (w/w)). CaLb release in the cheese digestibility experiments was lower than that observed in the microparticles in suspension. This could be due to the complexity of the food matrix.

4. Conclusions

Microparticles made with the different coat materials possessed good EE and morphological properties, although their high solubility in water limits their use to food products with a low a_w. All the microparticles showed a successful protective effect against LAB consumption when they were added to cottage cheese. NaCas and NaCas-TGase were selected for further analysis as the coat material is formed by casein, which is the major protein present in milk. No great differences were observed when TGase was employed. The functional dairy product developed revealed good texture and digestibility capacity. Thus, an innovative functional product with an increase in the quantity of CaLb of 61.4% with respect to the only such product

currently on the market, has been developed and could potentially be improved by adding a specific probiotic CaLb consumer in order to produce a new synbiotic product.

Acknowledgements

This work was financially supported by the Principality of Asturias, by the project FC-15-GRUPIN14-140. The authors thank Ricardo Alonso Herrero, from Caprinos del Nalón, for providing the milk necessary to manufacture the cottage cheeses.

5. References

Alonso, S., Rendueles, M. & Díaz, M., Bio-production of lactobionic acid: Current status, applications and future prospects, *Biotechnol. Adv*. 31 (2013) 1275–1291. doi:10.1016/j.biotechadv.2013.04.010.

Ashwell, M. Concepts of functional foods, 2002. doi:10.1108/nfs.2004.34.1.47.3.

Burgain, J., Gaiani, C., Cailliez-Grimal, C. Jeandel, C. & Scher, J. Encapsulation of Lactobacillus rhamnosus GG in microparticles: Influence of casein to whey protein ratio on bacterial survival during digestion, *Innov. Food Sci. Emerg. Technol*. 19 (2013) 233–242. doi:10.1016/j.ifset.2013.04.012.

Caleja, C. , Ribeiro, A. , Barros, L. , Barreira, J.C.M. , Antonio, A.L. , Oliveira, M.B.P.P. , Barreiro, M.F. & Ferreira, I.C.F.R. Cottage cheeses functionalized with fennel and chamomile extracts: Comparative performance between free and microencapsulated forms, *Food Chem.* 199 (2016) 720–726. doi:10.1016/j.foodchem.2015.12.085.

Chandrasekar, V., Coupland, J.N. & Anantheswaran, R. C. Characterization of nisin containing chitosan-alginate microparticles, *Food Hydrocoll*. 69 (2017) 301–307. doi:10.1016/j.foodhyd.2017.02.011.

Cilek, B., Luca, A., Hasirci, V., Sahin, S. & Sumnu, G. Microencapsulation of phenolic compounds extracted from sour cherry pomace: Effect of formulation, ultrasonication time and core to coating ratio, *Eur. Food Res. Technol*. 235 (2012) 587–596. doi:10.1007/s00217-012-1786-8.

da Rosa, C. G., Borges, C. D., Zambiazi, R. C., Nunes, M. R., Benvenutti, E. V., da Luz, S. R., D'Avila, R. F. & Rutz, J.K. Microencapsulation of gallic acid in chitosan, β-cyclodextrin and xanthan, *Ind. Crops Prod*. 46 (2013) 138–146. doi:10.1016/j.indcrop.2012.12.053.

Dias, M.I., Ferreira, I. C. F. R. & Barreiro, M. F. Microencapsulation of bioactives for food applications, *Food Funct*. 6 (2015) 1035–1052. doi:10.1039/c4fo01175a.

Everett, D. W. & Auty, M. A. E. Cheese structure and current methods of analysis, *Int. Dairy J.* 18 (2008) 759–773. doi:10.1016/j.idairyj.2008.03.012.

Ezhilarasi, P.N., Indrani, D. , Jena, B.S. & Anandharamakrishnan, C. Microencapsulation of Garcinia fruit extract by spray drying and its effect on bread quality, *J. Sci. Food Agric*. 94 (2014) 1116–1123. doi:10.1002/jsfa.6378.

Fatima, S. W. & Khare, S. K. Current insight and futuristic vistas of microbial transglutaminase in nutraceutical industry, *Microbiol. Res*. 215 (2018) 7–14. doi:10.1016/j.micres.2018.06.001.

FDA. Code of Federal Regulations, Title 21, 21 CFR 172.720. US Food and Drug Administration, 2017.

Foegeding, E. A. & Drake, M. A. Invited Review: Sensory and Mechanical Properties of Cheese Texture, *J. Dairy Sci*. 90 (2007) 1611–1624. doi:10.3168/jds.2006-703.

Francisco, C.R.L., Heleno, S.A. , Fernandes, I.P.M., Barreira, J.C.M., Calhelha, R.C. , Barros, L., Gonçalves, O.H., Ferreira, I.C.F.R. & Barreiro, M.F. Functionalization of yogurts with Agaricus bisporus extracts encapsulated in spray-dried maltodextrin crosslinked with citric acid, *Food Chem*. 245 (2018) 845–853. doi:10.1016/j.foodchem.2017.11.098.

García, G., Bautista, L., Rendueles, M. & Díaz, M. A new synbiotic dairy food containing lactobionic acid and Lactobacillus casei, *Int. J. Dairy Technol*. 70 (2018) 1–10. doi:10.1111/1471-0307.12558.

Głąb, T.K. & Boratyński, J. Potential of Casein as a Carrier for Biologically Active Agents, *Top. Curr. Chem.* 375 (2017). doi:10.1007/s41061-017-0158-z.

https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=172.720.

Jack, M. Cheese Analogues, 4 (2011) 814–821.

Jo, Y., Benoist, D.M., Ameerally, A. & Drake, M. A. Sensory and chemical properties of Gouda cheese, *J. Dairy Sci.* (2017) 1–23. doi:10.3168/jds.2017-13637.

Jyothi, N.V.N., Prasanna, P.M., Sakarkar, S.N., Prabha, K.S., Ramaiah, P.S. & Srawan, G. Y. Microencapsulation techniques, factors influencing encapsulation efficiency, *J. Microencapsul*. 27 (2010) 187–197. doi:10.3109/02652040903131301.

Kaushik, V. & Roos, Y.H. Limonene encapsulation in freeze-drying of gum Arabic-sucrose-gelatin systems, *LWT - Food Sci. Technol*. 40 (2007) 1381–1391. doi:10.1016/j.lwt.2006.10.008.

Kiryu, T., Kiso, T., Nakano, H., Ooe, K., Kimura, T. & Murakami, H. Involvement of Acetobacter orientalis in the production of lactobionic acid in Caucasian yogurt ("Caspian Sea yogurt") in Japan, *J. Dairy Sci*. 92 (2009) 25–34. doi:10.3168/jds.2008-1081.

Mihalcea, L., Turturică, M., Barbu, V., Ioniţă, E., Pătraşcu, L., Cotârleţ, M., Dumitraşcu, L., Aprodu, I., Râpeanu, G. & Stănciuc, N. Transglutaminase mediated microencapsulation of sea buckthorn supercritical CO2extract in whey protein isolate and valorization in highly value added food products, *Food Chem*. 262 (2018) 30–38. doi:10.1016/j.foodchem.2018.04.067.

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carrière, F., Boutrou, R., Corredig, M., Dupont, D., Dufour, C., Egger L., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A., Marze, S., McClements, D.J., Ménard, O., Recio, I., Santos, C.N., Singh, R.P., Vegarud, G. E., Wickham, M.S.J., Weiteschies, W. & Brodkorb, A. (2014). A standardised static in vitro digestion method suitable for food-an international consensus. *Food and Function*, *5*(6), 1113–1124. https://doi.org/10.1039/c3fo60702j

Morita, T., Horikiri, Y., Suzuki, T. & Yoshino, H. Preparation of gelatin microparticles by co-lyophilization with poly(ethylene glycol): Characterization and application to entrapment into biodegradable microspheres, *Int. J. Pharm*. 219 (2001) 127–137. doi:10.1016/S0378-5173(01)00642-1.

Ohama, H., Ikeda, H. & Moriyama H., Health Foods and Foods with Health Claims in Japan, *Nutraceutical Funct. Food Regul*. United States Around World Second Ed. 221 (2014) 265–299. doi:10.1016/B978-0-12-405870-5.00016-5.

Pang, Z., Deeth, H., Sopade, P., Sharma, R., & Bansal, N. Rheology, texture and microstructure of gelatin gels with and without milk proteins, *Food Hydrocoll*. 35 (2014) 483–493. doi:10.1016/j.foodhyd.2013.07.007.

Premi, M.& Sharma, H. K. Effect of different combinations of maltodextrin, gum arabic and whey protein concentrate on the encapsulation behavior and oxidative stability of spray dried drumstick (Moringa oleifera) oil, *Int. J. Biol. Macromol*. 105 (2017) 1232–1240. doi:10.1016/j.ijbiomac.2017.07.160.

Sarao, L. K. & Arora, M. Probiotics, prebiotics, and microencapsulation: A review, *Crit. Rev. Food Sci*. Nutr. 57 (2017) 344–371. doi:10.1080/10408398.2014.887055.

Schaafsma, G. Lactose and lactose derivatives as bioactive ingredients in human nutrition*, Int. Dairy J.* 18 (2008) 458–465. doi:10.1016/j.idairyj.2007.11.013.

Soleiman Abbasi M.A. & Somayeh R., Influence of microwave-microencapsulated citric acid on some sensory properties of chewing gum, *J. Microencapsul*. 26 (2009) 90–96.

Stratulat, I., Britten, M., Salmieri, S., Fustier, P., St-Gelais, D., Champagne, C. P. & Lacroix, M. Enrichment of cheese with bioactive lipophilic compounds, *J. Funct. Foods*. 6 (2014) 48–59. doi:10.1016/j.jff.2013.11.023.

Suguri S. S., Yanagidaria S. & Kobayashi T., Bifid bacteria multiplication-stimulating agent, 1995. doi:JP07277990A.

Tello, F., Prata, A.S., Rodrigues, A.R.F. & Sartoratto, C.R.F. Grosso, Improving the performance of transglutaminase-crosslinked microparticles for enteric delivery, *Food Res. Int*. 88 (2016) 153–158. doi:10.1016/j.foodres.2016.02.020.

Wilkinson, C., Dijksterhuis, G. B. & Minekus, M.From food structure to texture, *Trends Food Sci. Technol.* 11 (2001) 442–450. doi:10.1016/S0924-2244(01)00033-4.

Xing, G., Rui, X., Jiang, M., Xiao, M., Guan, Y., Wang, D. & Dong, M. In vitro gastrointestinal digestion study of a novel bio-tofu with special emphasis on the impact of microbial transglutaminase, *PeerJ*. 4 (2016) e2754. doi:10.7717/peerj.2754.

5.2. Alginato de sodio

En este apartado se muestran los dos trabajos desarrollados empleando alginato de sodio como matriz en la elaboración de *coatings* y cápsulas sinbióticas, con ácido lactobiónico como compuesto prebiótico y dos cepas diferentes del género *Lactobacillu*s como microorganismos probióticos.

5.2.1. *Coating* sinbiótico con ácido lactobiónico y *Lactobacillus plantarum* CECT 9567 en la elaboración y caracterización de un nuevo producto lácteo funcional

En este subapartado se recogen los resultados referentes al primer trabajo realizado empleando alginato de sodio como matriz en el desarrollo de *coatings* prebióticos, probióticos y sinbióticos.

En este caso, a los recubrimientos se les incorporó ácido lactobiónico comercial como compuesto prebiótico y *L. plantarum* CECT 9567 como microorganismo probiótico. Los *coatings* se analizaron en un modelo alimentario real, en este caso, queso fresco de cabra. Los quesos fueron recubiertos por el método de inmersión, que es uno de los más habituales en la industria alimentaria cuando el objetivo es recubrir alimentos (Suhag, Kumar, Petkoska, & Upadhyay, 2020).

En los quesos, se estudió la viabilidad del probiótico durante el periodo de almacenamiento, el consumo de ácido lactobiónico por su parte y las propiedades texturales de los quesos con *coatings*. Además, se realizaron pruebas de digestión *in vitro* simulada para conocer el efecto protector de los coatings en los probióticos y si el ácido lactobiónico también era capaz de conferir algún tipo de protección.

- **Publicación:** Sáez-Orviz, S., Puertas, C., Marcet, I., Rendueles, M., Díaz, M. 2020. Bioactive synbiotic coatings with lactobionic acid and *Lactobacillus plantarum* CECT 9567 in the production and characterization of a new dairy product. *Journal of Functional Foods*, 75, 104263, ISSN 1756-4646, (https://doi.org/10.1016/j.jff.2020.104263).
- **Estado:** publicada
- **Índice de impacto:** 4,451 (2020)

Bioactive synbiotic coatings with lactobionic acid and *Lactobacillus plantarum* CECT 9567 in the production and characterization of a new functional dairy product

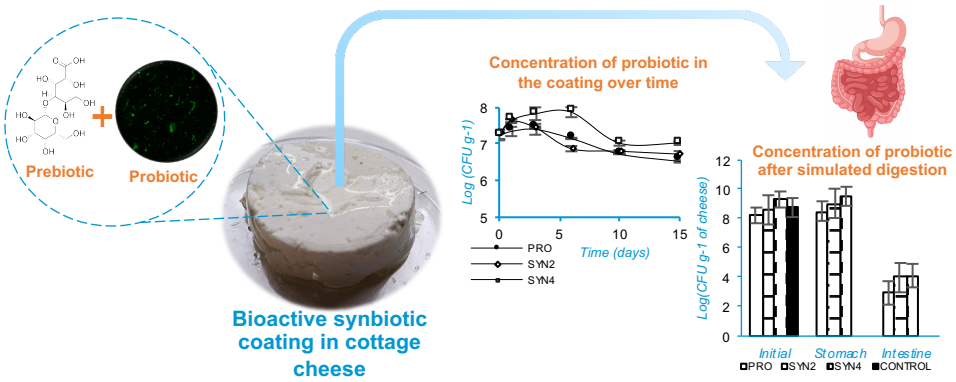
S. Sáez-Orviz[a], C. Puertas[a], I. Marcet[a], M. Rendueles[a], M. Díaz[a]*

[a]Department of Chemical and Environmental Engineering, University of Oviedo, C/Julián Clavería 8, 33006, Oviedo, Spain.

*Corresponding author

E-mail address: mariodiaz@uniovi.es (M. Díaz)

Graphical abstract



Abstract

Lactobionic acid (LBA), a prebiotic with beneficial health properties, can be mixed with a probiotic, *Lactobacillus plantarum* CECT 9567, to prepare novel synbiotic coatings. Coatings deposited on cottage cheese contained either probiotic (PRO, *L. plantarum* CECT 9567), prebiotic (PRE2 and PRE4; 20 and 40 g L^{-1} of LBA, respectively) or synbiotic compositions (SYN2 and SYN4). Coated cottage cheeses were analysed to determine probiotic and LBA concentration changes during storage, their textural properties and investigate the survival of bacteria during simulated digestion. Before the digestion test, PRO, SYN2 and SYN4 met minimal requirements to attain probiotic category and PRE2, PRE4, SYN2 and SYN4 contained adequate quantities of LBA throughout the experiment. Textural properties of cheese samples varied, with changes in the stickiness parameter. The digestion test showed that only SYN2 and SYN4

maintained acceptable probiotic values after simulated digestion, due to the presence of LBA, which increased probiotic survival.

Keywords: synbiotic coating, lactobionic acid, prebiotic, probiotic, functional dairy product, simulated digestion.

1. Introduction

Currently, consumers demand food products that, in addition to satisfying their nutritional demands, have the ability to improve their health and/or reduce the risk of certain diseases. This demand has led to the development and production of functional foods which incorporate bioactive compounds such as prebiotics and probiotics (Batista et al., 2017) (Ashwell, 2002).

Prebiotics comprise a wide range of compounds, within which, lactose derivatives such as lactobionic acid (LBA) have generated great interest. This compound is very useful in the food industry, as it is used as an antioxidant, moisturizer, chelating agent (Cardoso, Marques, Dagostin, & Masson, 2019) and as a prebiotic (Alonso, Rendueles, & Díaz, 2013). Furthermore, LBA is resistant to digestive enzymes and is badly absorbed in the small intestine, so it can be metabolised by gastrointestinal microflora (Saarela, Hallamaa, Mattila-Sandholm, & Mättö, 2003) (Schaafsma, 2008). There are very few studies into commercial food products with LBA (García, Bautista, Rendueles, & Díaz, 2018) (Sáez-Orviz, Camilleri, Marcet, Rendueles, & Díaz, 2019), although one of the most noteworthy investigates “Caspian Sea yogurt” (Kiryu et al., 2009), which was traditionally produced in the Caucasian region before its arrival in Japan, more than 30 years ago. The amount of LBA ingested when consuming this yogurt is between 0.5 and 1.0 g per year, and no harmful effects have been noted in the population (Kiryu et al., 2009). Nonetheless, at the moment, LBA has only been approved by the Food and Drug Administration (FDA) for its use in food in its salt form (*FDA. Code of Federal Regulations, Title 21, 21 CFR 172.720. US Food and Drug Administration*, 2017) (Cardoso et al., 2019) (Alonso et al., 2013), although future approval of LBA by the food authorities is expected.

Focussing now on probiotics, these can easily be added to a variety of food products, the most frequently chosen being those with a dairy matrix. Among the most commonly used probiotics are those belonging to the genera *Lactobacillus* and *Bifidobacterium* (Espitia, Batista, Azeredo, & Otoni, 2016). Furthermore, if probiotic strains and prebiotic compounds are mixed in the same product, the result is a synbiotic product. There are many dairy products in which probiotic and prebiotic are added together in milk before

the cheese is made, in order to obtain a synbiotic product directly (Langa et al., 2019). However, in the case of LBA this process is impractical due to its high solubility in water (Cardoso et al., 2019) (Alonso et al., 2013), which means that during the cheese curdling process this compound tends to be lost to the whey and does not get trapped in the curd. Therefore, an alternative means of obtaining a synbiotic functional food product through the incorporation of LBA in cheese is the use of bioactive coatings containing this prebiotic. Bioactive coatings allow different types of functional products to be developed, as they are very versatile (Espitia et al., 2016) (Pavli et al., 2017). These types of coatings are an excellent option for maintaining the viability of the probiotic strain and also allowing the preservation of LBA in the food product.

In this regard, there are very few studies on food products with LBA (García et al., 2018) (Sáez-Orviz et al., 2019), other than the few cases indicated previously. Therefore, the aim of this study is to develop and characterize a synbiotic coating, containing both a probiotic strain (*L. plantarum* CECT 9567) and a prebiotic compound (LBA), to take advantage of the properties of LBA. This coating is tested in cottage cheese, a real food model, with assessment of the LBA and probiotic concentrations in the coatings during storage time, the influence of the coating composition on the textural properties of the cheese and the effect of the LBA concentration in the coating on the protection offered to the probiotic bacteria as they pass through a simulated gastrointestinal tract (GIT).

2. Materials and methods

2.1. Probiotic microorganism and growth conditions

Lactobacillus plantarum CECT 9567 (from the Spanish Type Culture Collection, Valencia, Spain) was used as a probiotic strain and was maintained frozen (in 40% v/v glycerol solution at -20 ºC). It was propagated on MRS (de Man, Rogosa and Sharpe, Steinheim, Germany, Sigma-Aldrich) agar plates, incubated for 48 h at 37 ºC and then stored at 4 ºC. As the microorganism is aerobic, its culture was carried out in liquid medium in a 500 mL Erlenmeyer flask containing 100 mL of MRS broth (medium volume to air ratio of 1:4), which was incubated in an orbital shaker at 200 rpm and at a temperature of 37 ºC for 12 h.

2.2. Cottage cheese manufacture

Goat milk was supplied by a local farm from San Martín del Rey Aurelio (Asturias, Spain). Milk was pasteurised with a low temperature-long time (LTLT) procedure (at a temperature of 60 ºC for 25 min). After pasteurization, milk was cooled to 34 ºC and rennet was added (0.0025 g L^{-1}, Chy-Max®, CHR-Hansen, Denmark). No starter culture

was added due to the soft pasteurization process that allows some lactic acid bacteria to survive. After incubating milk at 34 ºC for 40 min, the curd was cut multiple times to stimulate syneresis. Finally, cottage cheese samples were made with 10 g of curd per piece.

2.3. Bioactive coating preparation

Cottage cheese was left for one hour at 4 ºC and then given different coatings by a dipping process. The film-forming solution was prepared as follows. Sodium alginate (Sigma-Aldrich) was dissolved in distilled water to a concentration of 20 g L^{-1} and this solution was heated at 70 ºC and kept in agitation at 700 rpm until a clear, homogeneous colour was obtained. Glycerol (Panreac S.A., Barcelona, Spain) was then added to reach a concentration of 15 g L^{-1} in the final volume and the coating-forming solution was cooled to room temperature. On the basis of previously performed preliminary experiments, for the prebiotic and synbiotic coatings LBA was added to the film-forming solution at a high concentration (40 g L^{-1}, PRE4 sample) and at a medium concentration (20 g L^{-1}, PRE2 sample), taking into consideration the amount of alginate in the film-forming solution. Then, this mixture was homogenized by stirring at 500 rpm at room temperature. In order to prepare the synbiotic coatings, after 12 hours of growth, the *L. plantarum* culture was centrifuged at 13200 rpm for 10 min and the pellet was added to the coating-forming solutions PRE2 and PRE4 to reach a concentration of 10^9 CFU mL^{-1} in the final volume, thus forming the coatings SYN2 and SYN4. In addition, a probiotic alginate-based coating (PRO) with a concentration of *L. plantarum* of 10^9 CFU mL^{-1} but without LBA, and a negative control with only the alginate coating (NC2), were prepared. All the prepared coatings and their formulations are shown in Table 5.3.

Table 5.3. The different cottage cheeses and their coatings.

Cottage cheese	Coating-forming solution	Lactobionic acid (LBA)	*L. plantarum* CECT 9567
Negative control 1 (NC1)	No	-	-
Negative control 2 (NC2)	Yes	-	-
Prebiotic cheese (PRE2)	Yes	20 g L^{-1}	-
Prebiotic cheese (PRE4)	Yes	40 g L^{-1}	-
Probiotic cheese (PRO)	Yes	-	10^9 CFU mL^{-1}
Synbiotic cheese (SYN2)	Yes	20 g L^{-1}	10^9 CFU mL^{-1}
Synbiotic cheese (SYN4)	Yes	40 g L^{-1}	10^9 CFU mL^{-1}

Once all the solutions were prepared, the cheese was submerged in the coating solution for 2 minutes, and afterwards it was left at room temperature for 1 minute to remove the excess solution. To harden the coating, the cottage cheese was immersed for 2 minutes in $CaCl_2$ solution (5% w/v, Merk KGaA, Darmstadt, Germany). Finally, the pieces were dried at room temperature for 30 minutes and then stored in a sterile container at 4 ºC for subsequent analysis (Bambace, Alvarez, & Moreira, 2019). A negative control cottage cheese sample with no kind of coating was also prepared (NC1). All the experimentation was carried out under sterile conditions to avoid external contamination. A total of 18 pieces of cottage cheese of each type of coating were prepared.

