Facultad de Farmacia
Departamento de Farmacia y Tecnología Farmacéutica
Programa de doctorado en Ciencias Farmacéuticas

Formas Farmacéuticas y microencapsulación para la administración oral de probióticos

Dosage forms and microencapsulation for the oral administration of probiotics

Memoria de Tesis presentada por la Licenciada en Farmacia Doña María José Martín Villena para optar al grado de Doctor por la Universidad de Granada, con mención de doctor internacional.

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SUMMARY

This thesis has been developed within the Andalusian Research Group CTS-205 "Practical pharmaceutics" in collaboration with NIZO Food Research (Ede, The Netherland). One of the research lines of the group CTS-205 is the development of different dosage forms and particles for the administration of probiotics. Specifically, this thesis has been focused in the preparation and characterization of microparticles for the protection of *L. fermentum* CECT 5716 and *Lactobacillus plantarum* WCSF-1. Furthermore, preparation of tablets for the administration of *L. fermentum* CECT5716 was also performed.

With these aims, different encapsulation technologies used with probiotics were firstly reviewed. Then, internal gelation technology was selected for the microencapsulation of *L. fermentum* CECT 5716. Results show that these particles were able to partially protect bacteria during one month at 4 ºC.

In the case of *L. plantarum* WCSF-1 spray dried technology using different carries was tested. The protection provide by the carriers was insufficient. However the food matrix (infant formula) selected seems to be adequate as a result of its low moisture content.

Concerning oral solid dosage forms, different gastro-resistant tablets were developed. The selected material was compatible with probiotic. These formulations maintain the viability after technological operations. The percentage of viability was reduced by 90% in all type of tablets after production. Tablets also meet the quality criteria required by the United States Pharmacopeia and Spanish Pharmacopeia referring to friability and disintegration test. Protection of probiotics through passage to the stomach and delivery of probiotic in the intestine were verified. The best viability after simulated stomach pH was for Eudragit® L100- sodium alginate tablets. This formulation was selected to the stability assays during 6 months. These tablets were found to be stable over a 6 months period when stored at 4ºC. However, at room temperature tablets started to lose viability after 90 days.

As a whole, the results of the present work provide evidence that probiotics can be protected by microencapsulation to be included in food and pharmaceutical matrices, but not all microencapsulation techniques are suitable. Internal gelation with alginate and starch has shown to be suitable, at least for *L.fermentum* CECT5716. Controlled-release tablets are a good dosage form for probiotics. Further studies are needed to prove the effects of these formulations *in vivo*.

INTRODUCCIÓN

En el intestino humano habitan alrededor de 100 billones de microorganismos, un número 10 veces mayor que el de todas nuestras células somáticas y germinales juntas. Hasta hace poco tiempo, las propiedades de esta microbiota eran prácticamente desconocidas, debido a la dificultad para estudiar su complejidad con la metodología disponible. Actualmente se sabe que ejerce una serie de efectos beneficiosos entre los que destacan la producción de vitaminas y anticuerpos, la prevención de la colonización por parte de patógenos, la estimulación del desarrollo de ciertos tejidos, la modulación de la respuesta inmunitaria, etc. Sin embargo, diferentes factores pueden afectarla, produciendo lo que se conoce como disbiosis y esto, a su vez, puede provocar la aparición de ciertas enfermedades. A ello cabe sumar el alarmante aumento de la resistencia a los antibióticos por diversas cepas, lo que revela la necesidad de adoptar un pensamiento nuevo y enfoques adecuados para desarrollar terapias alternativas efectivas.

En este sentido, el uso de probióticos ha manifestado tener utilidad en el tratamiento y prevención de diversas enfermedades**,** como han demostrado diversos estudios científicos. Fruto de esto se ha producido un aumento en el mercado de la elaboración y consumo de estos productos. No obstante, la fisiología intrínseca y la fragilidad tecnológica evidenciada por las cepas probióticas pueden hacerlas ineficaces para su uso clínico. Esto es debido, fundamentalmente, a su escasa resistencia a factores ambientales y tecnológicos, lo que limita su viabilidad, efectos beneficiosos y redunda negativamente en la percepción sobre la utilidad de estos productos por parte de los consumidores.

Con la intención de solventar estos inconvenientes, tradicionalmente se han desarrollado diversas estrategias como la sobredosificación o el almacenamiento en envases especiales, lo que encarece el precio del producto, limitando su acceso a un sector de la población. De ahí que, en los últimos años, el objetivo de la industria alimentaria y farmacéutica se haya centrado, en el desarrollo de alternativas que mejoren la estabilidad de los probióticos de forma eficiente y económicamente rentables. Como respuesta a esta necesidad, el trabajo desarrollado en la Tesis Doctoral se ha centrado, por un lado, en el estudio de la microencapsulación de probióticos y, por otro, en el desarrollo de formas farmacéuticas sólidas que contribuyan a mejorar su viabilidad durante el almacenamiento y su efectividad terapéutica tras la administración.

Para ello hemos trabajado con bacterias cuyas propiedades probióticas han sido ampliamente demostradas, *Lactobacillus fermentum* CECT 5716 y *Lactobacillus plantarum* WCFS 1, ambas son bacterias ácido lácticas que han manifestado presentar efectos beneficiosos frente a numerosas patologías como diarrea, alergias, enfermedad inflamatoria intestinal, gripe, etc.

OBJETIVOS

Como ya se ha indicado anteriormente, para que los probióticos puedan ejercer sus efectos beneficiosos deben llegar al lugar de acción en cantidad adecuada. Por lo que deben ser capaces de sobrevivir al paso por el tracto gastrointestinal superior, así como a las condiciones adversas que se dan durante los procesos de fabricación y almacenamiento de los productos que los contienen. El principal problema asociado a los probióticos es su escasa resistencia a factores como el pH, la post-acidificación en productos fermentados durante el almacenamiento, la producción de peróxido de hidrógeno, la toxicidad del oxígeno (permeación del oxígeno a través de los envases y durante el proceso de fabricación de los productos que los contienen), la humedad, o la temperatura, además de los diferentes obstáculos que se encuentran a su paso por el estómago como el pH, las enzimas y las sales biliares.