2.4. Effect of storage time on probiotic counts and lactobionic acid concentration in the coatings

The growth of microorganisms inside the coatings (PRO, SYN2 and SYN4 cottage cheese samples) and the LBA concentration (PRE2, PRE4, SYN2 and SYN4 cottage cheese samples) were followed for 15 days, sampling taking place at day 0, and on days 1, 3, 6, 10 and 15. Three independent cottage cheese samples with each type of coating were tested at each time. The sampling was adapted from Pavli *et al.* (2017) and performed as follows. A piece of coating (1 cm^2) was peeled from the cottage cheese and washed with sterile distilled water in order to eliminate other lactic acid bacteria. The coatings were placed in a Stomacher™ bag (Seward, UK) with 1 mL sodium citrate 1% (pH 6.0) (Sigma-Aldrich) and heated for 30 minutes to 40 ºC to break them down. The progress of microbial growth was analysed by making serial dilutions (1:10) (Pavli et al., 2017) employing NaCl 0.7% (w/v) (Sigma-Aldrich) and incubating on MRS agar plates for 48 h at 30 ºC. The size and weight of the cottage cheeses samples were previously measured, which allowed the grams of cottage cheese per cm^2 of coating to be calculated and the expression of the results in CFU g^{-1} of cheese. The LBA content was measured according to Sáez-Orviz *et al.* (2019) using High Performance Liquid Chromatography equipment (HPLC) (Agilent 1200, Agilent Technologies Inc., Santa Clara, CA, USA). A Coregel ION 300 column (Teknocroma, Barcelona, Spain), coupled to a refractive index detector (at a temperature of 40 ºC) was employed. Sulphuric acid solution (0.0450 mM L^{-1}, pH 3.1) was used with a flow rate of 0.3 mL min^{-1} and a column temperature of 75 ºC. Data acquisition and analysis were performed with ChemStation software (Agilent). The microorganism and LBA concentrations were expressed per gram of coated cheese.

2.5. Mechanical properties of the coated cheese

The texture was analysed with a TA.XTplus Texture Analyzer (Stable Systems, Godalming, Surrey, UK). Cottage cheese samples were subjected to a penetration test, at room temperature. The spherical probe employed was SMS P/0.5S with a test speed of 2.0 mm s^{-1} and a 5 kg load cell. Results are expressed in terms of firmness and stickiness values (grams). Three independent cottage cheese samples with each type of coating and NC1 were analysed in triplicate. The reported results correspond to the mean value.

2.6. Distribution of *Lactobacillus plantarum* CECT 9567 in the coatings

In order to determine the distribution of the probiotic organisms inside the bioactive coatings, they were analysed using fluorescence microscopy. For sampling purposes, a fragment of each of the different coatings (PRO, SYN2 and SYN4) was taken, as well as another from a control cheese covered exclusively with the coating-forming solution with no pro- or prebiotic (NC2). These fragments were washed with distilled water twice and then placed for 3 minutes in a solution containing 0.1% acridine orange (Sigma-Aldrich), previously dissolved in 67 mM phosphate buffer (pH 6.0). Samples were washed with phosphate buffer for 1 min and then placed for 30 s in 100 mM $CaCl_2$. Finally, the samples were dried, mounted on a slide and sealed and observed with a Leica TCS-SP-AOBS spectral confocal laser microscope ($\lambda_{excitation}$ 480 nm, $\lambda_{emitting}$ 508-603 nm). Photographs were taken at day 0 and day 7 (from day of cheese making), after which point no further differences were observed.

2.7. Simulated digestion test of the bioactive coatings

A simulated digestion test of the coatings of the PRO, SYN2 and SYN4 cheese samples was performed only to check the survival of the probiotic, as LBA is a non-digestible fibre, resistant to human digestive enzymes (Cardoso et al., 2019). The concentration of microorganisms in the coatings was determined after each digestive fluid test and calculated per gram of coated cheese. In addition, to determine the survival of the free bacteria in the digestive fluids, an MRS broth culture with a concentration of probiotic of 10^9 CFU mL^{-1} was also tested (control sample).

Gastric and intestinal conditions were simulated according to Minekus et al. (2014) with some modifications. The composition of the simulated gastric fluid (SGF) employed was 0.517 g L^{-1} KCl, 0.123 g L^{-1} KH_2PO_4, 2.106 g L^{-1} $NaHCO_3$ and 2.75 g L^{-1} NaCl. Simulated intestinal fluid (SIF) was prepared by mixing 0.509 g L^{-1} KCl, 0.110 g L^{-1} KH_2PO_4, 11.68 g L^{-1} $NaHCO_3$ and 2.24 g L^{-1} NaCl (all from Sigma-Aldrich).

Firstly, the concentration of microorganisms in the coatings was checked at time zero, before the gastric and intestinal digestion were carried out. For that purpose, an area of 10 cm^2 of coating was peeled from the cottage cheese samples and analysed as described below. Three independent samples with each type of coating were analysed.
In order to simulate the gastric digestion of the coatings, coating samples peeled from the surface of coated cheeses (~ 10 cm^2) were mixed with 18.4 mL SGF, 1.6 mL pepsin (863 U mg^{-1} protein, CAS 9001-75-6, Sigma-Aldrich) with a concentration of 15.15 g L^{-1}, using SGF as solvent, 5 µL of $CaCl_2$ 0.3 M (Sigma-Aldrich) and 0.696 µL of distilled water. The pH was adjusted to 3.0 with 1 M HCl (Sigma-Aldrich). Then, the mixtures were shaken at 300 rpm and 37 ºC for 90 minutes and coating samples (measuring 1 cm^2) were collected at the end of the gastric phase. In the case of the control sample, 1 mL of the bacterial suspension was added directly to the gastric fluid prepared as explained above and was incubated at the same conditions of agitation and temperature. After the simulated gastric digestion 1 mL of fluid was collected.
After the gastric simulation, intestinal simulation was carried out. For that purpose, 12.5 mL of SIF, bovine chymotrypsin (0.3% w/v) (60 U mg^{-1} protein, EC 232-671-2, Sigma-Aldrich), porcine pancreatin (0.1% w/v) (Sigma-Aldrich), 40 µL of 0.3 M $CaCl_2$ and 1.3 mL of distilled water were added to the fluids resulting from the gastric digestion. The pH was adjusted to 7.0 using 1 M NaOH (Sigma-Aldrich) and the mixtures were shaken at 300 rpm and 37 ºC for 2 h. At the end of the intestinal digestion, coating samples (1 cm^2) were collected and analysed. In the case of the control, 1 mL of the intestinal fluid was collected at the end of this treatment.
All coating samples (taken at time zero, and then after the gastric digestion and after the intestinal digestion) were broken down by immersion for 30 minutes at 40 ºC in 20 mL of a 1% (pH 6.0) sodium citrate solution. An aliquot of 1 mL was taken from every sample tested and serial dilutions (1:10) with NaCl 0.7% (w/v) were incubated on MRS agar plates (30 ºC for 48 h). Regarding the control, the 1 mL of sample of fluid collected after the gastric and intestinal digestion was centrifuged (10000 rpm, 10 min), the pellet resuspended in 20 mL of NaCl 0.7% (w/v) and incubated on MRS agar plates. Each sample was carried out in triplicate. Plates were incubated at 30ºC for 48 h. Results were expressed in CFU g^{-1}.

2.8. Statistical analysis

All experiments were carried out in triplicate with three independent batches of coated cottage cheese and the reported results correspond to the mean value. Analysis of variance (ANOVA) was applied. The method used to determine significant differences

between the data is Fischer's Least Significant Difference (LSD) procedure. The analysis was performed using Statgraphics 18® Centurion statistical software.

3. Results and discussion

3.1. Growth and viability of *Lactobacillus plantarum* CECT 9567 in the bioactive coating during storage

The number of viable bacteria in the coating during storage is of the greatest importance for the development of a functional product, because if their level of survival is not sufficiently high, the food product cannot be considered probiotic. There is no consensus on the specific number of microorganisms that must be consumed to obtain a beneficial effect, but the minimum estimated quantity is 10^6 CFU mL^{-1} or g^{-1} of product (Pavli et al., 2017) (Angiolillo, Conte, Faccia, Zambrini, & Nobile, 2014) (Kechagia et al., 2013) (Aureli et al., 2011).

Results for probiotic growth during storage are shown in Figure 5.6, which indicates the initial microbial growth observed in every sample tested, followed by a decrease stage and a final stabilisation stage at the end of the experiment. PRO, SYN2 and SYN4 cheese samples reached a final concentration of 6.53, 6.72 and 7.23 log CFU g^{-1}, respectively. The best result was obtained with SYN4, showing significant differences with PRO and SYN4 cheeses ($P < 0.05$). In addition, as they reach the established minimum required concentration, all these coated cheeses would attain the category of "probiotic" during the storage period analysed.

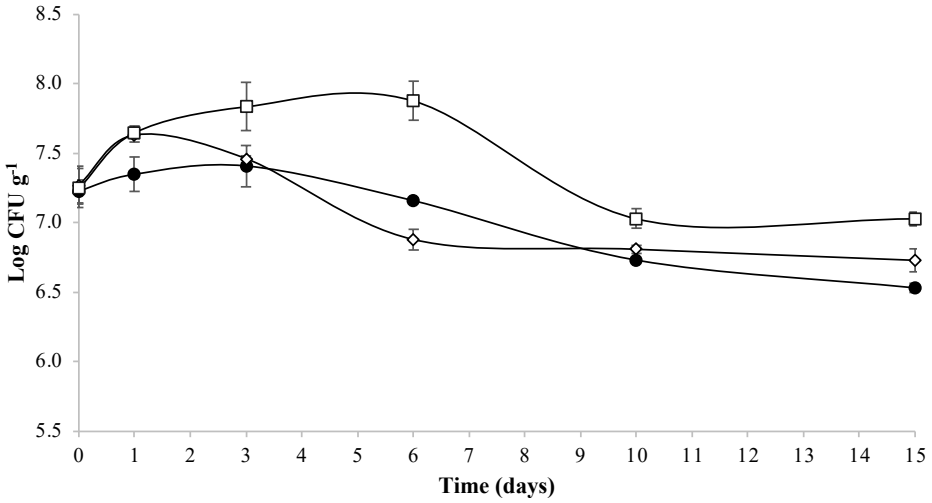


Figure 5.6. Growth of *L. plantarum* CECT 9567 in the coating during 15 days of storage. The concentration of microorganisms is represented as log CFU per gram of cheese. (●) probiotic cottage cheese (PRO), (◊) synbiotic cottage cheese with 2% of LBA (SYN2), (□) synbiotic cottage cheese with 4% LBA (SYN4). Experiments were carried out in triplicate and reported results correspond to the mean value.

Furthermore, it must be borne in mind that sodium alginate is a good matrix for maintaining the viability of the probiotic. There are a number of studies in which this polysaccharide has been used as a coating in different types of food products using different species of probiotic microorganisms. For example, different species of *Lactobacillus*, such as *L. plantarum* B282*, L. plantarum* L125 and *L. pentosus* L33, were added to an alginate-based film-forming solution to produce coatings that were used to cover ham, obtaining values over 6 log CFU g^{-1} after 70 days of sampling (Pavli et al., 2017).

The concentration of LBA in the coatings was analysed during 15 days of storage and the results are shown in Figure 5.7, where it can be observed that there is a rapid decline in the LBA concentration during the first three days of the experiment with the PRE2 and PRE4 samples, which may be due to the lactic acid bacteria present in the cottage cheese. If some of the LBA is accessible to these bacteria, they may be using it as a substrate. However, from day 4, the concentration remains constant in both these cases (0.95 mg LBA g^{-1} of cheese for PRE2 and 1.13 mg LBA g^{-1} of cheese for PRE4). In the synbiotic cheeses (SYN2 and SYN4), the concentration remains constant from the beginning of the experiment and throughout it in both coatings (0.912 mg LBA g^{-1} of cheese for SYN2 and 1.07 mg LBA g^{-1} of cheese for SYN4 at the end of this experiment). In any case, the amount of LBA that would be ingested with the coated cheese samples PRE2, PRE4, SYN2 and SYN4 at the end of the storage time would fall within the usual intake values measured in Japan with the “Caspian Sea yogurt” (Kiryu et al., 2009) and are far lower than the values that would cause lactose intolerance-like effects (24 g LBA day^{-1}) (Cardoso et al., 2019).

It must also be pointed out that the concentration of LBA is lower for SYN samples than for PRE samples at the beginning of storage time, with significant differences at zero time ($P < 0.05$) (Figure 5.7). These observed differences may occur due to the consumption of LBA by *L. plantarum* during the preparation of the coated cheese samples. The initial concentration of this bacteria in the coating solution could be considered high (9 log CFU mL^{-1}) and the LBA is the only carbon source that can be consumed by the microorganisms in the coat-forming solution. Although Figure 5.6 shows no difference in the initial concentration of the probiotic in PRO, SYN2 and SYN4 samples, it should be noted that only viable cultivable bacteria were being counted, and the improvement in the growth caused by the presence of LBA is particularly noticeable during the first day of storage. The following days, the concentration of LBA in the SYN2 and SYN4 coatings remains constant, which is likely to be because the remaining LBA

is not accessible to the bacteria, or because the bacteria cannot continue to grow because of space limitation in the coatings.

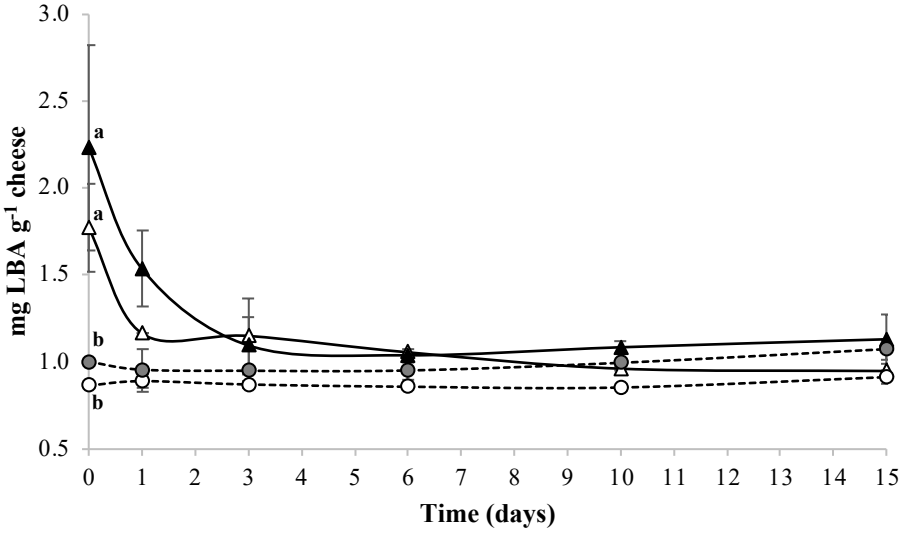


Figure 5.7. Changes in the concentration of LBA in the coating during 15 days of storage. LBA concentration is represented as mg of LBA per g of cheese. (△) prebiotic cottage cheese with 2% of LBA (PRE2), (▲) prebiotic cottage cheese with 4% LBA (PRE4), (○) synbiotic cottage cheese with 2% LBA (SYN2), (●) synbiotic cottage cheese with 4% LBA (SYN4). There are only significant differences ($P < 0.05$) between PRE2/PRE4 and SYN2/SYN4 at time 0. Different letters indicate significant differences at the same storage time. Experiments were carried out in triplicate and reported results correspond to the mean value.

These results confirm that prebiotics can be incorporated into the coatings to improve the stability and viability of the probiotic strains. In the case of the higher concentration (SYN4), the presence of LBA exerted a positive effect, since at the last sampling time of the experiment an increase was observed in the concentration of probiotic in SYN4 samples of 80.11%, as compared with the probiotic concentration in PRO cottage cheeses.

The increase in the viability of probiotics in coatings when prebiotics are present has been observed by other authors. In particular, similar results were obtained with cheeses coated with sodium alginate and FOS (fructo-oligosaccharides) as a prebiotic and using lactic acid bacteria as the probiotic bacteria. The presence of FOS made it possible to improve the survival of the probiotic, which is capable of using this compound as a substrate, maintaining a concentration of 10^9 CFU 100 g^{-1} of cheese when employing *Lactobacillus rhamnosus* GG as the probiotic (Angiolillo et al., 2014). There are also examples of the beneficial effect of prebiotics in sodium alginate bioactive coatings employed in non-dairy products, specifically, in products such as blueberries. In this case, the sodium alginate coatings were enriched with inulin and

oligofructose as FOS, which led to an increase in the viability of *L. rhamnosus* CECT 8361 compared to the results in coatings that lacked prebiotics (Bambace et al., 2019). In other food products, such as fresh-cut apples, sodium alginate bioactive coatings had inulin and oligofructose as prebiotics and *L. rhamnosus* GG as the probiotic. Using these coatings, pieces of apple with a bacterial concentration of 10^8 CFU g^{-1} were obtained, which is enough to attain a probiotic effect (Rößle, Brunton, Gormley, Ross, & Butler, 2010). Therefore, the mixture of prebiotics and probiotics in coatings makes it possible to obtain improved products that are beneficial to consumers because prebiotics enable higher persistence of probiotics in the foodstuffs. There are several studies on the preparation of synbiotic goat's milk products. Most of them investigate yoghurt and ice cream, with very little research having been devoted to cheese (Verruck, Dantas, & Schwinden, 2019), the subject of the work described here.

3.2. Textural characterization

To develop a new foodstuff, it is essential to study and analyse its structure in order to understand and improve its texture, which is an important factor in the sensory perception of consumers (Wilkinson, Dijksterhuis, & Minekus, 2001) (Zhong, Cavender, & Zhao, 2014). Texturometry allows different parameters, like firmness and stickiness, to be measured and in this case, the results of the textural characterization of the coated cheeses are shown in Figure 5.8.

Although there is variability in terms of firmness between the different types of coatings, the highest average values were found in NC1 coated cheeses (Figure 5.8-A). The mean value for this parameter for uncoated cottage cheese (NC1) increased after three days, and then remained constant over time, which suggests a higher loss of water during the first three days of the experiment for this uncoated sample in comparison with the coated cheese tested. This evidence corroborates the positive influence of such coatings on the preservation of moisture, and their role in delaying the hardening process when they are used to coat pieces of cheese (Zhong et al., 2014) (Ramos et al., 2012) (Costa, Maciel, Teixeira, Vicente, & Cerqueira, 2018). In the other coated samples, although there are statistically significant differences between some of the samples tested for this parameter, the similar average values obtained suggest that the presence of LBA or *L. plantarum* did not produce any clear differential effect on the firmness of the samples assessed (Figure 5.8-A).

Regarding the stickiness, there are differences depending on the sample and on the storage time (Figure 5.8-B). At zero-time there are no significant differences between the samples. As the period of storage time increased, the loss of water due to the

ripening of the cheese caused changes in the stickiness and it was observed that LBA and the probiotic noticeably affected this parameter. PRO showed the highest stickiness values obtained after 3 and 15 days of assay. When LBA was incorporated together with *L. plantarum*, the pieces of cheese assessed (SYN2 and SYN4) showed higher values for this parameter than PRE2 and PRE4 samples, but not as high as those values for the PRO coating. It suggests that the presence of the probiotic bacteria produced an increase in the sample's stickiness, maybe owing to the excretion of cellular metabolites or the growth of the microorganism population embedded within the coating, but this trend was at least partially limited by the incorporation of LBA in the coating composition.

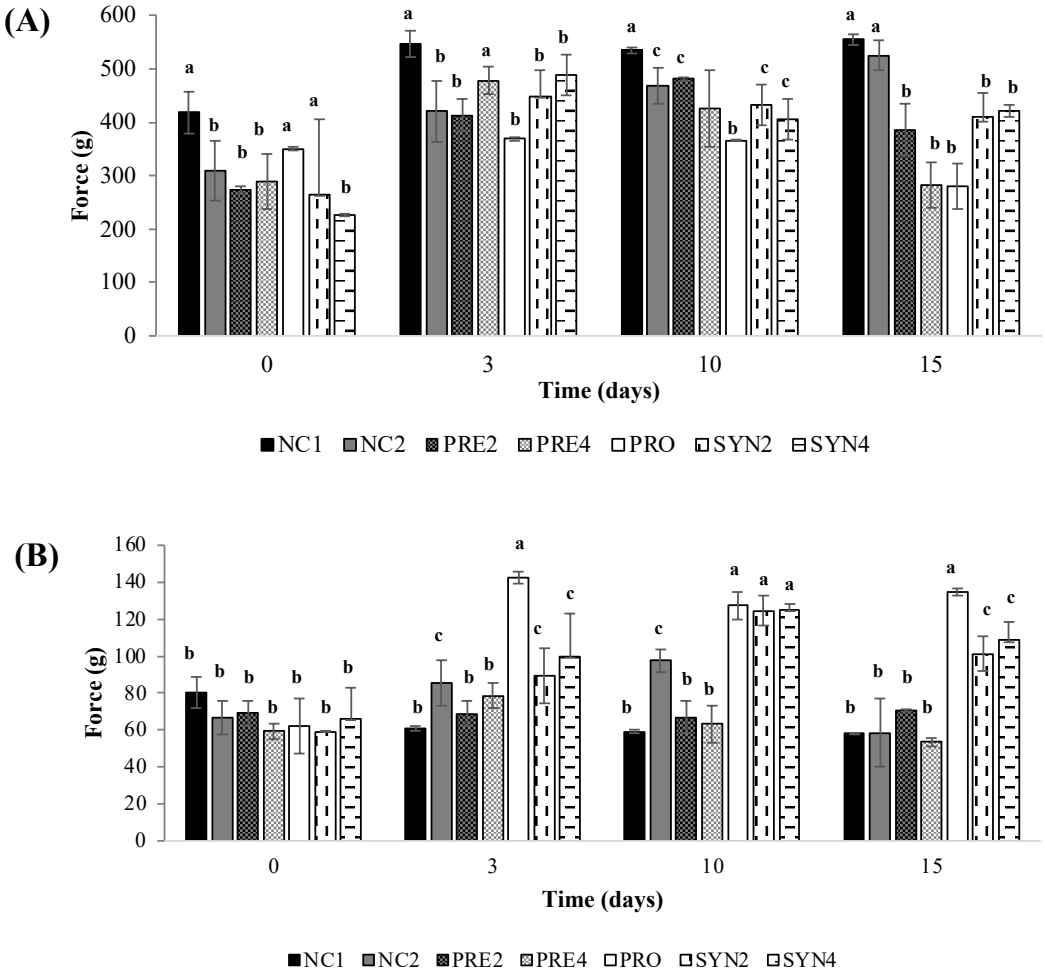


Figure 5.8. Textural analysis of coated cottage cheeses; (A) firmness analysis and (B) stickiness analysis (data represented as absolute value) with the standard deviation. Both are expressed in force units (g). Different letters indicate significant differences at the same storage time ($P < 0.05$). Experiments were carried out in triplicate and reported results correspond to the mean value.