La dosis de probiótico recomendada depende de la cepa o del producto, por lo que no es posible establecer una dosis general. Esta dosis, vendrá definida por las pruebas clínicas realizadas en humanos. Aunque normalmente las dosis empleadas en estudios de este tipo están alrededor de 10^9 unidades formadoras de colonias (ufc). Pero dada la sensibilidad de las bacterias probióticas a estos factores, diversos estudios han encontrado productos probióticos comercializados con un número de bacterias inferior al deseado, por lo que se hace necesario desarrollar técnicas que permitan proteger a los probióticos de estas condiciones adversas.

Por todo esto, el presente Trabajo de Investigación tiene como **objetivo principal** el diseño de técnicas que permitan proteger a los probióticos de agentes externos de manera que se garantice una viabilidad adecuada de los mismos al final del proceso tecnológico y la llegada en número suficiente al intestino.

En concreto, se pretenden alcanzar los siguientes **objetivos específicos:**

1. Revisar las diferentes técnicas de microencapsulación de probióticos que están descritas en la bibliografía, con objeto de utilizar las más adecuadas para la microencapsulación de las bacterias objeto de esta tesis doctoral.

2. En cuanto a la microencapsulación de *Lactobacillus plantarum* WCFS-1 y *Lactobacillus fermentum* CECT 5716:
   - Proponer polímeros que cumplan con los requisitos de compatibilidad de las bacterias, requerimientos de la tecnología utilizada, estabilidad en las

condiciones aplicadas, grado de protección de los microorganismos, condiciones de liberación de los mismos, etc.

- Seleccionar y desarrollar un método de microencapsulación adecuado a las cepas probióticas *Lactobacillus plantarum* WCFS-1 y *Lactobacillus fermentum* CECT 5716.

  La selección del método de microencapsulación se hará en función del tamaño medio de partícula requerida, las propiedades físicas del agente encapsulante, las aplicaciones que se persigan y del mecanismo de liberación deseado.

- Caracterizar las micropartículas obtenidas con la intención de determinar su forma, tamaño, superficie y estabilidad desde el punto de vista tecnológico.

- Comprobar la viabilidad de las cepas microencapsuladas a lo largo del tiempo y bajo diferentes condiciones de conservación con la intención de determinar las condiciones de almacenamiento más apropiadas.

- Estudiar la viabilidad de las micropartículas con *L. plantarum* WCFS-1 incluidas en matrices alimentarias.

3. Diseñar y elaborar formas farmacéuticas sólidas de administración oral de *L. fermentum* CECT 5716, para lo cual se seleccionarán materiales compatibles con las bacterias utilizadas y técnicas adecuadas, capaces de garantizar su viabilidad tras las operaciones tecnológicas necesarias. En concreto, se trabajará en la elaboración de comprimidos de liberación modificada.

   - Caracterizar las formas farmacéuticas diseñadas desde el punto de vista tecnológico con la intención de asegurar que cumplen con los criterios de calidad exigidos por la United States Pharmacopeia y la Real Farmacopea Española.

   - Estudiar la liberación del probiótico desde los sistemas propuestos, de modo que se consiga la protección de los probióticos en su paso por el estómago y llegue la mayor dosis posible a nivel intestinal.

- Evaluar la viabilidad de las bacterias a lo largo del tiempo de acuerdo con diferentes condiciones de conservación.

CONTRIBUCIÓN DEL TRABAJO

Como previamente se ha señalado, *Lactobacillus fermentum* CECT 5716 es una cepa probiótica de utilidad en el tratamiento de diversas patologías, que actualmente sólo se encuentra comercializada como componente de una fórmula infantil (Leche infantil Puleva Bebé, Lactalis, Barcelona, España) y en forma de cápsulas para el tratamiento de la mastitis (Lactanza Hereditum®, laboratorios Angelini Farmacéutica, S.A., Barcelona, España). Por su parte *Lactobacillus plantarum* WCSF-1 es una cepa con un alto potencial probiótico de la que no se tiene constancia de su comercialización como producto nutracéutico o farmacéutico.

Sin embargo, dada la importancia de las especialidades farmacéuticas probióticas en el tratamiento de diversas patologías y la necesidad de que dichas especialidades sean estables, efectivas y viables desde el punto de vista económico, pensamos que el trabajo desarrollado en la presente tesis doctoral aporta nuevas técnicas para la elaboración de formas farmacéuticas y alimentos probióticos más estables y con un mayor número de bacterias viables.

En definitiva, creemos que nuestra investigación puede contribuir en los siguientes aspectos:

- Dar un paso más en la tecnología y diseño de nuevas formas farmacéuticas de probióticos, lo cual podría tener un gran impacto en la Industria Farmacéutica, favoreciendo la incorporación de una amplia gama de productos y con ello un mayor acceso a estos por parte del consumidor.
- Desarrollar nuevas tecnologías para la protección de bacterias probióticas para permitir en el futuro la incorporación de estas a nuevas matrices alimentarias.
- Demostrar que es posible la liberación de *Lactobacillus fermentum* CECT 5716 desde las diferentes formas farmacéuticas y los beneficios aportados sobre la salud del consumidor.
- Al favorecer la incorporación de estas bacterias a nuevas formas farmacéuticas y a nuevas matrices alimentarias, se fomentará también la aceptación del paciente a la utilización de estos productos, con lo que se conseguiría favorecer el cumplimiento terapéutico o preventivo.