3.3. Status of *Lactobacillus plantarum* CECT 9567 in the bioactive coatings

A fluorescence microscopy technique was used to observe *L. plantarum* CECT 9567 inside the probiotic and synbiotic coatings. A coating without any bacteria was also analysed as a negative control. Results at time 0 and after 7 days of storage at 4 ºC are shown in Figure 5.9.

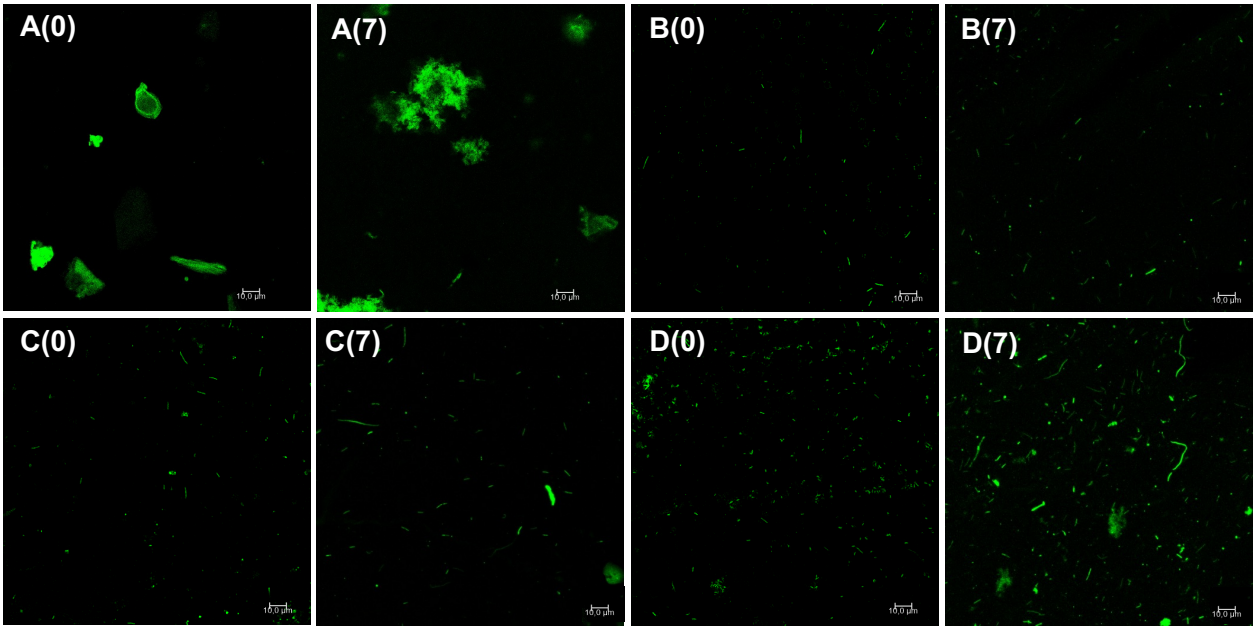


Figure 5.9. Fluorescence microscopy images of the coatings at zero time and after seven days of storage at 4 ºC. (A) Negative control. (B) PRO, (C) SYN2 and (D) SYN4 coatings. Numbers 0 and 7 in the images refer to each of the sampling times.

The visual appearance of the coatings was transparent and similar in all cases. As expected, no bacteria were observed in the negative control, but some large, amorphous structures were found. This is because acridine orange is capable of staining certain biological compounds, such as proteins and fat molecules (Yiu, 1985). Specifically, in the case of matrices such as cheese, acridine orange is used especially for dyeing proteins (Heilig, Göggerle, & Hinrichs, 2009). Therefore, the voluminous structures observed could be caseins that may remain attached to the film as these structures were also observed in the images of the probiotic and synbiotic coatings.

Fluorescent bacillus-shaped structures were differentiated, indicating the presence of *L. plantarum* inside the coatings. At time 0, the amount of *L. plantarum* observed in the PRO and SYN coating samples was quite similar. After 7 days of storage, a higher number of microorganisms were observed in the films. More bacteria were observed in the SYN coatings compared to the probiotic one, illustrating the synergic effect existing between *L. plantarum* CECT 9567 and LBA. Comparing the SYN2 and SYN4 coatings,

the one with 4% LBA showed a higher number of microorganisms, a result that agrees with the data in Figure 5.6.

3.4. Simulated digestion of the bioactive coatings

Probiotics and prebiotics have the capacity to modify the GIT microflora by enhancing the growth of beneficial bacteria. Probiotics act mostly in the small intestine, while prebiotics usually act in the colon (Lopez-Rubio, Gavara, & Lagaron, 2006). In order to be effective as a probiotic, bacteria must withstand gastric juices and be able to proliferate in the intestine. In this case, to test the protective effect on *L. plantarum* CECT 9567 of the alginate-based cheese coatings, the cottage cheese samples PRO, SYN2 and SYN4 were assessed in a simulated digestion test. *L. plantarum* CECT 9567 without coating (9 log CFU mL^{-1}) was also used as control (Figure 5.10).

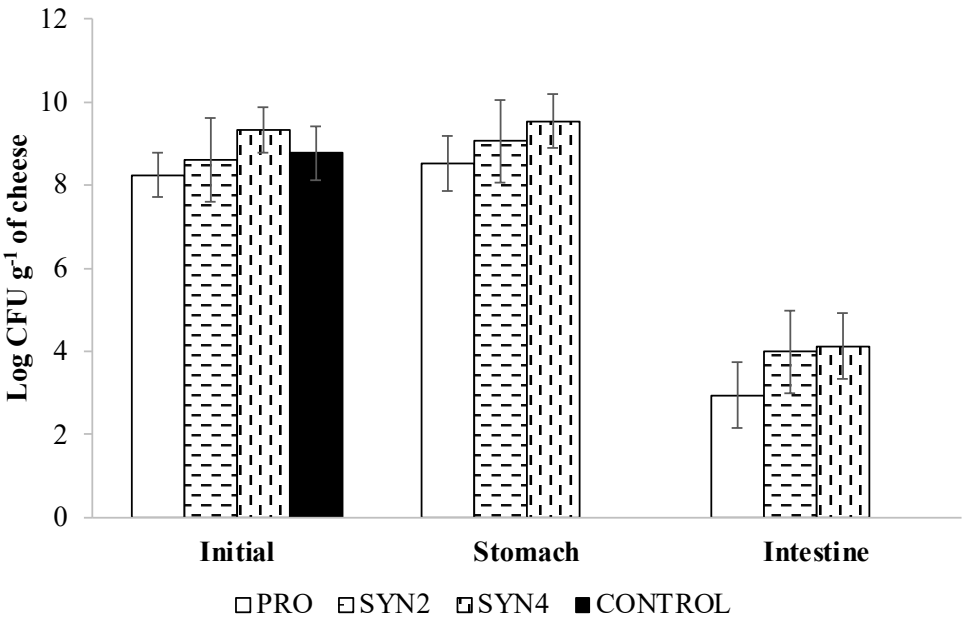


Figure 5.10. Concentration of probiotic (log CFU g^{-1} of cheese) throughout the digestion test. "Initial" refers the concentration of microorganism before the digestion trial. Experiments were carried out in triplicate and reported results correspond to the mean value.

At the initial time, the microbial concentration was similar for every coating, in the range of 8-9 log CFU g^{-1}. After the digestive simulation of the stomach, no survival of the microorganism without coating was observed. It is known that in the gastric phase, the long exposure to an acid pH, together with the presence of pepsin, cause the death of microorganisms (Chan & Zhang, 2005). However, the bacteria inside PRO, SYN2 and SYN4 coatings did survive, with a mean value of 8.53, 9.06 and 9.54 log CFU g^{-1},

respectively, showing that the coating exerted a protective effect. Sodium alginate is a water-soluble polymer but with a limited solubility at low pH values (Parreidt, Müller, & Schmid, 2018). The use of $CaCl_2$ could increase the insolubility of sodium alginate, due to the ability of calcium to bind cell wall polymers, making them stronger and allowing microorganisms to remain alive embedded in the coating during digestion in the stomach.

After the intestinal simulation, the probiotic load decreased abruptly. The change of the pH (~pH 7.0) and the presence of different enzymes may have affected the integrity of the coating. After 2 hours, the PRO coating showed a mean concentration of microorganisms of 2.95 log CFU g^{-1}, SYN2 of 3.99 log CFU g^{-1} and SYN4 of 4.13 log CFU g^{-1} (Figure 5.10). As the initial bacterial concentration in the PRO, SYN2 and SYN 4 coatings was different, the microbial reduction (in log CFU g^{-1}) between the gastric phase and the intestinal phase was statistically analysed. The average microbial reduction was 5.60± 0.12, 5.01± 0.23 and 5.21± 0.17 log CFU g^{-1} in PRO, SYN2 and SYN4, respectively. It was found that there were significant differences between the SYN2-SYN4 and PRO samples ($p < 0.05$). Therefore, the viability was higher for the coatings that included LBA in their composition (SYN2 and SYN4), which may be because the addition of prebiotics increases the resistance of microorganisms in the GIT environment (Lopez-Rubio et al., 2006). In support of this idea, there are many studies describing the improvement in the viability and in the tolerance of probiotics to simulated *in vitro* GIT conditions when prebiotics such as FOS and GOS (galacto-oligosaccharides) are included in the food composition (Langa et al., 2019) (Padilha, Morales, Vieira, Costa, & Saad, 2016) (Krasaekoopt & Watcharapoka, 2014) (Ranadheera, Baines, & Adams, 2010) (Orozco-Parra, Mejía, & Villa, 2020). In the experiments carried out here, LBA seems to have the same effect as the other more frequently studied prebiotics.

Furthermore, it is important to bear in mind that probiotics are only effective if the dosage is sufficiently high. Although there is no consensus within the international scientific community about a specific concentration necessary to obtain beneficial health effects, the minimum dosage is between 10^6-10^9 CFU per day (Espitia et al., 2016) (Saad, Delattre, Urdaci, Schmitter, & Bressollier, 2013), but these bacteria must resist passage through the digestive tract in order to modify the GIT microflora. The recommended daily fresh cheese intake is between 80 and 125 grams (Carcamo G. and Mena C., 2006), and therefore, taking into consideration the digestion testing results for the coatings and a portion of 125 g, the only coated cheeses tested here that meet the

conditions to qualify as probiotic after *in vitro* digestion are SYN2 and SYN4, with values of 9.24±0.56 and 9.56±0.55 log CFU portion^{-1} (before digestion) and 6.89±0.81 and 7.03±0.80 log CFU portion^{-1} (after *in vitro* digestion), respectively (Table 5.4). Regarding LBA, as it is a prebiotic, it is a non-digestible fibre and is resistant to human digestive enzymes (Cardoso et al., 2019), reaching the large intestine intact (Sáez-Orviz et al., 2019).

As can be seen from the results obtained, PRO, SYN2 and SYN4 would meet the minimum legal requirements to attain the probiotic category but only the synbiotic ones (SYN2 and SYN4) would also have the capacity to deliver the adequate probiotic dose to the GIT.

Table 5.4. Concentration of probiotic in a portion (125 g) of coated cottage cheese after and before *in vitro* digestion.

	Concentration of probiotic before *in vitro* digestion (log CFU portion^{-1})	Concentration of probiotic after *in vitro* digestion (log CFU portion^{-1})
PRO	8.95 ± 0.53	5.84 ± 0.80
SYN2	9.24 ± 0.56	6.89 ± 0.81
SYN4	9.56 ± 0.55	7.03 ± 0.80

4. Conclusions

The development of bioactive coatings to prepare synbiotic functional products with LBA and *L. plantarum* CECT 9567 was carried out successfully. PRO, SYN2 and SYN4 coated cottage cheeses meet the minimum legal requirements (10^6 CFU g^{-1} cheese) to attain the category of probiotic. PRE2, PRE4, SYN2 and SYN4 cheeses contain an adequate amount of LBA, equivalent to the regular intake of the only product containing LBA on the market ("Caspian Sea yogurt"). Texturometry experiments showed that the uncoated cottage cheeses were firmer and those with a probiotic coating showed slightly higher stickiness, although no great variations between samples were detected. After the simulated digestion tests, it was found that SYN2 and SYN4 cheeses were the only ones that would have the capacity to deliver an adequate number of probiotic organisms to the lower GIT, which leads to the conclusion that LBA increased the viability and tolerance of the probiotic as it passed through the GIT. With a view to further investigation, it is important to state that organoleptic properties, such as colour, taste, smell and texture are some of the most important parameters for new products to be accepted by consumers, so it would be of great interest to carry out a sensory study on these cheeses in the future. Therefore, the development of bioactive coatings,

in the case of SYN2 and SYN4, makes it possible to obtain synbiotic, functional and innovative cottage cheeses that could, from a nutritional point of view, have a high level of acceptance by consumers.

Declarations of interest

None.

Acknowledgements

This work was financially supported by the Principality of Asturias, by the project GRUPIN IDI/2018/000127 and by the grant *Programa Severo Ochoa de Ayudas Predoctorales para la investigación y docencia* (grant number BP19-127 to Sáez-Orviz, S.). The authors would like to thank Ricardo Alonso Herrero, from Caprinos del Nalón, for providing the milk necessary to manufacture the cottage cheeses. Marta Alonso is gratefully acknowledged for her technical assistance (Photonic Microscopy and Image Processing Area, Scientific-Technical Services, University of Oviedo).

5. References

Alonso, S., Rendueles, M., & Díaz, M. (2013). Bio-production of lactobionic acid: Current status, applications and future prospects. *Biotechnology Advances*, 31(8), 1275–1291. https://doi.org/10.1016/j.biotechadv.2013.04.010

Angiolillo, L., Conte, A., Faccia, M., Zambrini, A. V, & Nobile, M. A. Del. (2014). A new method to produce synbiotic Fiordilatte cheese. *Innovative Food Science and Emerging Technologies*, 22, 180–187. https://doi.org/10.1016/j.ifset.2013.09.010

Ashwell, M. (2002). Concepts of functional foods. *In IISI Europe Concise Monograph Series*. https://doi.org/10.1108/nfs.2004.34.1.47.3

Aureli, P., Capurso, L., Castellazzi, A. M., Clerici, M., Giovannini, M., Morelli, Poli A., Pregliasco F., Salvini F. & Zuccotti, G. V. (2011). Probiotics and health: An evidence-based review. *Pharmacological Research*, 63(5), 366–376. https://doi.org/10.1016/j.phrs.2011.02.006

Bambace, M. F., Alvarez, M. V., & Moreira, M. del R. (2019). Novel functional blueberries: Fructo-oligosaccharides and probiotic lactobacilli incorporated into alginate edible coatings. *Food Research International*, 122(December 2018), 653–660. https://doi.org/10.1016/j.foodres.2019.01.040

Batista, A. L. D., Silva, R., Cappato, L. P., Ferreira, M. V. S., Nascimento, K. O., Schmiele, M., Esmerino E. A., Balthazar C. F., Silva H. L. A., Moraes J., Pimentel T. C., Freitas M. Q., Raices R. S. L., Silva M. C. & Cruz, A. G. (2017). Developing a synbiotic fermented milk using probiotic bacteria and organic green banana flour. *Journal of Functional Foods*, 38, 242–250. https://doi.org/10.1016/j.jff.2017.09.037

Carcamo Vargas, G. I. & Mena Bastías, C. P., (2006). Alimentación saludable. *Horizontes educacionales*, (11). ISSN: 0717-2141.

Cardoso, T., Marques, C., Dagostin, J. L. A., & Masson, M. L. (2019). Lactobionic Acid as a Potential Food Ingredient: Recent Studies and Applications. *Journal of Food Science*, 84, 1672–1681. https://doi.org/10.1111/1750-3841.14686

Chan, E. S., & Zhang, Z. (2005). Bioencapsulation by compression coating of probiotic bacteria for their protection in an acidic medium. *Process Biochemistry*, 40(10), 3346–3351. https://doi.org/10.1016/j.procbio.2005.03.001

Costa, M. J., Maciel, L. C., Teixeira, J. A., Vicente, A. A., & Cerqueira, M. A. (2018). Use of edible films and coatings in cheese preservation: Opportunities and challenges. *Food Research International*, 107(November 2017), 84–92. https://doi.org/10.1016/j.foodres.2018.02.013

Espitia, P. J. P., Batista, R. A., Azeredo, H. M. C., & Otoni, C. G. (2016). Probiotics and their potential applications in active edible films and coatings. *Food Research International*, 90, 42–52. https://doi.org/10.1016/j.foodres.2016.10.026

FDA. Code of Federal Regulations, Title 21, 21 CFR 172.720. US Food and Drug Administration. (2017). Retrieved from https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=172.720

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. https://doi.org/10.1111/1471-0307.12558

Heilig, A., Göggerle, A., & Hinrichs, J. (2009). Multiphase visualisation of fat containing β-lactoglobulin-κ-carrageenan gels by confocal scanning laser microscopy, using a novel dye, V03-01136, for fat staining. *LWT - Food Science and Technology*, 42(2), 646–653. https://doi.org/10.1016/j.lwt.2008.08.006

Kechagia, M., Basoulis, D., Konstantopoulou, S., Dimitriadi, D., Gyftopoulou, K., Skarmoutsou, N., & Fakiri, E. M. (2013). Health Benefits of Probiotics: A Review. *ISRN Nutrition*, 2013, 1–7. https://doi.org/10.5402/2013/481651

Kiryu, T., Kiso, T., Nakano, H., Ooe, K., Kimura, T., & Murakami, H. (2009). Involvement of Acetobacter orientalis in the production of lactobionic acid in Caucasian yogurt ("Caspian Sea yogurt") in Japan. *Journal of Dairy Science*, 92(1), 25–34. https://doi.org/10.3168/jds.2008-1081

Krasaekoopt, W., & Watcharapoka, S. (2014). Effect of addition of inulin and galactooligosaccharide on the survival of microencapsulated probiotics in alginate beads coated with chitosan in simulated digestive system, yogurt and fruit juice. *LWT - Food Science and Technology*, 57(2), 761–766. https://doi.org/10.1016/j.lwt.2014.01.037

Langa, S., van den Bulck, E., Peirotén, A., Gaya, P., Schols, H. A., & Arqués, J. L. (2019). Application of lactobacilli and prebiotic oligosaccharides for the development of a synbiotic semi-hard cheese. *Lwt*, 114(April), 108361. https://doi.org/10.1016/j.lwt.2019.108361

Lopez-Rubio, A., Gavara, R., & Lagaron, J. M. (2006). Bioactive packaging: turning foods into healthier foods through biomaterials. *Trends in Food Science and Technology*, 17(10), 567–575. https://doi.org/10.1016/j.tifs.2006.04.012

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., F. Carrière, Boutrou R., Corredig M., Dupont D., Dufour C., Egger L., Golding M, KarakayaS., Kirkhus B., Le Feunteun S., Lesmes U., MacIerzanka A., MacKie A., Marze S., McClements D. J., Ménard O., Recio I., Santos C. N., Singh R. P., Vegarud G. E., Wickham M. S. J., Weitschies W. & Brodkorb, A. (2014). A standardised static in vitro digestion method suitable for food-an international consensus. *Food and Function*, 5(6), 1113–1124. https://doi.org/10.1039/c3fo60702j

Orozco-Parra, J., Mejía, C. M., & Villa, C. C. (2020). Development of a bioactive synbiotic edible film based on cassava starch, inulin, and Lactobacillus casei. *Food Hydrocolloids*, 104(January), 105754. https://doi.org/10.1016/j.foodhyd.2020.105754

Padilha, M., Morales, M. L. V., Vieira, A. D. S., Costa, M. G. M., & Saad, S. M. I. (2016). A prebiotic mixture improved: Lactobacillus acidophilus and Bifidobacterium animalis gastrointestinal in vitro resistance in petit-suisse. *Food and Function*, 7(5), 2312–2319. https://doi.org/10.1039/c5fo01592h

Parreidt, T. S., Müller, K., & Schmid, M. (2018). Alginate-based edible films and coatings for food packaging applications. *Foods*, 7(10), 1–38. https://doi.org/10.3390/foods7100170

Pavli, F., Kovaiou, I., Apostolakopoulou, G., Kapetanakou, A., Skandamis, P., Nychas, G. J. E., Tassou C. & Chorianopoulos, N. (2017). Alginate-based edible films delivering probiotic bacteria to sliced ham pretreated with high pressure processing. *International Journal of Molecular Sciences*, 18(9). https://doi.org/10.3390/ijms18091867

Ramos, Ó. L., Pereira, J. O., Silva, S. I., Fernandes, J. C., Franco, M. I., Lopes-da-Silva, J. A., Pintado M. E. & Malcata, F. X. (2012). Evaluation of antimicrobial edible coatings from a whey protein isolate base to improve the shelf life of cheese. *Journal of Dairy Science*, 95(11), 6282–6292. https://doi.org/10.3168/jds.2012-5478

Ranadheera, R. D. C. S., Baines, S. K., & Adams, M. C. (2010). Importance of food in probiotic efficacy. *Food Research International*, 43(1), 1–7. https://doi.org/10.1016/j.foodres.2009.09.009

Rößle, C., Brunton, N., Gormley, R. T., Ross, P. R., & Butler, F. (2010). Development of potentially synbiotic fresh-cut apple slices. *Journal of Functional Foods*, 2(4), 245–254. https://doi.org/10.1016/j.jff.2010.09.001

Saad, N., Delattre, C., Urdaci, M., Schmitter, J. M., & Bressollier, P. (2013). An overview of the last advances in probiotic and prebiotic field. *LWT - Food Science and Technology*, 50(1), 1–16. https://doi.org/10.1016/j.lwt.2012.05.014

Saarela, M., Hallamaa, K., Mattila-Sandholm, T., & Mättö, J. (2003). The effect of lactose derivatives lactulose, lactitol and lactobionic acid on the functional and technological

properties of potentially probiotic Lactobacillus strains. *International Dairy Journal*, 13(4), 291–302. https://doi.org/10.1016/S0958-6946(02)00158-9

Sáez-Orviz, S., Camilleri, P., Marcet, I., Rendueles, M., & Díaz, M. (2019). Microencapsulation of Calcium Lactobionate for Protection From Microorganisms in a Solid Phase Food. *Biochemical Engineering Journal*, 150(June), 107281. https://doi.org/10.1016/j.bej.2019.107281

Schaafsma, G. (2008). Lactose and lactose derivatives as bioactive ingredients in human nutrition. *International Dairy Journal*, 18(5), 458–465. https://doi.org/10.1016/j.idairyj.2007.11.013

Verruck, S., Dantas, A., & Schwinden, E. (2019). Functionality of the components from goat ' s milk , recent advances for functional dairy products development and its implications on human health. *Journal of Functional Foods*, 52(November 2018), 243–257. https://doi.org/10.1016/j.jff.2018.11.017

Wilkinson, C., Dijksterhuis, G. B., & Minekus, M. (2001). From food structure to texture. *Trends in Food Science and Technology*, 11(12), 442–450. https://doi.org/10.1016/S0924-2244(01)00033-4

Yiu, S. (1985). A fluorescence microscopic study of cheese . *Food Microstructure* , 4(1), 99–106.

Zhong, Y., Cavender, G., & Zhao, Y. (2014). Investigation of different coating application methods on the performance of edible coatings on Mozzarella cheese. *LWT - Food Science and Technology*, 56(1), 1–8. https://doi.org/10.1016/j.lwt.2013.11.006

5.2.2. Bacteria del ácido láctico co-encapsulada con ácido lactobiónico: viabilidad del probiótico durante pruebas de digestión *in vitro*

En este subapartado se recogen los resultados referentes al segundo trabajo desarrollado empleando alginato de sodio como matriz. En este caso, se elaboraron cápsulas sinbióticas como biomateriales de aplicación alimentaria. La bacteria probiótica empleada en este estudio fue *L. paracasei* CBA L74, patentada y proporcionada por Heinz Italia S.p.A. Se comprobó que este microorganismo es capaz de utilizar ácido lactobiónico como fuente exclusiva de carbono para su crecimiento. Se desarrollaron varios tipos de cápsulas, modificando las concentraciones de las soluciones de endurecimiento o añadiendo una capa extra de chitosano. Además, se emplearon dos métodos diferentes de secado (secado térmico o liofilización) con el objetivo de analizar diferencias en la morfología, supervivencia y rehidratación de estas. Las cápsulas que mostraron las mejores propiedades se sometieron a test de digestión *in vitro* simulada, tras ser rehidratadas en solución salina o yogurt como modelo alimentario real. Aunque se observaron diferencias en función del método de rehidratación empleado, ambas cápsulas se considerarían sinbióticas al lograr alcanzar la concentración mínima para que el producto pueda ser considerado probiótico.

Este trabajo se desarrolló en colaboración con el grupo *Food Engineering Lab*, del *Dipartimento di Ingegneria chimica, dei Materiali e della Produzione industriale* de la *Università degli Studi di Napoli Federico II*.

- **Publicación:** Sáez-Orviz, S., Passannanti, F., Gallo, M., Colucci Cante, R., Nigro, F., Budelli, A.L., Rendueles, M., Nigro, R., Díaz, M. 2021. Lactic acid bacteria co-encapsulated with lactobionic acid: probiotic viability during *in vitro* digestion. *Applied Sciences*, 11, 11404, (https://doi.org/10.3390/app112311404).
- **Estado**: publicada
- **Índice de impacto:** 2,679 (2020)

Lactic acid bacteria co-encapsulated with lactobionic acid: probiotic viability during *in-vitro* digestion

S. Sáez-Orviz[a§], F. Passannanti[b,c§*], M. Gallo[b,c,d*], R. Colucci Cante[b], F. Nigro[c], A. Budelli[b,c,e], M. Rendueles[a], R. Nigro[b] and M. Díaz[a]

[§] contributed equally to the work

[a]Department of Chemical and Environmental Engineering, University of Oviedo. C/Julián Clavería 8, 33006. Oviedo, Spain.

[b] Department of Chemical, Materials and Production Engineering, University of Naples Federico II, Piazzale Tecchio 80, 80121. Naples, Italy

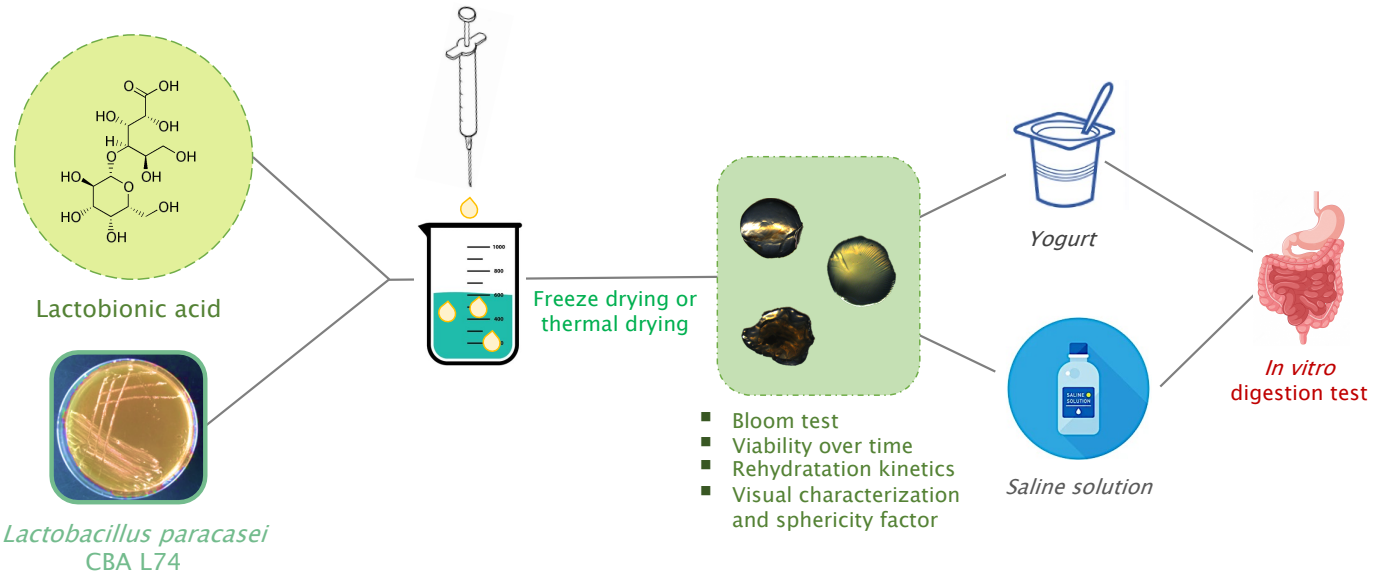
[c] Innovation & Technology Provider (ITP S.r.l.), via Bisignano a Chiaia 68, 80121. Naples, Italy

[d] Department of Engineering, University of Rome Niccolò Cusano, via Don Carlo Gnocchi 3, 00166. Rome, Italy

[e] Heinz Innovation Center, Nieuwe Dukenburgseweg 19, 6534 AD Nijmegen Postbus 57, NL-6500 AB Nijmegen, The Netherlands

*Corresponding authors: Marianna Gallo, marianna.gallo@unicusano.it; Francesca Passannanti, francesca.passannanti@itpna.it

Graphical abstract



Abstract

Synbiotic products are a type of functional foods with a great potential due to the consumer interest in foodstuff that improve their health and/or reduce the risk of certain diseases. In this study, synbiotic macrocapsules were developed using *Lactobacillus paracasei* CBA L74 as probiotic and lactobionic acid (LBA) as prebiotic. Firstly, the probiotic was proven to be able to use LBA as the only substrate source checking their growth and lactic acid production. Then, four different types of capsules were made using sodium alginate as matrix and different hardener solutions ($CaCl_2$ and chitosan). The macrocapsules were characterised regarding their strength and the best performing ones were used for further analysis. In order to obtain a synbiotic capsule, characterized by a longer stability time due to a low water activity, the capsules were dried using freeze and thermal drying. Successively, to revitalize the microorganisms, capsules were rehydrated in two different media (saline solution and yogurt), subjected to simulated *in vitro* digestion tests and visually characterised. Besides, their viability over time was assessed. As a result, *L. paracasei* was able to growth using LBA as only source of carbon with a better production of lactic acid in prolonged times. The more resistant freeze- and thermal-dried capsules showed differences in rehydration kinetics and visual changes were also observed. In simulated *in vitro* digestion tests capsules rehydrated in yogurt showed the best results in terms of survival. Regarding their viability over time, the importance of the use of chitosan was observed.

Keywords: synbiotic; prebiotic; probiotic; macrocapsules; *in vitro* digestion; drying processes; lactic acid bacteria; lactobionic acid; *Lactobacillus paracasei* CBA L74; viability

1. Introduction

Currently, consumers claim high quality food products which, apart from fulfilling their nutritional demands, have the potential to improve their health and/or reduce the risk of some diseases (Batista et al. 2017). In this context, functional foods have emerged, especially in the area of dairy products, where the greatest variety of functional food products is to be found (Ashwell, 2002) (Sáez-Orviz et al. 2020). These functional foodstuffs contain various bioactive compounds such as prebiotics and probiotics.

The group of prebiotics is broad and lactose derivatives, such as lactobionic acid (LBA), has generated strong interest in the food field (Alonso, Rendueles & Díaz, 2013). As other prebiotics, it is resistant to digestive enzymes (Cardoso, Marques, Dagostin & Massom, 2019) and can be metabolised by the microflora of the gastrointestinal tract

(GIT) (Saarela, Hallamaa, Mattila-Sandholm & Mättö, 2003) (Schaafsma, 2008). However, its use has only been approved by the Food and Drug Administration (FDA) in its salt form (FDA, 2017), although it is expected to be approved by other authorities in the near future. Prebiotic compounds have the ability to promote the growth of probiotic microorganisms. Probiotics are live microorganisms which, when administered in appropriate doses, have beneficial effects on consumers (Peng et al. 2020). These bacteria have the capacity to withstand the acidic environment of the stomach and reach the lower GIT, where they can convert carbohydrates into lactic acid and other essential nutrients. Besides, they prevent the development and growth of pathogenic microorganisms (Peng et al. 2020). The most common and widely used probiotics are lactic acid bacteria (LAB), especially bacteria belonging to the genera *Lactobacillus* and *Bifidobacterium* (Espitia, Batista, Azaredo & Otoni, 2016). Specifically, *Lactobacillus paracasei* CBA L74 has been tested as a probiotic in *in vivo* studies (Nocerino et al. 2017) and has showed various health benefits (Nocerino et al. 2017) (Gallo et al. 2019) (Labruna et al. 2019) (Sarno et al. 2014).

Besides, it has been successfully used in the development of probiotic non-dairy matrix foodstuff (Salameh et al. 2019) (Gallo et al. 2019) (Colucci Cante et al. 2020) (Gallo et al. 2018) (Gallo et al. 2020) (Colucci Cante et al. 2021). Moreover, when probiotics are combined with prebiotic compounds, synbiotic products are obtained (Langa et al. 2019) (Sáez-Orviz, Marcet, Rendueles & Díaz, 2021) and some authors have suggested that this synbiotic combination may increase the viability of the bacteria through the GIT (Sáez-Orviz, Marcet, Rendueles & Díaz, 2021).

These bioactive compounds are usually sensitive to chemical and physical factors (Dias, Ferreira & Barreiro, 2015). To protect them against external agents, different strategies have been explored, such as the development of films, coatings, or encapsulation techniques. Among the matrices that can be used, alginate gels are a suitable matrix to protect and immobilise cells as they also allow the diffusion of metabolisms through pores (Simó et al. 2017). The choice of the coating material and the production conditions of the capsules (hardening times, crosslinking agents and its concentrations) are all factors that can influence the viability of the probiotic and must be carefully investigated (Di Natale et al. 2021). Encapsulation techniques have the capacity to protect probiotic bacteria (Espitia et al. 2016) and also allow other bioactive products to be subjected to different drying techniques, such as heat drying and freeze-drying, which can extend their shelf life (Kaushik & Roos, 2007). In addition, this type of

techniques can increase the survival and viability of probiotics as they pass through the acidic environment of the stomach to reach the GIT.

Ultimate goal of the present work is to develop a dried synbiotic capsule with LBA as prebiotic and *L. paracasei* CBA L74 as probiotic, that allows a sufficient quantity of bacteria to be conveyed to the intestine for a functional effect and that has a certain stability over time.

For this purpose, the ability of the probiotic to consume LBA as a single substrate source will be first assessed. Then four synbiotic capsules, different for the coating, will be developed with sodium alginate and will be characterised in terms of strength. The capsule type with the best characteristics will be subjected to two different drying processes (thermal-drying and freeze-drying) and the ability to rehydrate in two different media (saline solution and yogurt) will be tested for both. The viability over time of the encapsulated probiotic will be assessed and, finally, the survival of the *L. paracasei* will be analysed in simulated *in vitro* digestion tests.

2. Materials and methods

2.1. Probiotic microorganism, growth conditions and lactic acid production

Lactobacillus paracasei CBA L74 (patented and provided by Heinz Italia S.p.A) was used as probiotic microorganism. This strain is a Gram-positive homo-fermentative, and facultative anaerobic bacteria. It was stored at -20ºC and revitalized in 10 mL of *Animal Free Broth (AFB)* (20 g/L Bacto Yeast Extract (BD Biosciences, Milan, Italy), 0.5 g/L $MgSO_4$ (Sigma-Aldrich, Milan, Italy), 50 g/L glucose (Sigma-Aldrich), 0.5 g/L citric acid (Sigma-Aldrich)) by incubation at 37ºC for 24 h. The bacterial load reached after revitalization was of approximately 8 log_{10} CFU/mL.

To test whether *L. paracasei* is able to use LBA as a substrate source, fermentation tests on three different *AFB* (different for the carbon source) were carried out. Briefly the revitalized strain was centrifuged (1600 rpm for 10 minutes), the supernatant discharged, and the pellet resuspended in 40 mL of fresh *AFB* and incubated at 37°C for 72 h. To verify the ability of the strain to use a carbon source other than glucose, three *AFB* were tested: *AFB-G*, in which the carbon source was glucose and *AFB-L* in which was LBA; *AFB-GL,* in which was a mix of the two (1:1 ratio) (all were purchased by Sigma-Aldrich). An initial bacterial load of approximately 6 log_{10} CFU/mL was observed for all. To monitor fermentation, samples were taken at the start of fermentation (T0) and after 2 (T2), 18 (T18), 24 (T24), 48 (T48) and 72 (T72) h from inoculation. Samples were analyzed for microbial growth and lactic acid production. After serial dilutions, samples were sowed on MRS agar (Sigma-Aldrich) plates, then

incubated at 37°C for 48 h. The lactic acid produced during fermentation was determined by high performance liquid chromatography (HPLC) (Agilent Technologies 1100), equipped with a C18 column (Agilent Zorbax C18 column – 4.6 mm x 150 mm and a pore size of 80A), with visible/UV detector. The eluent was 0.1M $NH_4H_2PO_4$, aqueous solution at pH of 2.7 with a flow rate of 0.8 mL/min; the detection wavelength and temperature were set at 218 nm and 30°C, respectively.

2.2. Co-encapsulation of the probiotic and the prebiotic and macrocapsule characterization

2.2.1. Encapsulation

Four different formulations were developed (Table 5.5). The probiotic was grown under optimal conditions in an animal free broth with LBA as an exclusive source of carbon (*AFB-L*). On the microbial culture, 20 g/L sodium alginate (Sigma-Aldrich) was added and mixed until a homogeneous solution was obtained. Then, the mixture was dropped into a $CaCl_2$ solution (0.1 or 0.5 M, Sigma-Aldrich) from a distance of 10 cm using a 1 mL sterile syringe. The capsules were left in the $CaCl_2$ solution for a set time (Table 5.5) at room temperature with gentle agitation. After this time, some of the capsules were collected and added to a chitosan solution (4.42 g/L chitosan (Sigma-Aldrich), 0.44% glacial acetic acid (Sigma-Aldrich), pH 5.7-6.0 with NaOH 1 M) in which they were kept at room temperature under gentle agitation for 40 minutes. Finally, the capsules were recovered and stored for further testing.

Table 5.5. Summary of the different capsules developed.

Capsules	$CaCl_2$ solution	Chitosan solution
A	0.1 M + 10 min	-
B	0.1 M + 30 min	-
C	0.1 M + 30 min	40 min
D	0.5 M + 10 min	40 min

2.2.2. Bloom test

The textural properties of the capsules were studied employing the Bloom test according to the International Standard ISO 9665 (ISO, 1998) using a TA.XTplus Texture Analyzer (Stable Micro Systems, UK). The test was conducted at room temperature using a P/0.5 1/2" probe with a test speed of 0.5 mm/s and a load cell of 5 kg.

Experiments were carried out in triplicate and reported results correspond to the mean value.

2.3. Drying processes

After production, capsules with the least (A) and the most (D) cross-linked coating were dried by two different technologies: freeze-drying and thermal-drying. Freeze-drying was carried out using a lab scale freeze-dryer (Christ Alpha 1-2 LDplus, Martin Christ, Osterode am Harz, Germany). It lasted 24 h, consisting of a main drying phase at a condenser temperature of -20°C, a pressure of 1.0 mbar for 12 h and a final drying phase for the removal of the residual moisture at a temperature of -56.5°C and a pressure of 0.017 mbar for 12 h. Thermal-drying was carried out using a dryer (CL252, Trevi, Rimini, Italy), with an air flow of 0.1-0.3 m/s, controlling the temperature at 37°C. The drying lasted 12 h. Capsules were evenly distributed on the surface to ensure homogeneous drying.

2.4. Viability over time

The impact on the viability of the probiotic over time of three different variables was assessed at changing carbon source, crosslinking of the capsules and the drying process used.

To test the impact of the carbon source and crosslinking, four different capsules were produced: two of them were made using *AFB-G,* as described above, with the least (A from Table 5.5) and the most (D from Table 5.5) crosslinked coating; the other two were produced using *AFB-L* and also in this case the two crosslinking levels were used.

In Table 5.6 are summarized the capsules formulations.

Table 5.6. Capsule formulations used to test probiotic viability over time, to study the impact of the carbon source used and of the crosslinking of the coating.

Capsules	Carbon source	Coating
I	AFB-G	0.1M 10 min (A from Table 5.5)
II	AFB-G	0.5M 10 min + chitosan (D from Table 5.5)
III	AFB-L	0.1M 10 min (A from Table 5.5)
IV	AFB-L	0.5M 10 min + chitosan (D from Table 5.5)

All were then subjected to freeze- or thermal-drying. The produced freeze-dried or thermal-dried capsules were stored at 20° C and at specific sampling times the microbial viability was tested. Briefly, the capsules were broken in 1% w/w sodium citrate solution at pH 6 and after serial dilution the sample was seeded on MRS agar as

previously described. The sampling times were (in days): day 0 (T0, the day the drying ended), 1, 2, 4, 6, 8, 11, 14, 18, 21 and 30.

2.5. Rehydration kinetics

The capsule with the best results in the Bloom test and with the higher viability over time was chosen for subsequent experiments. The chosen capsule, both in freeze-dried and thermal-dried forms, were tested for their ability to rehydrate. In particular, two conditions were tested: 1) rehydration in 0.9% NaCl solution at 37°C; 2) rehydration in natural yogurt (Danone, Danone Group SA, France) at 4°C. Briefly the capsules (freeze- or thermal-dried) were placed in 0.9% NaCl solution or in yogurt and collected at specific times (0, 0.25 min (15 sec), 6 min, 60 min, 180 min and 1440 min (24 h)). Their water content was measured using a volumetric titrator (Karl Fisher HI 903, Hanna instruments, Villafranca Padovana, Italy). Finally, the rehydration capacity of the capsules was evaluated as the ratio between the water content measured on the dried capsules compared to the initial water content of the fresh capsule, as described below:

$$(W_{rehydrate} / W_{fresh}) \times 100$$

where $W_{rehydrate}$ were the grams of water adsorbed by the dried capsule during the rehydration in 0.9% NaCl solution or in yogurt; W_{fresh} were the grams of water measured on the fresh capsules, before drying.

2.6. Simulated *in vitro* digestion of rehydrated freeze-dried and thermal-dried capsules

The *in vitro* digestion test was performed only with the aim to test the survival of *L. paracasei* since LBA is resistant to human digestive enzymes (Cardoso et al. 2019). For this purpose, the viability of the microorganisms was tested at several times during the digestion trial and calculated per gram of capsule. In addition, a sample of free bacteria, unencapsulated, was tested also as control, with an initial concentration of 7 $\log_{10}$ CFU/mL.

The three digestive conditions (salivary, gastric and intestinal phases) were prepared according to Passannanti et al. (2017) with some modifications. Simulated salivary fluid (SSF) was prepared by adding 1.12 g/L KCl, 0.50 g/L KH_2PO_4, 1.14 g/L $NaHCO_3$, 0.031 g/L $MgCl_2(H_2O)_6$ and 0.0058 g/L $(NH_4)_2CO_3$. Simulated gastric fluid (SGF) was prepared by mixing 0.51 g/L KCl, 0.12 g/L KH_2PO_4, 2.10 g/L $NaHCO_3$, 2.76 g/L NaCl, 0.020 g/L $MgCl_2(H_2O)_6$ and 0.048 g/L $(NH_4)_2CO_3$. Simulated intestinal fluid (SIF) was prepared by adding 0.51 g/L KCl, 0.11 g/L KH_2PO_4, 7.14 g/L $NaHCO_3$, 2.24 g/L NaCl and 0.067 g/L $MgCl_2(H_2O)_6$ (all reagents from Sigma-Aldrich).

Before the *in vitro* digestion test, the thermal-dried and freeze-dried capsules were rehydrated in two different ways: in a saline solution (0.9% NaCl, 1:100 (w/v) at 37ºC for 15 min) and in 120 g of natural yogurt (Danone, Danone Group SA, France) (at 4ºC for 24 h).

For the salivary simulation, 25 mL of SSF were mixed with $CaCl_2$ (Sigma-Aldrich) and α-amylase (1333 U/mg protein, CAS 900-90-2, Sigma-Aldrich) to obtain a concentration in the final volume of 0.083 g/L and 75 U/mL, respectively. Capsules rehydrated in saline solution were recovered and added directly while those rehydrated in yogurt were added with 5 g of yogurt. The oral mixture was incubated for 3 min at 37°C. Afterwards, 25 mL of SGF were added to the mixture. Porcine pepsin (2500 U/mg protein, CAS 9001-75-6, Sigma-Aldrich) and $CaCl_2$ were also added to this mixture to achieve a final concentration of 2000 U/mL and 0.0083 g/L. Then, the mixture was well homogenized, the pH was adjusted to 2.0 with HCl 5 M (Sigma-Aldrich) and incubated at 37 ºC for 2 h. Finally, porcine pancreatin (0.1% (w/v), CAS 9049-47-6, Sigma-Aldrich), bovine chymotrypsin (3% (w/v), CAS 9004-07-3, Sigma-Aldrich), amyloglucosidase (260 U/mL, CAS 9032-08-0), with a concentration in the final volume of 1.12 U/mL,(Warren et al. 2015)) and $CaCl_2$ (0.033 g/L) were mixed with 50 mL of SIF. This solution was added to the previous mixture and was well homogenized, the pH adjusted to 6.5 with NaOH 5 M (Sigma-Aldrich) and incubated at 37 ºC for 2 h.

The bacterial viability was assessed by taking samples at different times: one after the simulated salivary phase and one after each 30 minutes in the simulated gastric and intestinal phases (9 samples in total for the entire simulated digestion trial). For the sampling, a number of capsules was collected and weighed under sterile conditions. The capsules were homogenized and undone with a sterile sodium citrate solution (1% (w/w), pH 6.0, Sigma-Aldrich) in a 1:10 proportion (capsules weight/sodium citrate volume). Serial dilutions (1:10) were incubated on MRS agar plates (Sigma-Aldrich) for 48-72 h at 37ºC. Each sample was carried out in triplicate and results were expressed in $\log_{10}$ CFU/ g capsules (CFU/mL in the case of control).