PLAN DE TRABAJO

A tenor de los objetivos fundamentales de la presente Tesis Doctoral, el plan de trabajo propuesto se ha organizado en diferentes capítulos según se recoge a continuación:

CAPÍTULO I: MICROENCAPSULACIÓN DE BACTERIAS PROBIÓTICAS

I.1. Introducción sobre aspectos generales de la microbiota humana y los efectos de su manipulación mediante el uso de probióticos.

I.2. Revisión bibliográfica de las técnicas y de los materiales empleados en la microencapsulación de probióticos, así como de las ventajas e inconvenientes de los mismos, analizando también su posible aplicación y los efectos de la misma.

Microencapsulation of bacteria: a review of different technologies and their impact on the probiotic effects. M.J. Martín, F. Lara-Villoslada, M. A. Ruiz and M. E.Morales. (2015). *Innovative Food Science and Emerging Technologies***,** *27*:15-25.

I.3. Selección y puesta a punto de una técnica de microencapsulación adecuada para el probiótico *Lactobacillus fermentum* CECT 5716. En dicho trabajo, se ha procedido también a la verificación del tamaño y morfología de las partículas y a la valoración de la viabilidad del mismo a lo largo de un mes y tras la liofilización de las micropartículas.

Effect of unmodified starch on viability of alginate-encapsulated *Lactobacillus fermentum* CECT5716. M.J. Martin, F. Lara-Villoslada, M.A. Ruiz, M.E. Morales. (2013). *LWT - Food Science and Technology, 53*: 480-486.

I.4. Elección y puesta a punto de una técnica para la microencapsulación de *Lactobacillus plantarum* WFCS-1 (fruto de una estancia predoctoral en el centro NIZO Food Research Ede, Holanda). Se evaluó la viabilidad de las micropartículas a lo largo del tiempo. Así mismo, se incluyeron en una matriz alimentaria (leche infantil).

Survival of *Lactobacillus plantarum* WCFS1 to encapsulation by spray-drying with different carriers and the effect of infant formula as food matrice. M.J.

Martin, F. Lara-Villoslada, M.A. Ruiz, M.E. Morales, E. de Hoog. (Under review by Journal of Functional Food).

CAPÍTULO II: DISEÑO Y ELABORACIÓN DE COMPRIMIDOS DE LIBERACIÓN MODIFICADA CON PROBIÓTICOS.

II.1. Estudio teórico de formas farmacéuticas sólidas orales: comprimidos.

II.2. Desarrollo y caracterización tecnológica de comprimidos de liberación modificada con *L.fermentum* y evaluación de la viabilidad a lo largo del tiempo.

Development of gastro-resistant tablets for the protection and intestinal delivery of *Lactobacillus fermentum* CECT 5716. M.J. Martin, F. Lara-Villoslada, M.A. Ruiz, M.E. Morales. (Under review by International Journal of Pharmaceutics).

CAPÍTULO III: CONCLUSIONES

CAPÍTULO IV: BIBLIOGRAFÍA ADICIONAL

CAPÍTULO I.

Microencapsulación de bacterias probióticas

I.1. Aspectos generales de la microbiota humana y efectos de su manipulación mediante el uso de probióticos

1. Microbiota humana

La microbiota de cada individuo es como una huella dactilar. Existen sin embargo, por lo menos 57 especies de bacterias que pueden considerarse comunes a todos los humanos. Esta comunidad bacteriana está dominada por dos filos bacteriodes y firmicutes, que representan a más del 90% de los grupos filogenéticos presentes en el intestino (Aureli y col., 2011).

La adquisición de esta microbiota tiene lugar durante el parto, por la exposición vaginal y está relacionada, también, con la trasmisión durante el cuidado neonatal, debido principalmente a la alimentación con leche materna y después de esto, por la introducción de alimentos sólidos. De hecho, el descubrimiento de la presencia de bacterias ácido lácticas en la leche materna (Martín y col., 2003) ha puesto de manifiesto la importancia de ésta en la colonización inicial del intestino del neonato. Esta microbiota inicial es relativamente inestable y cambia durante el periodo inicial de la vida, llegando a ser similar a la de los adultos al año o dos de vida. Al principio, el tracto gastrointestinal de los recién nacidos es colonizado por anaerobios facultativos como las enterobacterias y cocos positivos, estos consumen el oxígeno del ambiente y crean un ambiente favorable para el desarrollo de bacterias anaerobias obligadas, como *Bacterioides, Clostridia, Eubacterias y Bifidobacterium* (Lannitti y Palmieri, 2010).

El tracto gastrointestinal de un humano adulto, por su parte, se caracteriza por ser un complejo ecosistema en continuo contacto con el medio externo. Este ecosistema incluye numerosos microorganismos que son capaces de promover efectos beneficiosos para la salud, pero también contiene otros considerados como potenciales patógenos, por su capacidad de invadir al hospedador. En concreto, en el tracto gastrointestinal, el número de los microorganismos aumenta longitudinalmente conforme se avanza desde el estómago hasta el colon. Así, el tracto gastrointestinal superior (estómago) contiene relativamente pocas bacterias (10^2ufc/mL), este número se incrementa progresivamente conforme se avanza distalmente, siendo máximo en el colon, en donde residen un total de 10^{12} ufc/mL bacterias vivas (Figura 1). Esto es consecuencia de la alta exposición a nutrientes, el lento tránsito, una temperatura estable y el bajo potencial redox del medio.