2.7. Visual characterization and sphericity factor (SF)

The visual characterization of the capsules was performed employing a LEICA M205Fa fluorescence stereo microscope (Leica Microsystems Inc., Heildelberg, Germany). A magnification 12x was used and Leica Application Suite v4.0 software platform was used to analyse the images and determine the size of the capsules.

The shape of the capsules was characterised using the spherification factor (SF). According to this parameter a 0 value is a perfect sphere, and a 1 value corresponds to an elongated sphere. SF parameter was calculated as follows:

$$SF = (d_{max} - d_{min})/(d_{max} + d_{min})$$

where d_{max} is the largest diameter and d_{min} is the smallest diameter perpendicular to d_{max}. SF of the capsules was calculated on fresh capsules, on dried capsules, on rehydrated capsules (saline solution and yogurt) and after the *in vitro* digestion tests.

2.8. Statistical analysis

Analysis of variance (ANOVA) was applied. Fischer's Least Significant Difference (LSD) was performed to determine significant differences between the data. A level of $p < 0.05$ was considered significant. Analyses were carried out using Statgraphics 18® Centurion statistical software.

3. Results and discussion

3.1. Analysis of the growth of *L. paracasei* using lactobionic acid as substrate and lactic acid production

L. paracasei CBA L74 was inoculated in three *Animal Free Broths (AFBs)*, to study its ability to use carbon sources other than glucose. In Figure 5.11 the bacterial growth (A) and the lactic acid produced (B) during the fermentation are shown.

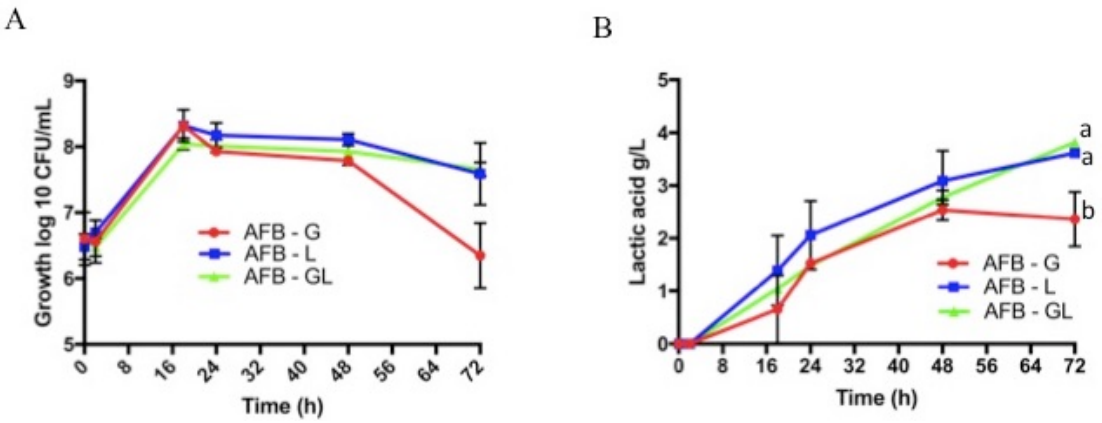


Figure 5.11. Microbial growth and lactic acid concentrations on three *AFBs*. (A) Bacterial growth in $\log_{10}$ CFU/mL and (B) lactic acid production in g/L, during fermentation on three different *AFB*s: *AFB-G*, with glucose as carbon source; *AFB-L*, with LBA as carbon source; *AFB-GL*, with a mix (1:1 ratio) of glucose and LBA. Bars represent standard deviation of three independent experiments. Different letters indicate significant differences ($p < 0.05$).

Starting for all with a bacterial concentration of about 6.5 $\log_{10}$ CFU/mL, a similar growth was obtained for all the broths. Only after 72 hours it seems that the growth of the microorganism on the *AFB-G* had a reduction in its viability (6.35 ± 0.49 $\log_{10}$ CFU/mL), but the difference with the other broths (*AFB-L:* 7.59 ± 0.47 $\log_{10}$ CFU/mL*; AFB-GL:* 7.66 ± 0.10 $\log_{10}$ CFU/mL) was not statistically significant. As for the lactic acid, it increased over time with a very similar trend for all the broths. Only after 72 hours a statistically significant difference ($p < 0.05$) was observed between the fermentations carried out on the broths with LBA (*AFB-L*: 3.62 ± 0.05 g lactic acid/L; *AFB-GL:* 3.82 ± 0.09 g lactic acid/L*)* and the fermentation on *AFB-G* (2.37 ± 0.52 g lactic acid/L). From these results it was possible to state that *L. paracasei* CBA L74 was able to use LBA as a carbon source and this substrate gave the same results as glucose in terms of bacterial growth and lactic acid production (for which a better production is observed in prolonged times). A similar test (Saarela, Hallamaa, Mattila-Sandholm & Mättö, 2003) was performed on different *Lactobacillus* strains: also, from their study *L. paracasei* seems to be able to grow using LBA (2% w/v) in the same way as using glucose.

3.2. Capsules characterization

After having tested the ability of the microorganism to use LBA as a substrate, *L. paracasei* and the prebiotic were co-encapsulated in four different types of capsules, the resistance of which was tested by Bloom test. The strength of the four types of capsules produced was analysed to choose the type of capsule with the best characteristics for further studies. In this type of test, Bloom gel strength is the mass in grams required to depress the surface gel 4 mm (García, Ranieri, Rendueles & Díaz, 2019). Results are shown in Table 5.7. The most resistant capsules were those that were made with the highest concentration of $CaCl_2$ (0.5 M) and had an extra layer of chitosan (D-capsules). With the rest of capsules developed, the $CaCl_2$ concentration employed (0.1 M) did not seem to harden them enough, as even with a chitosan layer (C-capsules) no significant differences were observed ($p > 0.05$). Therefore, the $CaCl_2$ concentration has an influence on the strength of the capsules. Other authors have found that the strength of the sodium alginate gel increases with the $CaCl_2$ concentration, as the formation of stronger and more crosslinked bound gels is favoured due to the presence of more available Ca^{2+} ions (Atencio et al. 2020) (Li et al. 2019). Besides that, the chitosan coating provides extra coverage in the capsules, which may influence their structure being able to make them to increase probiotic viability (Simó et al. 2017) and to withstand the digestive conditions and help the probiotic to survive and reach the

low gastrointestinal tract (GIT). Therefore, D-capsules were chosen for the rest of the analysis performed.

Table 5.7. Bloom values (g) for the different capsules. Different letters in the same column indicate significant differences ($p < 0.05$).

Capsules	A	B	C	D
Force (g)	253.7 ± 7.4 [a]	283.2 ± 68.3 [a]	304.3 ± 52.0 [a]	476.9 ± 38.7 [b]

3.3. Viability over time of the probiotic encapsulated in dried capsules

The viability of the microorganism is a key factor in ensuring the probiotic effect on the health of the host. Actually, there is no standard recommendation on the bacterial concentration to consume to have a benefit; however, it is generally accepted that the microorganism, to be defined as a probiotic, must have a concentration of at least 10^6 CFU for g of product (FAO/WHO, 2010). This bacterial concentration must be guaranteed at the time of consumption and considering that during storage the vital count can decrease, an assessment of the viability over time is essential. We evaluated the impact on viability over time of: 1) the carbon source used as substrate by the microorganism (testing two different broths: *AFB-G* and *AFB-L;* 2) the coating (testing the least (0.1 M $CaCl_2$ for 10 min) and the most (0.5 M $CaCl_2$ for 10 min and chitosan) cross-linked capsules); 3) the drying technology used (freeze- or thermal-dried, considering that for both a water content of about 0.1% on dry basis was measured immediately after the drying, while a value of less than 2% was measured after 30 days). In Figure 5.12 the microbial concentration observed over time (days) are shown. As it is possible to note, there were no differences due to the carbon source used: the same trend was obtained on capsules with the same structure but revitalized in different broths, and this can be affirmed for both the freeze- and the thermal-dried capsules. On the contrary, the resistance of the structure of the capsules seemed to be very important to guarantee stability: the capsules cross-linked in 0.1 M $CaCl_2$ for 10 minutes protect the microorganism less than the other type of capsules, with a decay of the microbial concentration of about 4 $\log_{10}$ at the end of time considered. The same reduction was indeed not present if the capsules were produced by crosslinking in 0.5 M $CaCl_2$ for 10 minutes and then coated with chitosan. Also, in this case the trend between the freeze- and the thermal- dried capsules was very similar and no difference statistically significant were found. Also, in the study of Mahmoud et al. (2020) the structure of the capsule seemed an important factor in determining the probiotic viability over time: it was found that chitosan-coated alginate capsules were more capable than

others (with skim milk, dextrin, or denatured whey protein) in protecting *L. plantarum* during storage. The importance of chitosan coating for probiotic viability during storage was also showed by Lopes et al. (2020). Based on these data, only the microorganisms encapsulated in the most cross-linked capsules, then coated with chitosan, had a useful microbial concentration to be considered probiotics, after 30 days of storage, while for the other capsules only half the storage time should be considered.

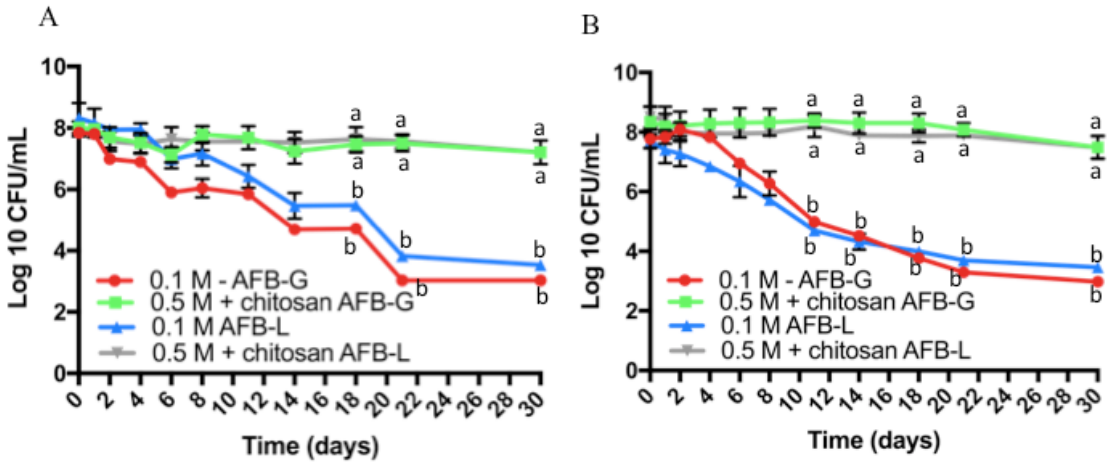


Figure 5.12. Probiotic viability over time. Microbial concentration measured for 30 days for the freeze-dried capsules (A) and for the thermal-dried capsules (B). For both cases two parameters were studied: the broth used (*AFB-G* or *AFB-L*) and the capsule structure (if crosslinked in 0.1 M $CaCl_2$ for 10 minutes or in 0.5 M $CaCl_2$ for 10 minutes and then coated with chitosan). Bars represent standard deviation of three independent experiments. Different letters indicate significant differences ($p < 0.05$).

3.4. Rehydration kinetic

Capsules produced with *AFB-L*, chosen as the best from the Bloom test and from the evaluation of the viability over time (D-capsules: 0.5M $CaCl_2$ and chitosan) were then freeze- or thermal-dried. The rehydration capacity over time of the capsules was evaluated as the ratio between the grams of water adsorbed by the dried capsules (freeze- or thermal-dried) compared to the grams of water measured in the fresh capsule, before drying, as written in the methods section. The results of the rehydration capacity are shown in Figure 5.13.

The freeze-dried capsules are more capable of rehydration than the thermal-dried ones, and the difference is statistically significant. On the contrary, there is no difference according to the medium used for rehydration (saline solution or yogurt). In all cases the rehydration reaches a maximum level after 3 h and lasts until 24 h, when the rehydration percentage observed were: 13.67 ± 3.10% for freeze-dried capsules rehydrated in saline solution; 14.12 ± 1.80% for the freeze-dried rehydrated in yogurt; 10.24 ± 1.50% for the thermal-dried rehydrated in saline solution and 10.00 ± 1.84% for the thermal-dried rehydrated in yogurt.

The difference related to the drying treatment used can be due to the physical alterations (shrinkage, altered porosity and microstructure and reduced ability in binding water) caused by thermal-drying (Chirife & Buera, 1995) (Stapelfeldt, Nielsen & Skibsted, 1997) (Witrowa-Rajchert & Lewicki, 2006).

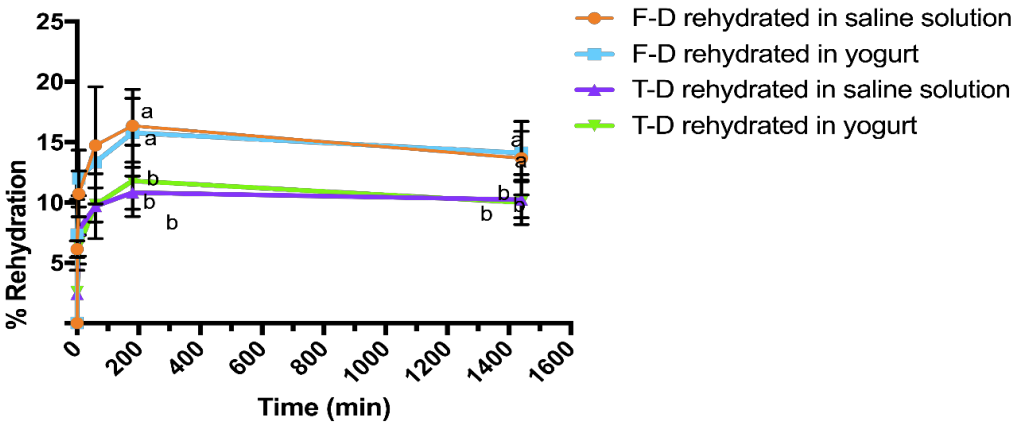


Figure 5.13. Rehydration capacity over time. Freeze- and thermal-dried capsules were placed in 0.9% NaCl solution (orange, purple, respectively) or in yogurt (light blue, green, respectively) and collected at specific sampling times (0, 0.25 min (15 sec), 6 min, 60 min, 180 min, and 1440 min (24 h)) to evaluate their rehydration capacity, as described in the methods section. Bars represent standard deviation of three independent experiments. Different letters indicate significant differences ($p < 0.05$).

3.5. Simulated *in vitro* digestion of rehydrated freeze-dried and thermal-dried and capsules

To check the protective capacity of the alginate capsules on the *L. paracasei* survival, freeze-dried and thermal-dried capsules rehydrated in saline solution and yoghurt were subjected to a simulated *in vitro* digestion test. As a control, *L. paracasei* unencapsulated (7 $\log_{10}$ CFU/mL) was used. Results are shown in Figure 5.14.

In the simulated salivary phase, the probiotic concentration was equal or greater than 6 $\log_{10}$ CFU/ g capsules or mL (in the case of control). The differences observed may be due to the initial concentration of microorganisms in each type of rehydrated capsules and not by the effect of salts and α-amylase present in the environment, as the differences are not related to the type of capsule (freeze- or thermal-dried) and the type of hydration (saline solution or yogurt). The main changes were observed in gastric simulation. After 30 min in the stomach simulation, no survival was observed in the case of the control. It is known that the acidic environment ($pH < 2.0$) as well as the presence of pepsin can cause the death of the microorganisms (Melchior et al. 2020). Significant differences ($p < 0.05$) were found in terms of the way the capsules were rehydrated but not between freeze- or thermal-dried capsules. Higher survival was obtained in

capsules rehydrated in yogurt than in those rehydrated in saline solution, with a difference of 57.75%. After the simulated gastric phase, there was a small reduction in the microbial load in the simulated intestinal phase. At the final point, the mean survival of capsules rehydrated in saline solution was 2.3 ± 0.1 $\log_{10}$ CFU/g capsules while those rehydrated in yogurt was 4.8 ± 0.1 $\log_{10}$ CFU/g capsules. Therefore, the way in which the capsules were rehydrated influences the survival of the probiotic throughout the simulated *in vitro* digestion test in both types of capsules (freeze-dried and thermal-dried). Yogurt, with its rich nutritional composition, could play a dual role in the protection of microorganisms: the first, by acting as a more complex matrix to digest, compared to saline solution, with more "engaged" digestive enzymes; the same results was obtained by Bove et al. (2013): different food carriers of probiotics were tested during *in vitro* digestion and higher survival was observed in complex and/or nutrient-rich ones. The second, by feeding microorganisms with nutrients: during rehydration in yogurt capsules can take water, enriched with nourishing molecules such as sugars, which can promote viability.

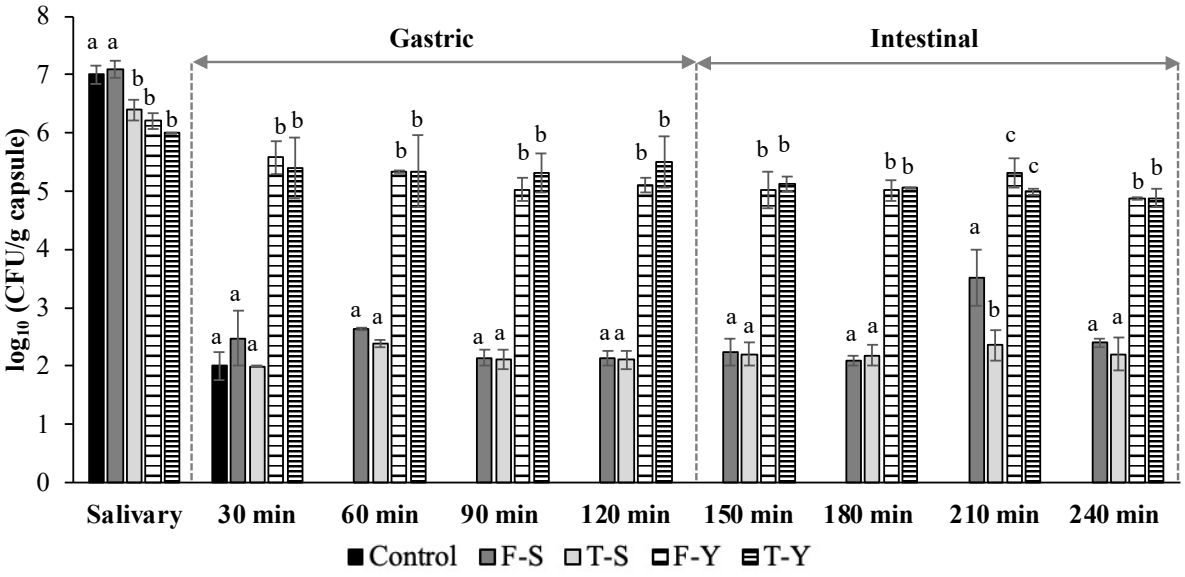


Figure 5.14. Variation of the concentration of *L. paracasei* ($\log_{10}$ CFU/g of capsule and $\log_{10}$ CFU/mL in the case of control sample) throughout the *in vitro* digestion test. (F-S) refers to freeze-dried capsules rehydrated in saline solution, (T-S) to thermal-dried capsules rehydrated in saline solution, (F-Y) to freeze-dried capsules rehydrated in yogurt and (T-Y) to thermal-dried capsules rehydrated in yogurt. Experiments were carried out in triplicate and reported results correspond to the mean value. Different letters indicate significant differences ($p < 0.05$).

For probiotic microorganisms to be effective, they must withstand the conditions of the digestive tract to reach the GIT. Although there is no scientific consensus on the probiotic concentration needed to obtain a beneficial effect, several authors suggest

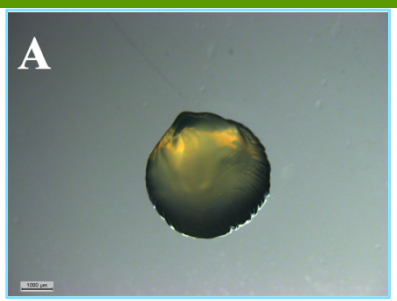
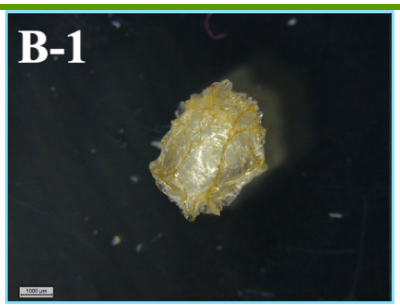
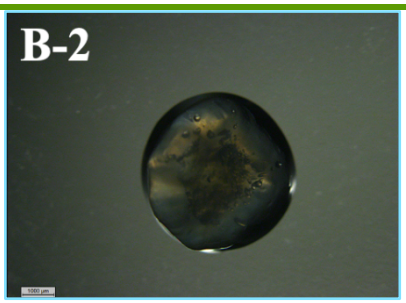
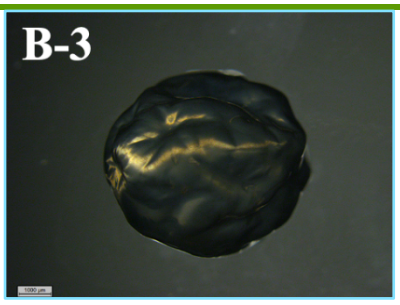
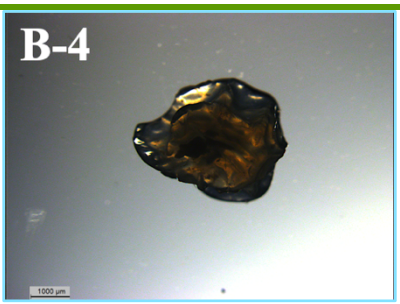
that this concentration would be between 6 and 9 log_{10} CFU/day (Espitia et al. 2016) (Saad et al. 2013). Regarding LBA, it was not measured during the simulated *in vitro* digestion test, as it is resistant to human digestive enzymes (non-digestible fibre) and it is capable to reach intact to the large intestine (Cardoso, Marques, Dagostin & masson, 2019) (Saad et al. 2013). In the case of yogurt, the recommended daily intake is between 200 and 250 g (Bonjour, Benoit, Payen & Kraenzlin, 2013), meaning two portions per day (120 g/portion). Considering the results obtained in the simulated *in vitro* digestion and adding an amount between 0.75 and 1 g of capsules per portion of yogurt, capsules rehydrated in saline solution would have a probiotic final mean value between 3.3 and 4.4 log_{10} CFU/day while capsules rehydrated in yogurt would have a mean value between 7.32 and 9.76 log_{10} CFU/day. As can be seen from these results, only capsules rehydrated in yogurt would have the capacity to deliver the adequate probiotic dose (a minimum concentration of 6 log_{10} CFU/day) to the lower GIT.

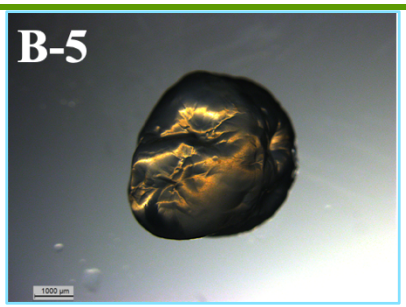
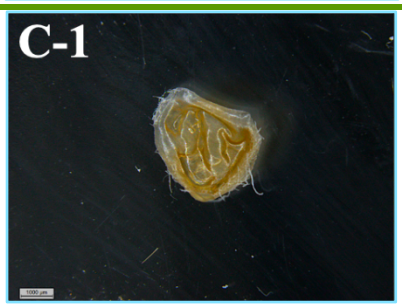
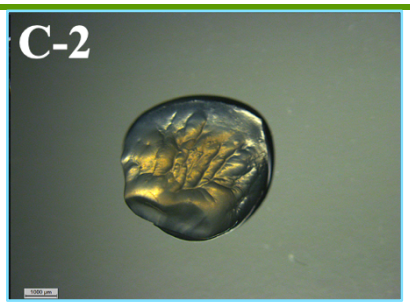
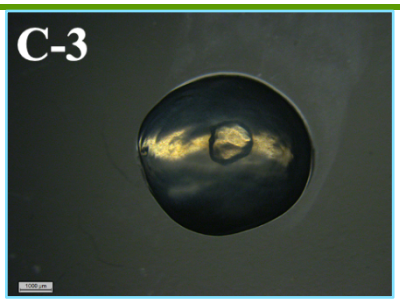
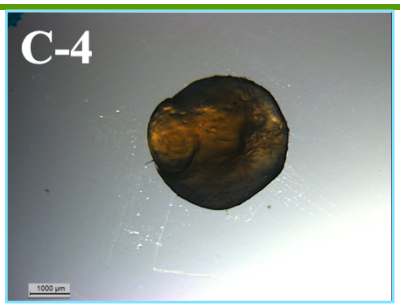
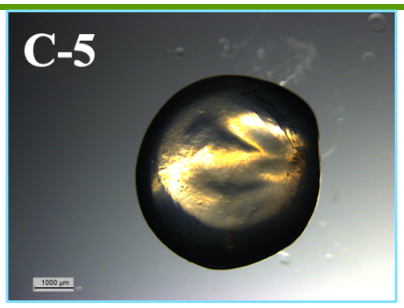
3.6. Visual characterization and sphericity factor (SF)

Stereo microscope images of the capsules (fresh, dried, rehydrated and digested) are shown in Table 5.8, together with the SF and the weight of the related capsule. In terms of size, freshly made capsules showed practically a spherical shape (SF < 0.05,(López-Córdoba, Deladino & Martino, 2013)). After the drying process, the capsules altered their shape. Even so, the SF values obtained (0.135 and 0.17 for the freeze-dried and thermal-dried capsules, respectively) were close to the value of a sphere. Significantly, the freeze-dried capsules were more rounded while the thermal-dried ones were flatter (0.135 ± 0.023 and 0.17 ± 0.082, respectively) due to the drying process carried out. This difference was also observed visually (B-1 and C-1).

After the rehydration process, significant differences were found between the weight of the capsules. Freeze-dried capsules showed a higher weight, both after hydration with saline and yogurt and after *in vitro* digestions tests. These results agreed with those obtained in the rehydration kinetics (section 3.4), as the freeze-dried capsules obtained a higher percentage of rehydration and therefore weighed more. In addition, differences in the capsules surface after the in vitro simulated digestion tests were observed. The freeze-dried capsules showed a surface roughness (B-3 and B-5) while the thermal-dried capsules showed smooth and uniform surface (C-3 and C-5). Besides, the size of the capsules increased, (as shown in B-5 and C-5) (1000 µm scale). Other authors have also found that after simulated *in vitro* digestion test, the size of sodium alginate capsules increased throughout the digestion process, up to three times the original size (Gorbunova, Evteev, Evdokimov & Bannikova, 2016).

Table 5.8. Sphericity factor values (SF), stereo microscope images (all with a magnification of 12x) and weight (mg) of the capsules. (D) refers to freshly made capsules, (F-D) to freeze-dried capsules and (T-D) to thermal-dried capsules.

Capsules	SF	Microscope Images	Weight (mg)
D	0.041 ± 0.021 [a]		71.1 ± 1.7 [a]
F-D	0.135 ± 0.023 [b]		2.6 ± 0.2 [b]
F-D rehydrated in saline solution	0.049 ± 0.03 [a]		9.0 ± 0.9 [c]
F-D rehydrated in saline solution after *in vitro* digestion	0.045 ± 0.01 [c]		13.7 ± 3.1 [d]
F-D rehydrated in yogurt	0.113 ± 0.04 [b]		10.8 ± 1.3 [e]

F-D rehydrated in yogurt after *in vitro* digestion	0.043 ± 0.023 [a]		13.1 ± 1.1 [d]
T-D	0.17 ± 0.082 [d]		2.3 ± 0.4 [b]
T-D rehydrated in saline solution	0.086 ± 0.042 [d]		7.3 ± 0.4 [f]
T-D rehydrated in saline solution after *in vitro* digestion	0.069 ± 0.045 [e]		8.8 ± 0.7 [c]
T-D rehydrated in yogurt	0.022 ± 0.013 [f]		6.8 ± 1.2 [f]
T-D rehydrated in yogurt after *in vitro* digestion	0.031 ± 0.008 [f]		10.6 ± 1.3 [e]

4. Conclusions

As other studied probiotic bacteria, *L. paracasei* CBA L74 was able to use LBA as only source of carbon, showing better results in lactic acid production over prolonged periods of time. Thus, synbiotic macrocapsules with *L. paracasei* CBA L74 as probiotic and LBA as prebiotic were developed and characterised. The most resistant capsules were those prepared using 0.5M $CaCl_2$ as hardener and with an extra layer of chitosan. The same capsules protected the microorganism over time better than the others tested during and were chosen for subsequent tests. From rehydration tests it emerged that, regardless of the medium used (saline solution or yogurt), the freeze-dried capsules incorporate water more easily than the thermal-dried ones, but in all cases the rehydration was partial. No differences were observed between the freeze- or thermal-drying processes in terms of probiotic viability, so that the composition of the capsules allows the survival of the probiotic to be maintained. The way in which capsules were rehydrated was a determining factor in the survival of the probiotic in the simulated *in vitro* digestion tests: capsules rehydrated in saline solution showed worse viability results compared to capsules rehydrated in yogurt, which can better protect microorganisms thanks to its rich nutritional composition. The synbiotic capsules produced (both freeze-dried and thermal-dried) with the recommended daily intake of yogurt would reach the minimum concentration for the product to be considered "probiotic".

Contributions: Marianna Gallo, Francesca Passannanti and Roberto Nigro designed the research; Sara Sáez-Orviz, Francesca Passannanti, Rosa Colucci Cante and Federica Nigro performed the experiments; Sara Sáez-Orviz, Francesca Passannanti and Marianna Gallo collected, analysed the data and contributed to the interpretation of the results; Andrea Budelli provided the raw materials and *Lactobacillus paracasei CBA L74*; Sara Sáez-Orviz, Francesca Passannanti and Marianna Gallo wrote the paper and have the primary responsibility for the final content. Manuel Rendueles, Roberto Nigro and Mario Díaz revised the original draft. All authors read and approved the final manuscript.

Acknowledgments: This work was financially supported by the Spanish Ministry of Science and Innovation through the project MCIU-19-RTI2018-094218- B-100 and by the grant *Programa Severo Ochoa de Ayudas Predoctorales para la Investigación y Docencia* (grant number BP19-127 to Sáez-Orviz, S.).

Conflicts of Interest: Dr Andrea Budelli is currently employed by Heinz BV, Netherlands. He provided *Lactobacillus paracasei CBA L74*. He did not have any additional role in the data collection and analysis, decision to publish, or preparation of the manuscript. Heinz BV did not provide any financial support to the authors for the experimental activity, did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

5. References

Alonso, S., Rendueles, M., & Díaz, M. (2013). Bio-production of lactobionic acid: Current status, applications and future prospects. *Biotechnology Advances*, 31(8), 1275–1291. https://doi.org/10.1016/j.biotechadv.2013.04.010

Ashwell, M. (2002). Concepts of functional foods. *In IISI Europe Concise Monograph Series*. https://doi.org/10.1108/nfs.2004.34.1.47.3

Batista, A. L. D., Silva, R., Cappato, L. P., Ferreira, M. V. S., Nascimento, K. O., Schmiele, M., Esmerino, E.A., Balthazar, C. F., Silva, H. L. A., Moraes, J., Pimentel, T.C., Freitas, M. Q., Raices, R. S. L., Silva, M. C. & Cruz, A. G. (2017). Developing a synbiotic fermented milk using probiotic bacteria and organic green banana flour. *Journal of Functional Foods*, 38, 242–250. https://doi.org/10.1016/j.jff.2017.09.037

Bonjour, J. P., Benoit, V., Payen, F., & Kraenzlin, M. (2013). Consumption of yogurts fortified in vitamin D and calcium reduces serum parathyroid hormone and markers of bone resorption: A double-blind randomized controlled trial in institutionalized elderly women. *Journal of Clinical Endocrinology and Metabolism*, 98(7), 2915–2921. https://doi.org/10.1210/jc.2013-1274

Cardoso, T., Marques, C., Dagostin, J. L. A., & Masson, M. L. (2019). Lactobionic Acid as a Potential Food Ingredient: Recent Studies and Applications. *Journal of Food Science*, 84(7), 1672–1681. https://doi.org/10.1111/1750-3841.14686

Dias, M. I., Ferreira, I. C. F. R., & Barreiro, M. F. (2015). Microencapsulation of bioactives for food applications. *Food and Function*, 6(4), 1035–1052. https://doi.org/10.1039/c4fo01175a

Espitia, P. J. P., Batista, R. A., Azeredo, H. M. C., & Otoni, C. G. (2016). Probiotics and their potential applications in active edible films and coatings. *Food Research International*, 90, 42–52. https://doi.org/10.1016/j.foodres.2016.10.026

FAO/WHO. (2010). Codex standard for fermented milks. N: Codex Stan (Eds.), Second Ed. Food and Agriculture Organization, United Nations, Rome, 1–5.

FDA. Code of Federal Regulations, Title 21, 21 CFR 172.720. US Food and Drug Administration. (2017). Retrieved from https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=172.720

García, C., Ranieri, G., Rendueles, M., & Díaz, M. (2019). Exploring encapsulation strategies as a protective mechanism to avoid amensalism in mixed populations of Pseudomonas

taetrolens and Lactobacillus casei. *Bioprocess and Biosystems Engineering*, (0123456789). https://doi.org/10.1007/s00449-019-02204-8

Gorbunova, N., Evteev, A., Evdokimov, I., & Bannikova, A. (2016). Kinetics of ascorbic acid transport from alginate beads during in vitro digestion. *Journal of Food and Nutrition Research*, 55(2), 148–158.

Intenational Standard ISO 9665. (1998). Adhesives-Animal glues-Methods of sampling and testing. 1998.

Kaushik, V., & Roos, Y. H. (2007). Limonene encapsulation in freeze-drying of gum Arabic-sucrose-gelatin systems. *LWT - Food Science and Technology*, 40(8), 1381–1391. https://doi.org/10.1016/j.lwt.2006.10.008

Khalf, M., Dabour, N., kheadr, E., & Fliss, I. (2010). Viability of probiotic bacteria in maple sap products under storage and gastrointestinal conditions. *Bioresource Technology*, 101(20), 7966–7972. https://doi.org/10.1016/j.biortech.2010.05.053

Li, L., Chen, Y., Wang, Y., Shi, F., Nie, Y., Liu, T., & Song, K. (2019). Effects of concentration variation on the physical properties of alginate-based substrates and cell behavior in culture. *International Journal of Biological Macromolecules*, 128, 184–195. https://doi.org/10.1016/j.ijbiomac.2019.01.123

Lopes, L., de Siqueira Ferraz Carvalho, R., Stela Santos Magalhães, N., Suely Madruga, M., Julia Alves Aguiar Athayde, A., Araújo Portela, I., Barao, C. E., Pimentel, T. C., Magnani, M. & Christina Montenegro Stamford, T. (2020). Microencapsulation of Lactobacillus acidophilus La-05 and incorporation in vegan milks: Physicochemical characteristics and survival during storage, exposure to stress conditions, and simulated gastrointestinal digestion. *Food Research International*, 135, 109295. https://doi.org/10.1016/j.foodres.2020.109295

López Córdoba, A., Deladino, L., & Martino, M. (2013). Effect of starch filler on calcium-alginate hydrogels loaded with yerba mate antioxidants. *Carbohydrate Polymers*, 95(1), 315–323. https://doi.org/10.1016/j.carbpol.2013.03.019

Mahmoud, M., Abdallah, N. A., El-Shafei, K., Tawfik, N. F., & El-Sayed, H. S. (2020). Survivability of alginate-microencapsulated Lactobacillus plantarum during storage, simulated food processing and gastrointestinal conditions. *Heliyon*, 6(3). https://doi.org/10.1016/j.heliyon.2020.e03541

Melchior, S., Marino, M., Innocente, N., Calligaris, S., & Nicoli, M. C. (2020). Effect of different biopolymer-based structured systems on the survival of probiotic strains during storage and in vitro digestion. *Journal of the Science of Food and Agriculture*, 100(10), 3902–3909. https://doi.org/10.1002/jsfa.10432

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., F. Carrière, Boutrou R., Corredig M., Dupont D., Dufour C., Egger L., Golding M, KarakayaS., Kirkhus B., Le Feunteun S., Lesmes U., MacIerzanka A., MacKie A., Marze S., McClements D. J., Ménard O., Recio I., Santos C. N., Singh R. P., Vegarud G. E., Wickham M. S. J., Weitschies W. & Brodkorb, A. (2014). A standardised static in vitro digestion method suitable for food-an

international consensus. *Food and Function*, 5(6), 1113–1124. https://doi.org/10.1039/c3fo60702j

Nocerino, R., Paparo, L., Terrin, G., Pezzella, V., Amoroso, A., Cosenza, L., Cecere, G., De Marco, G., Micillo, M., Albano, F., Nugnes, R., Ferri, P., Ciccarelli, G., Giaccio, G., Spadaro R., maddalena, Y., Berni Canani, F. & Berni Canani, R. (2017). Cow's milk and rice fermented with Lactobacillus paracasei CBA L74 prevent infectious diseases in children: A randomized controlled trial. *Clinical Nutrition*, 36(1), 118–125. https://doi.org/10.1016/j.clnu.2015.12.004

Peng, M., Tabashsum, Z., Anderson, M., Truong, A., Houser, A. K., Padilla, J., Akmel, A., Bhatti, J., Rahaman, S. O. & Biswas, D. (2020). Effectiveness of probiotics, prebiotics, and prebiotic-like components in common functional foods. *Comprehensive Reviews in Food Science and Food Safety*, 19(4), 1908–1933. https://doi.org/10.1111/1541-4337.12565

Saarela, M., Hallamaa, K., Mattila-Sandholm, T., & Mättö, J. (2003). The effect of lactose derivatives lactulose, lactitol and lactobionic acid on the functional and technological properties of potentially probiotic Lactobacillus strains. *International Dairy Journal*, 13(4), 291–302. https://doi.org/10.1016/S0958-6946(02)00158-9

Schaafsma, G. (2008). Lactose and lactose derivatives as bioactive ingredients in human nutrition. *International Dairy Journal*, 18(5), 458–465. https://doi.org/10.1016/j.idairyj.2007.11.013

Simó, G., Fernández-Fernández, E., Vila-Crespo, J., Ruipérez, V., & Rodríguez-Nogales, J. M. (2017). Research progress in coating techniques of alginate gel polymer for cell encapsulation. *Carbohydrate Polymers*, 170, 1–14. https://doi.org/10.1016/j.carbpol.2017.04.013

Warren, F. J., Zhang, B., Waltzer, G., Gidley, M. J., & Dhital, S. (2015). The interplay of α-amylase and amyloglucosidase activities on the digestion of starch in in vitro enzymic systems. *Carbohydrate Polymers*, 117, 185–191. https://doi.org/10.1016/j.carbpol.2014.09.043

Capítulo 6.

Discusión general

La presente tesis doctoral aborda la incorporación de ácido lactobiónico como compuesto prebiótico y *L. plantarum* CECT 9567 y *L. paracasei* CBA L74 como microorganismos probióticos durante el desarrollo y caracterización de materiales bioactivos de uso alimentario empleando diferentes matrices proteicas y polisacáridas. Los resultados obtenidos, que se discutirán a continuación, pueden resultar de interés para la industria alimentaria, en el contexto del empleo de materiales alimentarios sostenibles y del desarrollo de alimentos funcionales al incorporar estos materiales bioactivos y funcionales.

El proceso biotecnológico para el desarrollo de materiales bioactivos alimentarios comienza con la selección del compuesto prebiótico y de las bacterias probióticas. En el caso del prebiótico, se seleccionó el ácido lactobiónico, además de por sus numerosas propiedades detalladas en el **Capítulo 2-Apartado 2.1**, por sus características fisicoquímicas. Las moléculas de ácido lactobiónico son muy pequeñas, tienen un bajo peso molecular (358,296 g/mol) y además son muy higroscópicas (Cardoso, Marques, Sotiles, Dagostin, & Masson, 2019). A la hora de desarrollar biomateriales de uso alimentario, es una característica favorable, ya que permite que se retenga una mayor cantidad de agua en los mismos, ayudando a mantener la humedad y la textura de los productos alimentarios. Además, puede considerarse como un plastificante, al tener mayor polaridad que la sacarosa y ya se ha empleado como tal en el desarrollo de algunos *films* (Wan, Moon, & Lee, 2005). Por otro lado, se trata de un prebiótico novedoso (Cardoso, Marques, Dagostin, et al., 2019) (Alonso, Rendueles, & Díaz, 2013b). A pesar de la falta de estudios *in vivo* en poblaciones humanas (Van Dokkum, Wezendonk & van Aken-Schneider, 1994), su uso en Japón desde hace décadas (Kiryu et al., 2009) (Kiryu et al., 2012) y la aprobación de su empleo por parte de autoridades alimentarias como la FDA (FDA,2017) muestran que tiene un gran potencial en la industria alimentaria.

Sólo uno de los tipos de biomateriales desarrollados, los *films* de gelatina (**Capítulo 4,-Subapartado 4.1.1**) incluyeron ácido lactobiónico producido por síntesis biológica a partir de la fermentación de suero lácteo con *P. taetrolens* LMG2336. En ese trabajo, se quería aprovechar un subproducto de la industria alimentaria y obtener un producto prebiótico con un alto valor añadido que pudiese tener una aplicación directa en el campo alimentario. No se encontraron diferencias entre el ácido lactobiónico producido por síntesis biológica y el ácido lactobiónico comercial. La presente tesis doctoral no se centra en los procesos de separación y purificación si no en la elaboración de biomateriales activos y el estudio de la interacción que hay entre estos compuestos.

Una de las futuras líneas de investigación sería el acoplamiento de este proceso de producción para obtener, a partir de un subproducto de la industria alimentaria, ácido lactobiónico purificado que se pueda emplear directamente en la elaboración de materiales de uso alimentario. Para el resto de las investigaciones desarrolladas en los **Capítulos 4 y 5**, se empleó ácido lactobiónico comercial, a excepción de las micropartículas, en las que se añadió como su sal más habitual (lactobionato de calcio, E-399), observando que las características fisicoquímicas eran prácticamente idénticas.

En cuanto a los microorganismos probióticos, se seleccionaron dos cepas del género *Lactobacillus*, ya que se trata de unos de los más empleados en la industria alimentaria (Zendeboodi, Khorshidian, Mortazavian, & da Cruz, 2020). En ambos casos, se verificó que las dos cepas eran capaces de consumir ácido lactobiónico como única fuente de carbono para su crecimiento, ya que no todos los probióticos son capaces de metabolizar cualquier compuesto prebiótico. Se obtuvieron resultados positivos en ambos casos, destacando que *L. paracasei* CB L74 es capaz de producir más ácido láctico de manera prolongada en el tiempo usando como sustrato ácido lactobiónico en vez de glucosa.

Una vez seleccionados los compuestos bioactivos, el segundo paso fue la elección de los diferentes biopolímeros para desarrollar los materiales alimentarios. Uno de los factores clave en el desarrollo de estos biomateriales, es la caracterización de los mismos, atendiendo a sus propiedades fisicoquímicas y mecánicas, así como al estudio de su comportamiento en productos alimentarios.

En el caso de las proteínas, se seleccionaron dos diferentes: gelatina **(apartado 4.1)** y proteína de yema de huevo delipidada **(apartado 4.2)**.

La gelatina se seleccionó como matriz en la elaboración de *films* con ácido lactobiónico producido a través de la síntesis biológica por parte de *P. taetrolens* LMG 2366 empleando permeado de suero dulce como sustrato por ser una proteína fácil de incorporar y ser una de las más estudiadas en el ámbito alimentario (Nilsen-Nygaard et al., 2021) (Calva-Estrada et al., 2019) (**subapartado 4.1.1**). En este caso, se partió de un subproducto de la industria lechera con el objetivo de intentar aprovecharlo y revalorizarlo en términos de economía circular. Además, también se añadió en la formulación *L. plantarum* CECT 9567 con el objetivo de desarrollar un material sinbiótico. Tras la producción por medio de un biorreactor con agitación mecánica, fue necesaria la eliminación de las endotoxinas producidas por parte de la bacteria Gram

negativa usando técnicas de microfiltración, obteniéndose una concentración < 0,25 EU/mL, pudiendo usarse por tanto en la industria alimentaria (Wassenaar & Zimmermann, 2018). Los *films* elaborados mostraron buenas propiedades mecánicas, siendo el parámetro PS influenciado por la presencia de ácido lactobiónico y el PD por la presencia del probiótico, al igual que sucedió con los *films* elaborados con proteína de yema de huevo delipidada. Sin embargo, estos *films* mostraron una mayor solubilidad en agua, muy probablemente debido a la alta solubilidad del ácido lactobiónico (Cardoso, Marques, Sotiles, et al., 2019). Además, también se observó que la presencia del ácido lactobiónico mejoró la viabilidad del probiótico.