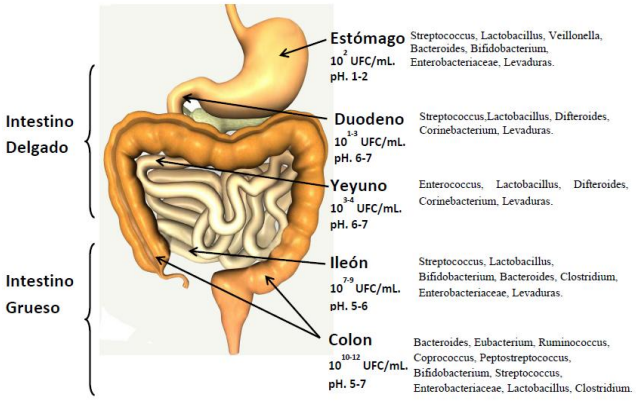

Figura 1. (Lannitti, T, Palmieri, 2010)

La microbiota normal que coloniza el tracto gastrointestinal ejerce diversas funciones: síntesis de vitaminas (K y ciertas del grupo B), previene la colonización por patógenos, secreta sustancias que inhiben o destruyen a especies no indígenas, estimula el desarrollo de ciertos tejidos (ciego y tejido linfáticos), así como la producción de anticuerpos, genera una gran variedad de sustancias (ácidos grasos, peróxidos, bacteriocinas, etc.) que inhiben o matan otras bacterias. Por último, también influyen en el balance de energía, de hecho hay evidencia demostrada de la importancia de la microbiota intestinal en la fisiopatología de la obesidad (Lannitti y Palmieri, 2010).

Diferentes factores pueden afectar a la microbiota (Figura 2), produciendo una alteración del balance microbiano, que se conoce con el nombre de disbiosis y esto puede provocar la aparición de enfermedades. Por ello, la manipulación de esta microbiota, a través de probióticos, tiene un gran interés en la prevención y tratamiento de diferentes patologías:

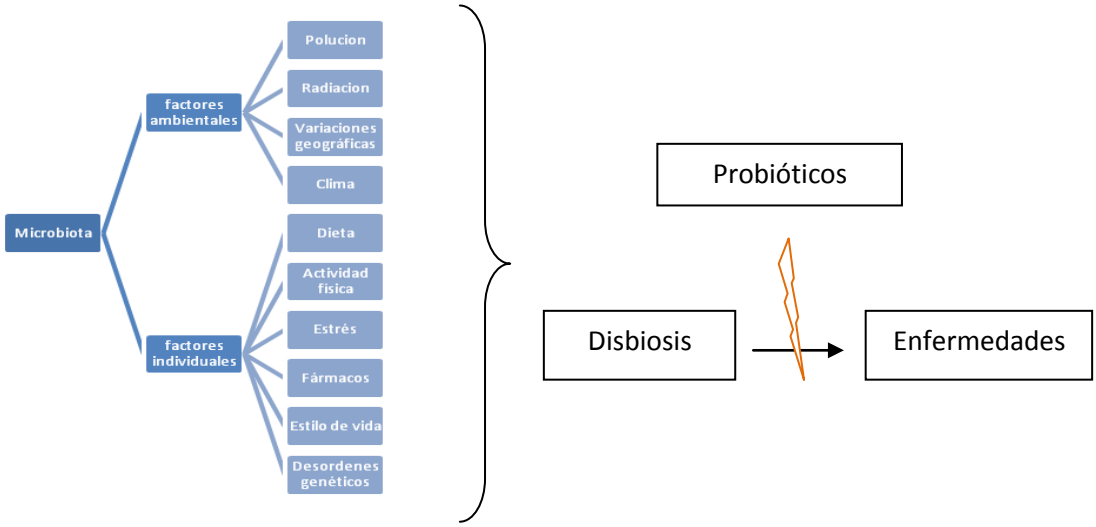

Figura 2. Factores que afectan a la microbiota (Pande, Bagad, Dubey, & Ghosh, 2012).

2. Probióticos

El término probiótico, procede etimológicamente del griego pro bios (por la vida). La OMS los define como "organismos vivos que administrados en cantidades adecuadas ejercen un efecto beneficioso sobre la salud del hospedador". En este sentido, varios científicos han demostrado que algunos microrganismos inactivados, e incluso sus componentes celulares, pueden ejercer un efecto beneficioso en la salud (Ouwehand y Salminene, 1998; Isolauri y col., 2002) por lo que todos estos hallazgos deberán considerarse en futuras revisiones del concepto de probiótico.

Entre los microorganismos utilizados como probióticos, las bacterias acido-lácticas y las bifidobacterias ocupan el lugar más destacado, pero también se utilizan con este fin bacterias que pertenecen a otros géneros, como *Escherichia coli* y *Bacillus cereus*, así como levaduras, principalmente *Saccharomyces cerevisiae* (Tabla 1).

PROBIOTICOS	**ESPECIES**
Lactobacillus sp	*L. acidophilus, L. casei, L. delbrueckii ssp, L. cellobiosus, L.curvatus, L. fermentum, L. lactis, L. plantarum, L.reuteri, L. brevis*
Bifidobacterium sp	*B. bifidum, B. adolescentis, B.animalis, B. infantis. B.thermophilum, B. longum*
Enterococcus sp	*Ent. Faecalis, ent. Faecium*
Streptococcus sp	*S. cremoris, S. salivarius, S. diacetylactis, S. intermedius*
Bacterias no ácido lácticas	*B. cereus, B. subtilis*
Saccaromyces	S. bourlardii, S. cerevisiae

Tabla 1. Probioticos usados en preparaciones comerciales (Mombelli y Gismondo, 2000;Tripathi y Giri, 2014)

2.1. Género Lactobacillus

Dentro de las bacterias ácido lácticas el grupo más numeroso es el género Lactobacillus. Los Lactobacilos son bacilos o coco-bacilos Gram-positivos que frecuentemente forman cadenas. Son catalasa negativos, no esporulados y en general inmóviles (unas pocas especies presentan flagelos peritricos). Al ser un género tan amplio, que abarca a tantas

especies distintas, sus propiedades son muy heterogéneas. Esta diversidad se ve reflejada en el contenido en Guanina + Citosina de sus miembros, que varía desde un 32% hasta un 52%. En cuanto a su temperatura óptima de crecimiento, pueden ser mesófilos o termófilos. El pH adecuado para su desarrollo oscila entre 5,5 y 6,2. Son anaerobios aerotolerantes y su crecimiento se ve favorecido en atmósfera microaerófila con un 5-10% de CO_2. Desde el punto de vista fenotípico y atendiendo a sus características metabólicas, se pueden clasificar en tres grupos en función de la presencia o ausencia de los enzimas fructosa-1,6- difosfato aldolasa y fosfocetolasa (Kandler y Weiss, 1986):

- **Grupo I.** Homofermentadores estrictos: comprende el grupo de *Lactobacillus acidophilus* formado por seis especies*: L. acidophilus, L. gasseri, L. johnsonii, L. crispatus, L. amilovorus y L. gallinarum*. También se incluyen en esta categoría las especies *L. delbrueckii, L. helveticus, L. leichmanii, L. salivarius y L. jensenii.*

- **Grupo II.** Heterofermentadores facultativos: las especies principales de este grupo son: *L. casei, L. plantarum, L. sakei y L. curvatus.*

- **Grupo III.** Heterofermentadores estrictos: se incluyen en este *grupo L. reuteri, L. fermentum, L. cellobiosus, L. brevis, L. buchneri y L. viridescens.*

Los lactobacilos se encuentran presentes en una amplia variedad de nichos ecológicos, siempre que en ellos exista abundante fuente de carbohidratos hidrosolubles, productos de degradación de proteínas, vitaminas y una tensión de oxígeno reducida. De hecho, podemos encontrarlos en hábitats tan variados como la leche y sus derivados, vegetales, carnes, bebidas fermentadas y formando parte de la microbiota gastrointestinal y genitourinaria del hombre y los animales.

Por lo general son las bacterias acido lácticas las que mejor toleran la acidez y muchas de sus especies se utilizan habitualmente en la industria alimentaria. Se emplean en la elaboración de productos fermentados, ya que contribuyen a la conservación, sabor y textura de los mismos. La conversión de azúcares en ácido láctico, la producción de péptidos antimicrobianos, exopolisacáridos y una gran variedad de metabolitos son otras de sus importantes propiedades. A pesar de que, como ya se ha indicado anteriormente, algunas de las especies del género *lactobacillus* forman parte de la microbiota humana, solo unas cuantas de estas especies se utilizan en la industria alimentaria para producir

productos fermentados, algunas de estas especies son *L. bulgaricus*, *L. crispatus, L. gasseri, y L. plantarum* (de Vries y col., 2006).

2.2. Género Bifidobacterium

Las bifidobacterias son microorganismos Gram-positivos, anaerobios estrictos (aunque el grado de sensibilidad al oxígeno depende de la especie), inmóviles, no esporulados y catalasa negativos. Presentan una gran variedad de formas: cocoide, con protuberancias y/o bifurcaciones, con extremos espatulados o cadenas estrelladas. Su nombre hace referencia a las formas bífidas con dos ramas en Y o V que presentan en ocasiones. Aunque poseen algunas características comunes con las bacterias ácido lácticas, y en muchas ocasiones se incluyen dentro de éstas por razones prácticas, el género Bifidobacterium pertenece en realidad a la subdivisión Actinomycetes con un alto contenido cromosómico en G+C (>50%).

El metabolismo de los azúcares difiere también del de las verdaderas bacterias lácticas, ya que las bifidobacterias carecen de aldolasa y glucosa-6-fosfato deshidrogenasa. Las hexosas son degradadas exclusivamente por la ruta de la fructosa-6-fosfato, caracterizada por la presencia de la enzima fructosa-6-fosfato fosfocetolasa (F6PPK) típica de este género. Esta vía metabólica de fermentación de carbohidratos conduce a la formación de ácido láctico y ácido acético en una proporción 2:3, sin producción de CO_2. Además, las bifidobacterias no pueden utilizar los ácidos grasos u otros ácidos orgánicos como fuentes de carbono y son negativas en la reducción de nitrato, la formación de indol, la licuefacción de gelatina y la fermentación de glicerol. La cisteína es un aminoácido esencial para estos microorganismos y actúa adicionalmente en los medios de cultivo como agente reductor. El pH adecuado para el desarrollo de las bifidobacterias se sitúa entre 6 y 7 y la temperatura óptima de crecimiento alrededor de los 37°C. Actualmente, el género está formado por unas 32 especies, aisladas del tracto gastrointestinal humano y de diversos animales, así como de aguas residuales. Estas especies forman una unidad filogenética coherente con un grado de identidad superior al 93% en las secuencias del gen del ARNr 16S. La especie tipo del género es *Bifidobacterium bifidum*.

2.3. Características de los probióticos

De acuerdo con lo comentado anteriormente, los probióticos son microorganismos que promueven la salud de quienes los consumen y, para que puedan considerarse como tales, es necesario que cumplan una serie de características o requisitos (Figura 3), entre los que se incluyen (Butel, 2014):

1. Ser una cepa segura, es decir, que pueda ser utilizada en humanos sin riesgos para la salud. La Food and Drug Administration (FDA) americana define a este tipo de microorganismos como (*Generally Recognized As Safe*). La Autoridad Europea de Seguridad Alimentaria (EFSA) los define como QPS (*Qualified Presumption of Safety*). Por ejemplo, el ser de origen humano es una propiedad deseable, ya que, en teoría, las cepas aisladas de seres humanos sanos presentaran una mayor facilidad para colonizar el intestino humano y probablemente no sean patógenas
2. Deben poseer tolerancia a las condiciones del tracto gastroientestinal, ya que los microorganismos probióticos han de llegar viables al intestino en un número significativo, y para ello es preciso que resistan el pH gástrico, las enzimas digestivas y la acción detergente e inhibidora de las sales biliares.
3. Han de ser capaces de colonizar el intestino, con un tiempo corto de replicación, así como, de adherirse a la mucosa intestinal para que tenga lugar la modulación de la respuesta inmune, tanto como la exclusión de microorganismos patógenos (si bien esto último, puede deberse también, a su capacidad de producir compuestos antimicrobianos). Este criterio está actualmente en discusión, porque se ha comprobado que probióticos con baja capacidad de colonización tienen también efectos positivos para la salud.
4. Cuando se incorporan en alimentos, deben mantener sus características y permanecer estables durante la facturación y durante la conservación, además de no conferir desviaciones de sabor.
5. Los efectos beneficiosos que producen a nivel de la salud deben estar clínicamente testados y validados.