La proteína de yema de huevo se seleccionó como una forma de revalorizar una fracción con menos uso en la industria alimentaria (Marcet et al., 2014) ya que está compuesta casi de manera exclusiva por proteínas, con muy poca cantidad de lípidos y colesterol (Marcet, Paredes, & Díaz, 2015). Esta fracción puede ser muy atractiva para la industria alimentaria debido a su capacidad como agente emulsificante, su gran cantidad de folatos y su potencial para ser usada como matriz en el desarrollo de materiales alimentarios, tal y como se detalla en el **subapartado 4.2.1**.Tras delipidar la proteína de yema de huevo con el objetivo de eliminar los pocos lípidos presentes en esta fracción, estas proteínas se usaron como matriz en el desarrollo de *films* **(subapartado 4.2.2)**. Los *films* desarrollados mostraron buenas propiedades ópticas y de transparencia, permitiendo la inspección ocular de los productos alimentarios. En cuanto al color, mostraron una ligera tonalidad amarilla, que puede ser causada por la presencia de carotenoides liposolubles, como el β-caroteno, que es soluble en solventes como el hexano (Kovalcuks & Duma, 2016) y puede no haber sido eliminado por completo con la delipidación con etanol (Li-Chan, E.C.Y and Kim, 2008). En cuanto a las propiedades mecánicas, mostraron menores valores de PS y PD que los obtenidos para los *films* elaborados con gelatina o caseína. Como se ha comentado en la presente memoria, las interacciones que tienen lugar a nivel molecular definen, entre otras, las propiedades mecánicas de los *films* resultantes. Esta matriz al estar compuesta por lipoproteínas de alta densidad de tipo globular hace que las interacciones sean más débiles. Sin embargo, el tratamiento con transglutaminasa permitió mejorar estas propiedades, tal y como han observado otros autores con otras matrices proteicas (Weng & Zheng, 2015). Una característica a destacar de estos *films* es su baja solubilidad en agua, a pesar de tratarse de una matriz proteica. Esto se debe a que los gránulos de la proteína de yema de huevo solo son solubles en agua con una alta fuerza iónica (< 0.3 M) o a pH muy básicos. Estas condiciones no son fáciles de encontrar en

la industria alimentaria, por lo que estos *films* serían óptimos para el recubrimiento durante largos periodos de tiempo de una gran gama de productos alimentarios. Tras obtener estos resultados, estos *films* se elaboraron incorporando ácido lactobiónico y *L. plantarum* CECT 9567 (**subapartado 4.2.3**). La incorporación de ambos causó cambios a nivel molecular que dieron lugar a modificaciones de las propiedades mecánicas. Se observó una disminución de los parámetros de PS por la presencia del prebiótico, fenómeno que ha sido descrito previamente por otros autores (Fernandes et al., 2020b) (Orozco-Parra, Mejía, & Villa, 2020) (Oliveira-Alcântara et al., 2020) (Odila Pereira, Soares, J.P. Monteiro, Gomes, & Pintado, 2018), mientras que el probiótico aumentó los valores de PD. De esta investigación cabe destacar la interacción que tuvo lugar entre el prebiótico y *L. plantarum*. La formulación en la que estaban ambos compuestos conjuntamente fue la que permitió obtener una mayor viabilidad del probiótico durante el periodo de almacenamiento, tanto en su formulación en *film* como recubriendo gelatina como modelo alimentario. Este efecto sinérgico ha sido observado por otros autores con diferentes prebióticos y especies probióticas (Orozco-Parra et al., 2020) (Oliveira-Alcântara et al., 2020) (Odila Pereira et al., 2018).

Los siguientes biopolímeros que se seleccionaron para desarrollar otros materiales de uso alimentario fueron los polisacáridos (**Capítulo 5**). Utilizando una mezcla de matrices (**apartado 5.1**) y alginato de sodio (**apartado 5.2**) se desarrollaron otros tipos de biomateriales de aplicación alimentaria diferentes a los *films*.

En una primera aproximación, se empleó una mezcla de varios polisacáridos diferentes como matriz para elaborar micropartículas prebióticas con lactobionato de calcio (**Subapartado 5.1.1**). Se empleó la microencapsulación ya que es una técnica muy habitual en la industria alimentaria para encapsular diferentes compuestos bioactivos (Sarao & Arora, 2017). En la caracterización de las micropartículas se observó que, independientemente de la matriz empleada (maltodextrina, goma arábiga, caseinato de sodio o gelatina), todas las micropartículas mostraron una alta solubilidad en agua, lo que limitaría su uso en productos alimentarios con un alto contenido en humedad. Todas ellas mostraron una buena eficacia de encapsulación y morfología. Para estudiar el comportamiento de las micropartículas en un modelo alimentario real, éstas se introdujeron en quesos frescos de cabra. El prebiótico fue encapsulado por dos objetivos. Primero, porque el ácido lactobiónico no puede ser añadido directamente a la leche durante la manufacturación del queso. Al ser un compuesto altamente soluble en agua (Cardoso, Marques, Sotiles, et al., 2019), surgen problemas tecnológicos, ya que permanecía en la fracción del suero y no en la red de caseína. En segundo lugar,

con la encapsulación del prebiótico se evitó la pérdida de este hace la matriz del queso, impidiendo su consumo por parte de las BAL presentes de manera natural en el queso de cabra. Los quesos de cabra con micropartículas mostraron buenas propiedades texturales y las micropartículas mostraron capacidad de protección del prebiótico.

Empleando como matriz alginato de sodio se desarrollaron *coatings* (**subapartado 4.2.1**.) y cápsulas (**subapartado 4.2.2**.). En el caso de los coatings prebióticos, probióticos y sinbióticos, se utilizó queso de cabra como modelo alimentario que fue recubierto con la técnica de inmersión, una de las más usadas en la industria alimentaria (Suhag et al., 2020). Al incluir el prebiótico en el coatings, también se solventó el problema tecnológico generado por la alta solubilidad del ácido lactobiónico en agua (Cardoso, Marques, Sotiles, et al., 2019). Además, se añadió el probiótico en el *coating* y no en la matriz del queso con el resto de BAL con el objetivo de desarrollar un recubrimiento sinbiótico. Los quesos recubiertos elaborados mostraron buenas propiedades texturales, con apenas diferencias respecto al control. La caracterización textural es un parámetro clave, ya que es uno de los principales factores en la percepción sensorial por parte de los consumidores (Wilkinson, Dijksterhuis, & Minekus, 2001) (Zhong, Cavender, & Zhao, 2014). Al igual que en los estudios discutidos anteriormente, la presencia de ácido lactobiónico mejoró la viabilidad del probiótico durante el periodo de almacenamiento. Con el objetivo de desarrollar otro tipo de materiales alimentarios que no tuviesen un uso exclusivo como recubrimiento, como es el caso de los *films* y *coatings*, se elaboraron cápsulas de alginato de sodio (**subapartado 5.2.2**), de uso habitual en la industria alimentaria (Espitia, Batista, Azeredo, & Otoni, 2016). Estas cápsulas pueden ser consumidas solas o añadidas a diferentes productos alimentarios con el objetivo de convertirlos en alimentos funcionales. En este caso, se empleó *L. paracasei* CB L74 como microorganismo probiótico. Las cápsulas se endurecieron con soluciones de diferentes concentraciones de $CaCl_2$ y con quitosano, observándose que las endurecidas a mayor concentración de $CaCl_2$ y con la capa extra de quitosano mostraron los mejores valores de resistencia en términos de Bloom. Las cápsulas se secaron térmicamente y con métodos de liofilización sin observarse diferencias significativas debido al método de secado en cuanto a la viabilidad del probiótico, pero sí en cuanto a la composición de la cápsula. Sin embargo, sí se observaron diferencias debido al método de secado en cuanto a la rehidratación de las cápsulas, siendo óptima en las cápsulas liofilizadas, independientemente de si se rehidrataron en solución salina o yogurt.

Por último, cabe destacar y matizar dos puntos importantes de la investigación desarrollada en la presente tesis doctoral.

En primer lugar, la cantidad de ácido lactobiónico (o su sal más común) añadida a los diferentes materiales de uso alimentario elaborados y caracterizados. Como se ha comentado a lo largo de la memoria, en ninguna normativa se ha concretado todavía una cantidad límite para su consumo en humanos. Las cantidades añadidas en los diferentes materiales alimentarios han variado, no excediendo nunca los 50 g/L. Hay que tener en cuenta que las cantidades de *film*, *coating*, cápsulas o micropartículas que se ingerirían serían pequeñas. En todos los casos, la cantidad de ácido lactobiónico que se ingeriría se encontraría dentro de los valores habituales de ingesta medidos en Japón por el consumo del "Caspian Sea yogurt" (Kiryu et al., 2009) y se encontrarían bastante alejados de los valores que podrían llegar a causar efectos similares a la intolerancia por lactosa (24 g de ácido lactobiónico por día (Van Dokkum, Wezendonk, van Aken-Schneider, 1994)).

En segundo lugar, la caracterización de los materiales probióticos y sinbióticos en cuanto a las pruebas de digestión *in vitro* simuladas. A la hora de desarrollar materiales bioactivos con microorganismos vivos, no es importante únicamente la viabilidad de estos durante los procesos de producción y almacenamiento, si no también durante su consumo. A pesar de que no hay un consenso científico internacional sobre la concentración de probióticos necesaria para obtener efectos beneficiosos sobre la salud, varios autores consideran que esta concentración está entre 6 y 9 $\log_{10}$ UFC por día (Espitia et al., 2016) (Saad, Delattre, Urdaci, Schmitter, & Bressollier, 2013). Los productos con esas concentraciones pueden considerarse probióticos. En las investigaciones detalladas en la presente memoria, se ha puesto hincapié en la diferencia de viabilidad del probiótico durante el almacenamiento y tras las pruebas de digestión *in vitro* simulada. Todos los materiales desarrollados con microorganismos probióticos (con y sin presencia de ácido lactobiónico) entran dentro de la categoría de "probiótico", a excepción de los *films* elaborados empleando gelatina como matriz. Además, algunos de ellos, como los recubrimientos sinbióticos con proteína de yema de huevo delipidada como matriz *(subapartado 4.2.3)*, los dos recubrimientos sinbióticos de alginato de sodio *(subapartado 5.2.1)*, y las cápsulas rehidratadas en yogurt *(subapartado 5.2.2)*, tendrían la categoría de "probiótico" (mínimo de 10^6 UFC/día) tras las pruebas de digestión *in vitro* simulada, siendo capaces de proporcionar una cantidad adecuada de microorganismos probióticos al colon. Por otro lado, también se analizó la posibilidad de un efecto protector del ácido lactobiónico

durante las pruebas de digestión *in vitro* simulada, ya que otros autores detectaron este tipo de efecto en otros prebióticos, como con los FOS y GOS (Krasaekoopt & Watcharapoka, 2014) (Padilha, Morales, Vieira, Costa, & Saad, 2016) (Langa et al., 2019) (Orozco-Parra et al., 2020) (Ranadheera, Baines, & Adams, 2010). En este caso, en la investigación llevada a cabo sólo se vio una influencia positiva por parte del ácido lactobiónico en la fase oral de las pruebas de digestión *in vitro* simuladas.

Capítulo 7.

Conclusiones

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

- Las dos cepas probióticas utilizadas en esta investigación (*L. plantarum* CECT 9567 y *L. paracasei* CB L74) fueron capaces de emplear de manera exclusiva como fuente de carbono ácido lactobiónico. Por tanto, la combinación de este prebiótico con ambas bacterias probióticas es adecuada.
- La incorporación de ácido lactobiónico como prebiótico y *L. plantarum* CECT 9567 como bacteria probiótica en *films* elaborados con proteínas como matriz (gelatina y proteína de yema de huevo delipidada) fue óptima.
- Los *films* prebióticos elaborados a partir de permeado de suero lácteo como subproducto de la industria alimentaria utilizando gelatina como matriz proteica pueden ser usados en el ámbito alimentario tras la eliminación de las endotoxinas producidas por *P. taetrolens* LMG 2336 hasta niveles adecuados para el consumo. La incorporación de *L. plantarum* CECT 9567 no permitió obtener *films* sinbióticos, debido a la pobre composición de la matriz en cuanto a nutrientes.
- Los *films* desarrollados con proteína de yema de huevo delipidada mostraron buenas propiedades fisicoquímicas y mecánicas, destacando por su baja solubilidad en agua lo que los hace idóneos para recubrir diferentes tipos de productos alimentarios con un alto nivel de humedad durante tiempos prolongados.
- Los *films* elaborados con proteína de yema de huevo delipidada como matriz mostraron buenas propiedades fisicoquímicas y mecánicas tras la incorporación del prebiótico y el probiótico, considerando los cambios que se produjeron a nivel molecular por la adición de dichos compuestos.
- La integración de ácido lactobiónico y *L. plantarum* CECT 9567 y *L. paracasei* CB L74 en biomateriales desarrollados usando polisacáridos como matriz fue apropiada.
- Las micropartículas permitieron mantener el lactobionato de calcio como prebiótico en el interior de quesos frescos de cabra, impidiendo su consumo por parte de las BAL, pudiendo llegar de manera intacta al tracto gastrointestinal tras las pruebas de digestión *in vitro* simulada.
- Los *coatings* prebióticos, probióticos y sinbióticos mostraron un buen comportamiento y propiedades al emplearse para recubrir quesos frescos de cabra. No se apreciaron cambios significativos en las propiedades texturales de los mismos.

- Las cápsulas sinbióticas con ácido lactobiónico y *L. paracasei* CB L74 mostraron buenas propiedades y permitieron mantener la viabilidad del probiótico tras los procesos de secado empleados (secado térmico y liofilización). La rehidratación con yogurt fue mejor que la rehidratación en solución salina para la viabilidad del microorganismo en las pruebas de digestión *in vitro* simulada.
- En todos los materiales desarrollados, se comprobó el efecto sinérgico del ácido lactobiónico sobre los dos probióticos, mejorando su viabilidad durante el periodo de almacenamiento. De esta forma, todos los materiales probióticos y sinbióticos desarrollados (a excepción de los *films* de gelatina) se les puede otorgar la categoría de “probiótico”.
- Los biomateriales desarrollados que fueron sometidos a pruebas de digestión *in vitro* simuladas, confirieron protección a las bacterias probióticas, manteniendo la viabilidad de éstas.
- A diferencia de otros prebióticos, el ácido lactobiónico no mostró un efecto protector por sí mismo durante las pruebas de digestión *in vitro* simuladas. Solamente se observó cierto efecto protector en la fase oral de estas pruebas.

Capítulo 8.

Bibliografía

Alonso, S., Rendueles, M., & Díaz, M. (2011). Efficient lactobionic acid production from whey by Pseudomonas taetrolens under pH-shift conditions. *Bioresource Technology*, *102*(20), 9730–9736. https://doi.org/10.1016/j.biortech.2011.07.089

Alonso, S., Rendueles, M., & Díaz, M. (2013a). Bio-production of lactobionic acid: Current status, applications and future prospects. *Biotechnology Advances*, *31*(8), 1275–1291. https://doi.org/10.1016/j.biotechadv.2013.04.010

Alonso, S., Rendueles, M., & Díaz, M. (2013b). Bio-production of lactobionic acid: Current status, applications and future prospects. *Biotechnology Advances*, *31*(8), 1275–1291. https://doi.org/10.1016/j.biotechadv.2013.04.010

Alonso, S., Rendueles, M., & Díaz, M. (2013c). Feeding strategies for enhanced lactobionic acid production from whey by Pseudomonas taetrolens. *Bioresource Technology*, *134*, 134–142. https://doi.org/10.1016/j.biortech.2013.01.145

Amin, U., Khan, M. U., Majeed, Y., Rebezov, M., Khayrullin, M., Bobkova, E., Shariati, M. A.,, Chung, I. M. & Thiruvengadam, M. (2021). Potentials of polysaccharides, lipids and proteins in biodegradable food packaging applications. *International Journal of Biological Macromolecules*, *183*(May), 2184–2198. https://doi.org/10.1016/j.ijbiomac.2021.05.182

Anton, M. (2013). Egg yolk: Structures, functionalities and processes. *Journal of the Science of Food and Agriculture*, *93*(12), 2871–2880. https://doi.org/10.1002/jsfa.6247

Ashwell, M. (2002). Concepts of functional foods. In *IISI Europe Concise Monograph Series*. https://doi.org/10.1108/nfs.2004.34.1.47.3

Barlow, S., Chesson, A., Collins, J. D., Dybing, E., Flynn, A., Fruijtier-Pölloth, C., Hardy, a., Knaap, A., Kuiper, H., Le Neindre, P., Schlatter, J., Silano, V., Skwefving, S. & Vannier, P. (2007). Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA - Opinion of the Scientific Committee. *EFSA Journal*, *5*(12), 1–16. https://doi.org/10.2903/j.efsa.2007.587

Batista, A. L. D., Silva, R., Cappato, L. P., Ferreira, M. V. S., Nascimento, K. O., Schmiele, M., esmerino, E.A., Balthazar, C.F., Silva, H.L.A., Moraes, J., Pimentel, T.C., Freitas, M.Q., Raices, R.S.L, Silva, M.C. & Cruz, A. G. (2017). Developing a synbiotic fermented milk using probiotic bacteria and organic green banana flour. *Journal of Functional Foods*, *38*, 242–250. https://doi.org/10.1016/j.jff.2017.09.037

Calva-Estrada, S. J., Jiménez-Fernández, M., & Lugo-Cervantes, E. (2019). Protein-Based Films: Advances in the Development of Biomaterials Applicable to Food Packaging. *Food Engineering Reviews*, (2019), 78–92. https://doi.org/10.1007/s12393-019-09189-w

Cardoso, T., Marques, C., Dagostin, J. L. A., & Masson, M. L. (2019). Lactobionic Acid as a Potential Food Ingredient: Recent Studies and Applications. *Journal of Food Science*, *84*(7), 1672–1681. https://doi.org/10.1111/1750-3841.14686

Cardoso, T., Marques, C., Sotiles, A. R., Dagostin, J. L. A., & Masson, M. L. (2019). Characterization of lactobionic acid evidencing its potential for food industry application. *Journal of Food Process Engineering*, *42*(7), 1–11. https://doi.org/10.1111/jfpe.13277

Cazón, P., Velazquez, G., Ramírez, J. A., & Vázquez, M. (2017). Polysaccharide-based films and coatings for food packaging: A review. *Food Hydrocolloids*, *68*, 136–148. https://doi.org/10.1016/j.foodhyd.2016.09.009

Chiralt, A., Menzel, C., Hernandez-García, E., Collazo, S., & Gonzalez-Martinez, C. (2020). Use of by-products in edible coatings and biodegradable packaging materials for food preservation. *Sustainability of the Food System: Sovereignty, Waste, and Nutrients Bioavailability*, 101–127. https://doi.org/10.1016/B978-0-12-818293-2.00006-9

De Giorgi, S., Raddadi, N., Fabbri, A., Gallina Toschi, T., & Fava, F. (2018). Potential use of ricotta cheese whey for the production of lactobionic acid by Pseudomonas taetrolens strains. *New Biotechnology*, *42*(April 2017), 71–76. https://doi.org/10.1016/j.nbt.2018.02.010

Dias, M. I., Ferreira, I. C. F. R., & Barreiro, M. F. (2015). Microencapsulation of bioactives for food applications. *Food and Function*, *6*(4), 1035–1052. https://doi.org/10.1039/c4fo01175a

Espitia, P. J. P., Batista, R. A., Azeredo, H. M. C., & Otoni, C. G. (2016). Probiotics and their potential applications in active edible films and coatings. *Food Research International*, *90*, 42–52. https://doi.org/10.1016/j.foodres.2016.10.026

FAO/WHO. (2002). *FAO/WHO Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food.* London. Ontario. Canada.

FAO. (2008). FAO Technical Meeting on Prebiotics. *Advance Analytical Technologies*. Rome.

FDA. Code of Federal Regulations, Title 21, 21 CFR 172.720. US Food and Drug Administration. (2017).https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=172.720

FDA. Code of Federal Regulations, title 21, CFR 20, 25, 170, 184, 186 and 570. Substances Generally Recognized as Safe. (2016). *81*(159), 54960–55055.

Fernandes, Leonardo M. Guimaraes Jonas T., Pimentel Tatiana C., Esmerino Erick A., Freitas Mônika Q., Carvalho Carlos Wanderlei P., C. A. G. & S. M. C. (2020). Chapter 7. Edible whey protein films and coatings added with prebiotic ingredients. In *Agri-Food Industry Sytrategies for Healthy Diets and sustainability* (pp. 177–193). https://doi.org/https://doi.org/10.1016/B978-0-12-817226-1.00007-2

Fernandes, L. M., Guimarães, J. T., Silva, R., Rocha, R. S., Coutinho, N. M., Balthazar, C. F., Cavalcanti, R.N., Piler, C.W., Pimentel, T.C., Neto, R.P.C, Tavares, M.I.B., Esmerino, E.A., Freitas, M.Q., Silva, M.C. & Cruz, A. G. (2020a). Whey protein films added with galactooligosaccharide and xylooligosaccharide. *Food Hydrocolloids*, *104*(February). https://doi.org/10.1016/j.foodhyd.2020.105755

Figueroa-González, I., Quijano, G., Ramírez, G., & Cruz-Guerrero, A. (2011). Probiotics and prebiotics-perspectives and challenges. *Journal of the Science of Food and Agriculture*, *91*(8), 1341–1348. https://doi.org/10.1002/jsfa.4367

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*(5), 1250–1256. https://doi.org/10.1002/btpr.2507

Gibson, G. R., Hutkins, R., Sanders, M. E., Prescott, S. L., Reimer, R. A., Salminen, S. J., Scott, K., Stanton, C., Swanson, K.S., Cani, P.D., Verbeke, K. & Reid, G. (2017). Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nature Reviews Gastroenterology and Hepatology*, *14*(8), 491–502. https://doi.org/10.1038/nrgastro.2017.75

Gibson, G. R., & Roberfroid, M. B. (1995). Critical Review Dietary Modulation of the Human Colonic Microbiota: Introducing the Concept of Prebiotics. *Journal of Nutrition*, *125*(June 1995), 1401–1412. https://doi.org/10.1093/jn/125.6.1401

Gopalakrishnan, S., Xu, J., Zhong, F., & Rotello, V. M. (2021). Strategies for Fabricating Protein Films for Biomaterial Applications. *Advanced Sustainable Systems*, *5*(1), 1–12. https://doi.org/10.1002/adsu.202000167

Hassan, B., Chatha, S. A. S., Hussain, A. I., Zia, K. M., & Akhtar, N. (2018). Recent advances on polysaccharides, lipids and protein based edible films and coatings: A review. *International Journal of Biological Macromolecules*, *109*, 1095–1107. https://doi.org/10.1016/j.ijbiomac.2017.11.097

Hellebois, T., Tsevdou, M., & Soukoulis, C. (2020). Functionalizing and bio-preserving processed food products via probiotic and synbiotic edible films and coatings. In *Advances in Food and Nutrition Research* (1st ed., Vol. 94). https://doi.org/10.1016/bs.afnr.2020.06.004

Jeya Jeevahan, J., Chandrasekaran, M., Venkatesan, S. P., Sriram, V., Britto Joseph, G., Mageshwaran, G., & Durairaj, R. B. (2020). Scaling up difficulties and commercial aspects of edible films for food packaging: A review. *Trends in Food Science and Technology*, *100*(February), 210–222. https://doi.org/10.1016/j.tifs.2020.04.014

Kiryu, T., Kiso, T., Nakano, H., Ooe, K., Kimura, T., & Murakami, H. (2009). Involvement of Acetobacter orientalis in the production of lactobionic acid in Caucasian yogurt ("Caspian Sea yogurt") in Japan. *Journal of Dairy Science*, *92*(1), 25–34. https://doi.org/10.3168/jds.2008-1081

Kiryu, T., Yamauchi, K., Masuyama, A., Kenichi, O., Kimura, T., Kiso, T., Nakano, H. & Murakami, H. (2012). Optimization of Lactobionic Acid Production by *Acetobacter orientalis* Isolated from Caucasian Fermented Milk, "Caspian Sea Yogurt." *Bioscience, Biotechnology, and Biochemistry*, *76*(2), 361–363. https://doi.org/10.1271/bbb.110608

Kovalcuks, A., & Duma, M. (2016). Distribution of Phospholipids, Cholesterol and Carotenoids in Two-Solvent System during Egg Yolk Oil Solvent Extraction. *World Academy of Science, Engineering and Technology, International Journal of Biological, Biomolecular, Agricultural, Food and Biotechnological Engineering*, *10*(5), 323–328.

Krasaekoopt, W., & Watcharapoka, S. (2014). Effect of addition of inulin and galactooligosaccharide on the survival of microencapsulated probiotics in alginate beads coated with chitosan in simulated digestive system, yogurt and fruit juice. *LWT - Food Science and Technology*, *57*(2), 761–766. https://doi.org/10.1016/j.lwt.2014.01.037

Langa, S., van den Bulck, E., Peirotén, A., Gaya, P., Schols, H. A., & Arqués, J. L. (2019). Application of lactobacilli and prebiotic oligosaccharides for the development of a synbiotic semi-hard cheese. *Lwt*, *114*(April), 108361. https://doi.org/10.1016/j.lwt.2019.108361

Li-Chan, E.C.Y and Kim, H. . (2008). Composition of Eggs. *In Egg Bioscience and Biotechnology*, 1–95.

Marcet, I., Álvarez, C., Paredes, B., & Díaz, M. (2014). Inert and Oxidative Subcritical Water Hydrolysis of Insoluble Egg Yolk Granular Protein, Functional Properties, and Comparison to Enzymatic Hydrolysis. *Journal of Agricultural and Food Chemistry*, *62*(32), 8179–8186.

Marcet, I., Paredes, B., & Díaz, M. (2015). Egg yolk granules as low-cholesterol replacer of whole egg yolk in the preparation of gluten-free muffins. *Lwt*, *62*(1), 613–619. https://doi.org/10.1016/j.lwt.2014.08.031

Moreno, F. J., Corzo, N., Montilla, A., Villamiel, M., & Olano, A. (2017). Current state and latest advances in the concept, production and functionality of prebiotic oligosaccharides. *Current Opinion in Food Science*, *13*, 50–55. https://doi.org/10.1016/j.cofs.2017.02.009

Nilsen-Nygaard, J., Fernández, E. N., Radusin, T., Rotabakk, B. T., Sarfraz, J., Sharmin, N., Sivertsvik, M., Sone, I. & Pettersen, M. K. (2021). Current status of biobased and biodegradable food packaging materials: Impact on food quality and effect of innovative processing technologies. *Comprehensive Reviews in Food Science and Food Safety*, *20*(2), 1333–1380. https://doi.org/10.1111/1541-4337.12715

Odila Pereira, J., Soares, J., J.P. Monteiro, M., Gomes, A., & Pintado, M. (2018). Impact of whey protein coating incorporated with Bifidobacterium and Lactobacillus on sliced ham properties. *Meat Science*, *139*(October 2017), 125–133. https://doi.org/10.1016/j.meatsci.2018.01.016

Oh, Y. R., Jang, Y. A., Lee, S. S., Kim, J. H., Hong, S. H., Han, J. J., & Eom, G. T. (2020). Enhancement of Lactobionic Acid Productivity by Homologous Expression of Quinoprotein Glucose Dehydrogenase in Pseudomonas taetrolens. *Journal of Agricultural and Food Chemistry*, *68*(44), 12336–12344. https://doi.org/10.1021/acs.jafc.0c04246

Ohama, H., Ikeda, H., & Moriyama, H. (2014). Health Foods and Foods with Health Claims in Japan. *Nutraceutical and Functional Food Regulations in the United States and Around the World: Second Edition*, *221*, 265–299. https://doi.org/10.1016/B978-0-12-405870-5.00016-5

Oliveira-Alcântara, A. V., Abreu, A. A. S., Gonçalves, C., Fuciños, P., Cerqueira, M. A., Gama, F. M. P., Pastrana, L. M., Rodrigues, S. & Azeredo, H. M. C. (2020). Bacterial cellulose/cashew gum films as probiotic carriers. *Lwt*, *130*(March), 109699. https://doi.org/10.1016/j.lwt.2020.109699

Orozco-Parra, J., Mejía, C. M., & Villa, C. C. (2020). Development of a bioactive synbiotic edible film based on cassava starch, inulin, and Lactobacillus casei. *Food Hydrocolloids*, *104*(January), 105754. https://doi.org/10.1016/j.foodhyd.2020.105754

Padilha, M., Morales, M. L. V., Vieira, A. D. S., Costa, M. G. M., & Saad, S. M. I. (2016). A prebiotic

mixture improved: Lactobacillus acidophilus and Bifidobacterium animalis gastrointestinal in vitro resistance in petit-suisse. *Food and Function*, *7*(5), 2312–2319. https://doi.org/10.1039/c5fo01592h

Parreidt, T. S., Müller, K., & Schmid, M. (2018). Alginate-based edible films and coatings for food packaging applications. *Foods*, *7*(10), 1–38. https://doi.org/10.3390/foods7100170

Paulo, A. F. S., Baú, T. R., Ida, E. I., & Shirai, M. A. (2021). Edible coatings and films with incorporation of prebiotics —A review. *Food Research International*, *148*(March). https://doi.org/10.1016/j.foodres.2021.110629

Pereira, J. O., Soares, J., Costa, E., Silva, S., Gomes, A., & Pintado, M. (2019). Characterization of edible films based on alginate or whey protein incorporated with Bifidobacterium animalis subsp. lactis BB-12 and prebiotics. *Coatings*, *9*(8). https://doi.org/10.3390/coatings9080493

Ranadheera, R. D. C. S., Baines, S. K., & Adams, M. C. (2010). Importance of food in probiotic efficacy. *Food Research International*, *43*(1), 1–7. https://doi.org/10.1016/j.foodres.2009.09.009

Ribeiro, A. M., Estevinho, B. N., & Rocha, F. (2021). Preparation and Incorporation of Functional Ingredients in Edible Films and Coatings. *Food and Bioprocess Technology*, *14*(2), 209–231. https://doi.org/10.1007/s11947-020-02528-4

Saad, N., Delattre, C., Urdaci, M., Schmitter, J. M., & Bressollier, P. (2013). An overview of the last advances in probiotic and prebiotic field. *LWT - Food Science and Technology*, *50*(1), 1–16. https://doi.org/10.1016/j.lwt.2012.05.014

Saarela, M., Hallamaa, K., Mattila-Sandholm, T., & Mättö, J. (2003). The effect of lactose derivatives lactulose, lactitol and lactobionic acid on the functional and technological properties of potentially probiotic Lactobacillus strains. *International Dairy Journal*, *13*(4), 291–302. https://doi.org/10.1016/S0958-6946(02)00158-9

Sarao, L. K., & Arora, M. (2017). Probiotics, prebiotics, and microencapsulation: A review. *Critical Reviews in Food Science and Nutrition*, *57*(2), 344–371. https://doi.org/10.1080/10408398.2014.887055

Sarenkova, I., Sáez-Orviz, S., Ciprovica, I., Rendueles, M., & Díaz, M. (2022). Lactobionic acid production by Pseudomonas taetrolens in a fed-batch bioreactor using acid whey as substrate . *International Journal of Dairy Technology*, *0*, 1–11. https://doi.org/10.1111/1471-0307.12841

Schaafsma, G. (2008). Lactose and lactose derivatives as bioactive ingredients in human nutrition. *International Dairy Journal*, *18*(5), 458–465. https://doi.org/10.1016/j.idairyj.2007.11.013

Suhag, R., Kumar, N., Petkoska, A. T., & Upadhyay, A. (2020). Film formation and deposition methods of edible coating on food products: A review. *Food Research International*, *136*(July), 109582. https://doi.org/10.1016/j.foodres.2020.109582

Van Dokkum W, Wezendonk LJW, van Aken-Schneider P, K. C. (1994). Tolerance of lactobionic

acid in man. TNO Report. *The Netherlands: TNO Nutri- Tion and Food Research*.

Wan, V. C. H., Moon, S. K., & Lee, S. Y. (2005). Water vapor permeability and mechanical properties of soy protein isolate edible films composed of different plasticizer combinations. *Journal of Food Science*, *70*(6), e387–e391. https://doi.org/10.1111/j.1365-2621.2005.tb11443.x

Wassenaar, T. M., & Zimmermann, K. (2018). Lipopolysaccharides in food, food supplements, and probiotics: should we be worried? *European Journal of Microbiology and Immunology*, *8*(3), 63–69. https://doi.org/10.1556/1886.2018.00017

Weng, W., & Zheng, H. (2015). Effect of transglutaminase on properties of tilapia scale gelatin films incorporated with soy protein isolate. *Food Chemistry*, *169*, 255–260. https://doi.org/10.1016/j.foodchem.2014.08.012

Wilkinson, C., Dijksterhuis, G. B., & Minekus, M. (2001). From food structure to texture. *Trends in Food Science and Technology*, *11*(12), 442–450. https://doi.org/10.1016/S0924-2244(01)00033-4

Zendeboodi, F., Khorshidian, N., Mortazavian, A. M., & da Cruz, A. G. (2020). Probiotic: conceptualization from a new approach. *Current Opinion in Food Science*, *32*(April), 103–123. https://doi.org/10.1016/j.cofs.2020.03.009

Zhong, Y., Cavender, G., & Zhao, Y. (2014). Investigation of different coating application methods on the performance of edible coatings on Mozzarella cheese. *LWT - Food Science and Technology*, *56*(1), 1–8. https://doi.org/10.1016/j.lwt.2013.11.006

Zoghi, A., Khosravi-Darani, K., & Mohammadi, R. (2020). Application of edible films containing probiotics in food products. *Journal Fur Verbraucherschutz Und Lebensmittelsicherheit*, *15*(4), 307–320. https://doi.org/10.1007/s00003-020-01286-x

Capítulo 9.

Anexos

A1. Lista de figuras

A2. Lista de tablas

A3. Lista de ecuaciones

A4. Lista de abreviaturas

5-MTHF	5-methyltetrahydrofolate
ABTS	2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
AFB	Animal-free broth
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
BAL	Bacteria del ácido láctico
BCCM	Colección Coordenada Belga de Microorganismos
BSA	Bovine serum albumin
CaLb	Calcium lactobionate
CaLb	Lactobionato de calcio
CAS	Chemical Abstracts Service
CECT	Colección Española de Cultivos Tipo
CFU	Colony forminf units
Copr.	Corporation
DO	Densidad óptica
DPPH	2,2-difenil-1-picrilhidrazilo
DTG Curves	Derivative thermogravimetric curve
EAC	Cantidad del compuesto activo encapsulado
EDTA	Ácido etilendiaminotetraacético
EE	Eficacia de encapsulación
EEUU	Estados Unidos
EFSA	European Food Safety Authority
EU	Unidades de endotoxina
EU	European Union
FAO	Organización de las Naciones Unidas para la Agricultura y la Alimentación
FDA	Food and Drug Administrarion
FOS	Fructo-oligosaccharides
FOSHU	Food for Spefic Health Use
FTIR	Fourier transform infrared
GA	Goma arábiga
GIT	Gastrointestinal tract
GIT	Gastrointestinal tract
GOS	Galacto-oligosaccharides
GRAS	Generally Recognized As Safe

GTT	Transglutaminase
HDL	High-density lipoproteins
HHP	High hydrostatic pressure
HPLC	High Performance Liquid Cromatography
IgY	Inmunoglobulins from yolk
ILAS S.A.	Industrias Lácteas Asturianas S. A.
ILSI	International Life Sciences Institute
IMO	Isomalto-oligosaccharides
iTRAQ	Isobaric tag for relative and absolute quantitation
LAB	Lactic Acid Bacteria
LBA	Lactobionic Acid
LDL	Lipoproteins
LMG	Laboratorium voor Microbiologie Universiteit Gent
LPS	Lipopolisacáridos
LSD	Fischer's Least Significant Difference
LTLT	Low temperatura-long time
MBC	Minimum bactericidal concentration
MC	Moisture content
MD	Maltodextrina
MIC	Minimum inhibitory concentration
MRS	Caldo de cultivo DeMan Rogosa and Sharpe
MRSA	*Staphyloccous aureus* resistente a la meticilina
NA	Not available
NaCas	Caseinato de sodio
NB	Caldo de cultivo Nutrient Broth
OD	Optical density
PBS	Tampón fosfato salino
PD	Puncture Deformation
PRE	Prebiótico
PRO	Probiótico
PS	Puncture Strenght
PVDF	Fluoruro de polivinilideno
RID	Refraction index detector
ROS	Reactive oxygen species
rpm	Revoluciones por minuto
SAC	Cantidad del compuesto activo en superficie

SACS	stearic acid conjugated chitosan
SACS-HDL	Stearic acid conjugated chitonan
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
SF	Spherification factor
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
SOS	Soya-oligosaccharides
SSF	Simulated salivary fluid
SYN	Sinbiótico
TAC	Cantidad total del compuesto activo
TE	Tampón Tris + EDTA
TEM	Transmission electron microscopy
TG	Transglutaminasa
TGA	Thermo-gravimetric analyses
TGase	Transglutaminase
U.S.	United States
UFC	Unidades formadoras de colonia
UV	Ultravioleta
WHO	World Healt Organization
WS	Water solubility
WVP	Water vapour permeability
WVTR	Water vapour transmission rate
XOS	Xylo-oligosaccharides
YI	Yellowness index

A5. Lista de símbolos

~	Aproximadamente
a*	Parámetro de color que mide el enrojecimiento y verdor
A_{600}	Abosrbancia a una longitud de onda de 600 nm
b*	Parámetro de color que mide la amarillez y el tono azul
D	Diámetro
d_{max}	Diámetro mayor de la cápsula (mm)
d_{min}	Diámetro menor de la cápsula (mm)
F_m	Máxima fuerza aplicada (N)
g	Gramo
h	Hora
H_0	Surface hydrophobicity
kDa	Kilodalton
L	Litro
L*	Parámetro de color que mide la luminosidad o brillo
log_{10}	Logaritmo de base 10
m1	Peso de film secado al horno
m2	Peso de film no disuelto secado
mBar	Milibares
MDa	Megadalton
mg	Miligramo
min	Minuto
mL	Mililitro
N	Newton
nm	nanómetros
p/p	Peso/peso
p/v	Peso/volumen
R	Radio
s	Segundo
Th	Thickness (mm)
U	Unidad enzimática
v/v	Volumen/volumen
w/v	Weight/volume
w/w	Weight/weight
$W_{frescas}$	Peso de las cápsulas frescas antes de ser secadas (g)

$W_{rehidratación}$	Peso de agua absorbido por las cápsulas secas durante la rehidratación (g)
x	Grosor de los films (mm)
ΔE	Diferencia de color
ΔE	Diferencia total de color
λ	Longitud de onda
μL	Microlitro
μm	Micrómetro

A6. Difusión de la tesis

A6.1-Artículos científicos derivados de la tesis doctoral

- Marcet, I., Sáez, S., Rendueles, M., Díaz, M. Edible films from residual delipidated egg yolk proteins. *Journal of Food Science and Technology*, **2017**, 54(12), 3969–3978.
- Sáez-Orviz, S., Camilleri, P., Marcet, I., Rendueles, M., Díaz, M. Microencapsulation of calcium lactobionate for protection from microorganisms in a solid phase food. *Biochemical Engineering Journal*, **2019**, 150, 107281.
- Sáez-Orviz, S., Puertas, C., Marcet, I., Rendueles, M., Díaz, M. Bioactive synbiotic coatings with lactobionic acid and *Lactobacillus plantarum* CECT 9567 in the production and characterization of a new functional dairy product. *Journal of Functional Foods*, **2020**, 75, 104263.
- Sáez-Orviz, S., Marcet, I., Rendueles, M., Díaz, M. Bioactive packaging based on delipidated egg yolk protein edible films with lactobionic acid and *Lactobacillus plantarum* CECT 9567: Characterization and use as coating in a food model. *Food Hydrocolloids*, **2021**, 119, 106849.
- Sáez-Orviz, S., Passannanti, F., Gallo, M., Colucci Cante, R., Nigro, F., Budelli, A.L., Rendueles, M., Nigro, R., Díaz, M. Lactic acid bacteria co-encapsulated with lactobionic acid: probiotic viability during *in vitro* digestion. *Applied Sciences*, **2021**, 11, 11404.
- Sáez-Orviz, S.; Marcet, I.; Rendueles, M.; Díaz, M. Preparation of edible films with *Lactobacillus plantarum* and lactobionic acid produced by sweet whey fermentation. *Membranes*, **2022**, 12, 115.
- Marcet, I., Sáez-Orviz, S., Rendueles, M., Díaz, M. Egg yolk granules and phosvitin. Recent advances in food technology and applications. *LWT*, **2022**, 153, 112442.
- Sáez-Orviz, S., Marcet, I., Rendueles, M., Díaz, M. The antimicrobial and bioactive properties of lactobionic acid. *Journal of the Science of Food and Agriculture*, **2022**.

A6.2-Comunicaciones a congresos

Comunicaciones orales

- Marcet, I., Sáez-Orviz, S., Rendueles, M., Díaz, M. Edible films from residual delipidated egg yolk proteins. Comunicación oral. *Congreso Nacional de*

Biotecnología (Biotec 2017). Murcia (España). Junio, 19-21, **2017**.

- Sáez-Orviz, S., García, C., Rendueles, M., Díaz, M. Interaction of *Lactobacillus casei* with *Pseudomonas taetrolens* and other microorganisms of food interest. Comunicación oral. *Congreso Nacional de Biotecnología* (Biotec 2017). Murcia (España). Junio, 19-21, **2017**.
- Sáez-Orviz, S., Marcet, I., Laca, A., Paredes, B., Rendueles, M., Díaz, M. Preparación de materiales alimentarios y no alimentarios a partir de yema de huevo. Comunicación oral (ponencia). *7ma Feria Internacional del Huevo.* Tepatiplán de Morelos (Jalisco, México). Octubre, 18-19, **2018**.
- Sáez-Orviz, S. Retos y perspectivas del mundo avícola. Ponente en el foro de ovoproductos. *7ma Feria Internacional del Huevo.* Tepatiplán de Morelos (Jalisco, México). Octubre, 18-19, **2018**.
- Sáez-Orviz, S., Rendueles, M., Díaz, M. Innovative functional yogurt incorporating microencapsulated calcium lactobionate. Comunicación oral. *Congreso Nacional de Biotecnología* (Biotec 2019). Vigo (España). Junio, 10-13, **2019**.
- Sarenkova, I., Sáez-Orviz, S., Ciprovica, I., Rendueles, M., Díaz, M. Lactobionic acid production from acid whey under different fermentative conditions. Comunicación oral. *6th International Conference on Food and Agricultural Engineering* (ICFAE 2020). Barcelona (España). Abril, 13-14, **2020**.
- Weng, S., Sáez-Orviz, S., Marcet, I., Rendueles, M., Díaz, M. Novel bovine plasma protein film reinforced with nanofibrillated cellulose fiber as edible food packaging material. *3º Congreso Iberoamericano de Biotecnología.* Braga (Portugal). Abril, 7-9, **2022**.

Pósteres

- Sáez-Orviz, S., Rendueles, M., Díaz, M. Microencapsulación de lactobionato de calcio para la elaboración de un nuevo alimento funcional. Póster. *VII Jornadas Doctorales y I Congreso de Investigación*. Mieres (España). Julio, 4-5, **2018**.
- Díaz, M., Sáez-Orviz, S., Rendueles, M. Encapsulación y control de la liberación de ácido lactobiónico en micropartículas de caseína. Póster. *XXXV Jornadas Nacionales de Ingeniería Química*. Salamanca (España). Julio, 4-6, **2018**.
- Sáez-Orviz, S., Marcet, I., Rendueles, M., Díaz, M. Preparation and characterization of bioactive edible films based on delipidated egg yolk protein with lactobionic acid as prebiotic and *Lactobacillus plantarum* CECT 9567 as probiotic. Póster. *3º*

Congreso Iberoamericano de Biotecnología. Braga (Portugal). Abril, 7-9, **2022**.

Premios

- 1st Best Poster Award: Bioactive edible films based on delipidated egg yolk protein with lactobionic acid as prebiotic and *Lactobacillus plantarum* CECT 9567 as probiotic. *3º Congreso Iberoamericano de Biotecnología.* Braga (Portugal). Abril, 7-9, **2022**.

A6.3-Artículos científicos no derivados de la tesis doctoral

- Sáez-Orviz, S., Laca, A., Rendueles, M., Díaz, M. Approaches for casein film uses in food stuff packaging. *Afinidad-Barcelona*, **2017**, 74(577), 26-29.
- Marcet, I., Weng, S., Sáez-Orviz, S., Rendueles, M., Díaz, M. Production and characterisation of biodegradable PLA nanoparticles loaded with thymol to improve its antimicrobial effect. *Journal of Food Engineering*, **2018**, 239, 26-32.
- Sáez-Orviz, S., Marcet, I., Weng, S., Rendueles, M., Díaz, M. PLA nanoparticles loaded with thymol to improve its incorporation into gelatine films. *Journal of Food Engineering*, **2020**, 269, 109751.
- Weng, S., López, A., Sáez-Orviz, S., Marcet, I., García, P., Rendueles, M., Díaz, M. Effectiveness of bacteriophages incorporated in gelatine films against *Staphylococcus aureus*. *Food Control*, **2021**, 121, 107666.
- Sarenkova, I., Sáez-Orviz, S., Ciprovica, I., Rendueles, M., Díaz, M. Lactobionic acid production from acid whey under different fermentative conditions. *Journal of Advanced Agricultural Technologies*, **2021**, 8(2), 35-40.
- Weng, S.; Sáez-Orviz, S.; Marcet, I.; Rendueles, M.; Díaz, M. Novel bovine plasma protein film reinforced with nanofibrillated cellulose fiber as edible food packaging material. *Membranes,* **2022**, 12, 31.
- Sarenkova, I., Sáez-Orviz, S., Ciprovica, I., Rendueles, M., Díaz, M. Lactobionic acid production by *Pseudomonas taetrolens* in a fed-batch bioreactor using acid whey as substrate. *International Journal of Dairy Technology*, **2022**, 1354-727X.
- Sarenkova, I., Sáez-Orviz, S., Rendueles, M., Ciprovica, I., Zagorska, J., Díaz, M. Downstream approach routes for the purification and recovery of lactobionic acid. *Foods*, **2022**, 11, 583.