Figura 3. Características de las probióticos (Saarela y col., 2000).

2.4. Efectos de los probióticos

Como se ha indicado en el epígrafe anterior la capacidad de los probióticos para colonizar la mucosa intestinal, posibilita que se modifique la composición de la misma, pudiendo ejercer los siguientes efectos beneficiosos:

1. Competición con bacterias nocivas por:
   - Desplazamiento de su sitio de unión al epitelio.
   - Inhibición de su crecimiento y/o muerte mediante la producción de compuestos antibacterianos o reducción del pH.
2. Mejora de la función de la barrera intestinal.
3. Producción de nutrientes importantes para la función intestinal.
4. Inmunomodulación.

Por todo ello, los probióticos se han utilizado para la prevención y tratamiento de diversas patologías como las que aparecen recogidas en la siguiente tabla (tabla 2):

Enfermedad	Cepa	Referencia
Eczema	*Escherichia coli, Bifidobacterium bifidum* *Bifidobacterium lactis, Lacotococcus lactis*	Niers y col.. (2009), Soh y col., (2009), Viljanen y col., (2005a and 2005b)
Alergias alimentarias	*Escherichia coli, Bacilus circulans PB7, Lactobacillus plantarum DSMZ 12028*	Lodinova- Zadnikova y col., (2003) Bandyopadhyay y Das Mohapatra (2009), Cammarota y col., (2009)
Regeneración de la microbiota beneficiosa tras el tratamiento con antibióticos	*Enterococcus mundtii ST4 SA,Lactobacillus plantarum 423, Lactobaciulls brevis KB290*	Botes y col., (2008), Fukao y col., (2009) y Zhou y col., (2005)
Gastroenteritis	*Lactobacillus casei*	Yamada y col., (2009)
Hiperpermeabilidad intestinal	*Lactobacillus plantarum 299* *Lactobacillus plantarum LP299*	Kennedy y col., (2000), Strowski y Wiedenmann (2009) y White y col., (2006)
Candidiasis vaginal	*Lactobacillus rhamnosus GR-1* *Lactobacillus reuteri RC-14*	Martínez y col., (2009)
Infecciones del tracto urinario	*Lactobacillus rhamnosus GR-1* *Lactobacillus reuteri RC-13*	Anukam y col., (2009)
Intolerancia a la lactosa	*Lactobacillus acidophilus*	Hawrelak (2003)
Disbiosis intestinal	*Lactobacillus johnsonii La1* *Lactobacillus GG*	Hawrelak (2003), Silva y col. (1987) y Bennett y col. (1996)
Enfermedad inflamatoria intestinal	*Bifidobacterium infantis 35624* *Escherichia coli DSM 17252* *Bifidobacterium infantis 35624*	Brenner y Chey (2009), Enck y col., (2009), Whorwell y col., (2006)
Diarrea del viajero	*Lactobacillus GG* *Lactobacillus plantarum*	Hawrelak (2003); Michail y Abernathy (2002)
Diarrea inducida por radiación	*Lactobacillus casei DN-144 001*	Giralt y col., (2008)
Enfermedad de Crohn	*Escherichia coli strain Nissle 1917*	Boudeau y col., (2003)
Prevención del cáncer de colon	*Enterococcus faecium M-74*	Mego y col., (2005); Thirabunyanon y col., (2009)
Colitis ulcerosa	*Lactobacillus acidophilus* *Escherichia coli Nissle 1917* *Bifidobacterium*	Abdin y Saeid (2008); Adam y col., (2006); Imaoka y col., (2008)
Ulcera péptica	*Lactobacillus acidophilus*	Iaroven y col., (2007)
Prevención atopia	*Lactobacillus rhamnosus GG*	Huurre y col., (2008); va der Aa y col., (2008)
Hipercolesteriolemia y enfermedad cardiovascular	*Enterococcus faecium M-74* *Lactobacillus plantarum* *Orpionibacterium Freudenreichii* *Lactobacillus plantarum PH04*	Hlivak y col., (2005), Kiatpapan y col., (2001); Nguyen y col.,. (2007)

Tabla 2. Probióticos utilizados en diversas patologías (Amara y Shibl, 2013).

3. *Lactobacillus fermentum* CECT 5716

3.1. Características

Lactobacillus fermentum CECT 5716 es una cepa aislada de la leche de madres lactantes. El mecanismo por el cual estas bacterias pasan a la leche materna no es del todo conocido, pero parece que las células dendríticas podrían estar implicadas. Estas células actúan como centinelas y, a través de sus dendritas, son capaces de hacer un selección de las bacterias del lumen intestinal. En función de las moléculas que estas bacterias tengan en su membrana, las células dendríticas son capaces de discriminar entre bacterias potencialmente patógenas y bacterias comensales, destruyendo a las primeras e internalizando a las segundas. De esta forma, las bacterias podrían llegar a diferentes localizaciones, como por ejemplo a la glándula mamaria, a través de la vía enteromamaria (Rescigno y col., 2001).

Como ya se ha indicado anteriormente para que un microorganismo pueda considerarse probiótico debe cumplir una serie de requisitos. En este sentido, *Lactobacillus fermentum* se caracteriza por cumplir todos y cada uno de los requisitos (Martín y col., 2005) expuestos en el apartado 2.3.:

- Es una cepa de origen humano
- Posee resistencia a los ácidos gástricos y biliares. En un estudio en el que se administraban conjuntamente con leche, demostraron mantener una viabilidad del 70% cuando son expuestas a las condiciones que se encuentran a nivel del tracto gastrointestinal (Martín, y col., 2005). Por lo que una elevada fracción de bacterias alcanzará el compartimento intestinal vivas. En el mismo estudio, se indicó que la presencia de la leche puede tener un efecto protector para el probiótico.
- Capacidad para adherirse a la mucosa intestinal. Este es un prerrequisito para la colonización y para llegar a ejercer sus efectos beneficiosos. Los resultados *in vitro* utilizando líneas celulares HT -29 y Caco-2 muestran unas excelentes propiedades de adhesiónsimilares e incluso mejores a las de algunas cepas como son *L. rhamnosus* GG, *L. Johnsosnii* La1 *y L. Casei inmunitas*.
- Estas bacterias no son capaces de producir compuestos antibacterianos (bacteriocinas o reuterina), pero sí generan H_2O_2 que inhibe el crecimiento de

Staphylococcus aureus, un microorganismo que generalmente se asocia a la producción de mastitis.

- *Lactobacillus fermentum* es una especie incluída en la lista QPS de la EFSA (EFSA, 2004). Por tanto, su uso en humanos es seguro y no produce reacciones adversas. En todo caso, diferentes estudios de tolerancia y ausencia de efectos adversos se han llevado a cabo con la cepa *Lactobacillus fermentum* CECT5716. A este respecto, las aminas biogénicas son ácidos orgánicos que se producen en diferentes tipos de alimentos debido a la actividad descarboxilasa de algunos microorganismos. La ingesta de estos productos ha sido asociada con varios efectos tóxicos. Así, la histamina y la tiramina tiene efectos vasoactivos y psicoactivos mientras que la diamina, putrecina y la cadaverina pueden potenciar la toxicidad de las citadas aminas y son conocidas por ser potenciales precursores de nitrosaminas carcinógenas. *Lactobaciulls fermentum CECT 5716* no produjo ninguna de las aminas enumeradas anteriormente (Martín y col., 2005). Por otro lado, esta bacteria no degrada la mucina gástrica en ensayos *in vitro* (Martín y col., 2005). Así mismo, *L.fermentum* CECT5716 ha demostrado ser segura en un modelo de toxicidad aguda en animales de experimentación (expresadas en kg de peso del cuerpo)(Lara-villoslada y col., 2009). Por otro lado, Gil-Campos y col. (2012) verificaron la seguridad y la tolerancia de una fórmula infantil suplementada con *L. fermentum* por parte de niños de 1 a 6 meses. La fórmula fue bien tolerada por los niños, además se observó una reducción en el incidencia de infecciones gastrointestinales.

Otro factor a tener en cuenta es la resistencia a antibióticos. Los probióticos deben ser sensibles a los antibióticos o, en el caso de que sean resistentes, no deben ser capaces de transferir esta resistencia a otras bacterias. En concreto, *L .fermentum CECT 5716* es sensible a antibióticos betalactámicos (penicilina), a aminoglicósidos como la gentamicina, macrólidos (eritromicina) y antibióticos de gran espectro como el cloranfenicol y la tetraciclina (tabla 3).

RESISTENTE	SENSIBLE
Ciprofloxacino	Ampicilina
Gentamicina	Amoxicilina
Kanamicina	Cefalotina
Acido nalidixico	Cloranfenicol
Trimetoprim +sulfametoxazol	Eritromicina
Cefotoxina	Penicilina
	Tetraciclina

Tabla 3. Sensibilidad de *L. fermentum* a diversos antibióticos.

3.2. Efectos beneficiosos

L. fermentum CECT 5716 ha demostrado inhibir la adhesión de *Salmonella cholerasuis* a la mucina e incrementar la supervivencia de los ratones infectados por este patógeno (Olivares y col., 2006). Así mismo, manifestó tener un efecto beneficioso en un modelo animal de inflamación intestinal, reduciendo la respuesta inflamatoria y el daño intestinal (Mañé y col., 2009). Otros efectos (tabla 4) consisten en modular la respuesta inmunitaria aumentando la actividad fagocítica, el número de células *natural killer* y la concentración de inmunoglobulina A. En concreto, el consumo de *L. fermentum* aumenta la respuesta en voluntarios de 26-40 años a la vacuna de la gripe, reduciendo la incidencia de esta enfermedad (Olivares y col., 2007). Por otro lado, la administración de una fórmula infantil de continuación a niños de entre seis y doce meses redujo la incidencia de infecciones tanto gastrointestinales (46%) como respiratorias (27%) (Maldonado y col., 2012). Por último, su suministro a madres lactantes mejoró y disminuyó la recurrencia y los síntomas de la mastitis, con un efecto similar e incluso superior al de los antibióticos (amoxicilina-clavulánico, amoxicilina, clotrimazol, cloxacilina y eritromicina) (Arroyo y col., 2010).

L. fermentum CECT 5716	**Efectos antialérgicos**	**Lara- Villoslada y col., 2007; Olivares y col., 2005**
	Colonización intestinal	Martín y col., 2005; Olivares y col., 2006.
	Producción de compuestosantimicrobianos	Martín y col., 2005
	Efectos antimicrobianos	Díaz-Ropero y col., 2006
	Aumenta los efectos de las vacunas	Olivares y col., 2007
	Efectos antinflamatorios	Diaz-Ropero y col., 2006; Peran y col., 2005; Peran y col., 2007
	Mastititis	(Arroyo y col., 2010) NCT00716183

Tabla 4. Efectos beneficiosos de *L. fermentum* en modelos animales y en ensayos clínicos(Lara-Villoslada y col., 2007).

4. *Lactobacillus plantarum* WCFS 1

4.1. Características

Lactobacillus plantarum es una bacteria acido láctica que se encuentra en una gran variedad de nichos, incluyendo productos fermentados, carnes y vegetales. También puede encontrarse, como ya se ha indicado anteriormente, en el tracto gastrointestinal humanos. Diversos estudios clínicos indican la seguridad del uso de *L. plantarum* en seres humanos (de Vries y col., 2006).

En concreto, *Lactobacillus plantarum* WCFS1, una cepa probiótica aislada de la saliva humana de la que se conoce su genoma completo, tiene la capacidad para codificar la adquisición y la utilización de un gran número de azúcares, péptidos y la formación de la mayoría de los aminoácidos. El gran número de proteínas ancladas a su superficie sugiere que *L. plantarum* WCSF1 tiene el potencial de asociarse con muchas superficies

diferentes. Además, el elevado número de genes que codifican funciones reguladoras indica su capacidad de adaptarse a muchas condiciones diferentes. *Lactobacillus plantarum* WCFS1, al igual que *L. fermentum* CECT 5716 se caracteriza por cumplir los requisitos expuestos en el apartado 2.3.:

- Es una cepa de origen humano.
- Clasificada como segura (Qualified presumption of safety (QPS)) por la EFSA (European Food Safety Authority). Como ocurría con *L. fermentum,* no degrada la mucina gástrica, aunque se piensa que puede codificar proteínas que se unan a la misma.
- Se trata de una cepa robusta, con una adecuada supervivencia en el tracto gastrointestinal y con buena actividad metabólica.
- Con susceptibilidad a antibióticos y capacidad para hidrolizar lactosa y formar biofilms (Guidone y col., 2014).

4.2. Efectos beneficiosos

Dentro de sus posibles aplicaciones destaca el tratamiento de personas con niveles elevados de colesterol, con permeabilidad intestinal incrementada o con enfermedades en las que la estimulación de células TH1 o células reguladoras T sea beneficiosa (datos aun no publicados).

Otras aplicaciones son:

- Diarrea.
- Síndrome metabólico.
- Colitis ulcerosa.
- Enfermedad inflamatoria intestinal.
- Gripe y resfriado.
- Desordenes intestinales y cerebrales.
- Autismo.

I.2. Microencapsulation of bacteria: A review of different technologies and affect their impact on the probiotics

Microencapsulación de bacterias: una revisión sobre diferentes métodos de encapsulación y su impacto en los efectos de los probióticos.

El uso de productos probióticos está asociado a numerosos beneficios sobre la salud. Sin embargo, el principal problema con el que nos encontramos es su baja supervivencia en productos alimentarios y la baja carga bacteriana que alcanza el tracto gastrointestinal. En nuestra opinión, la microencapsulación es uno de los métodos que de manera más eficiente nos permitiría conseguir una protección adicional que garantice la viabilidad de las bacterias probióticas y su liberación a nivel intestinal. Sin embargo, todavía hay muchos retos que superar en lo que a la microencapsulación de probióticos se refiere. Por ello, en este trabajo de revisión bibliográfica hemos realizado una exhaustiva recopilación de los diversos métodos que, hasta el momento, se han utilizado en la microencapsulación de bacterias probióticas, así como de los materiales utilizados

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Microencapsulation of bacteria: A review of different technologies and their impact on the probiotic effects

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ABSTRACT

Probiotic based products are associated with many health benefits. However, the main problem is the low survival of these microorganisms in food products and in gastrointestinal tract. Providing probiotics with a physical barrier is an efficient approach to protect microorganisms and to deliver them into the gut. In our opinion, microencapsulation is one of the most efficient methods, and has been under especial consideration and investigation. However, there are still many challenges to overcome with respect to the microencapsulation process. This review focuses mainly on the methodological approach of probiotic encapsulation including materials and results obtained using encapsulated probiotic in food matrices and different pathologies in animal models.

Industrial relevance: The inclusion of probiotics into food matrices is one of the most challenging lines of research in food technology. Probiotics in general, and some strains in particular, have a low resistance to different environmental conditions, such as oxygen, light or temperature. Thus, the protection and isolation of the microorganism from the food matrix and the environmental condition are crucial for the development of new probiotic food. In this sense, microencapsulation has gained an increasing interest, since it has been demonstrated that it could protect the bacteria not only during its production process but also during its incorporation into the food matrix, also with protective effects during storage. In conclusion, microencapsulation is of great interest since it could allow a wider application of probiotics in the food market, actually restricted to fresh or powder products.

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1. Introduction

Probiotics are described by the World Health Organization (WHO) as "live organism, which when administered in adequate amounts confer health benefits to the host" (FAO/WHO, 2001). In this sense, probiotics have shown in some studies, to be effective in the treatment of several intestinal disorders and to have an impact on the immune system (Kurmann & Rasic, 1991). Considering that these microorganisms are mainly consumed orally, it would be reasonable to believe that its beneficial effects would be mainly apparent in these intestinal pathologies. However, their modulatory effects on systemic immune response may lead to positive effects in systemic disorders such as allergy (Majamaa & Isolauri, 1997) or inflammatory diseases (Malchow, 1997) and they also have demonstrated a beneficial effect in the treatment of vaginitis (Reid, 2000).

The most used probiotic microorganisms are *Lactobacillus* and *Bifidobacteria* strains (Solanki et al., 2013). However, other species, such as *Escherichia coli* and *Bacillus cereus* have also been used to achieve the same objectives, together with some yeast, mainly *Saccharomyces cerevisiae* (Burgain, Gaiani, Linder, & Scher, 2011). Some of these species have been incorporated in foods converting them in functional food (Champagne, Gardner, & Roy, 2005). These kinds of aliments are defined as modified food or food ingredient that provides a health benefit beyond satisfying traditional nutrient requirements (Sanders, 1998).

To produce these beneficial effects in health, probiotics have to be able to survive and multiply in the host. In this respect, probiotics should be metabolically stable and active in the product, survive passage through the stomach and reach the intestine in large amounts (Sanz, 2007). However, several factors have been reported to affect the viability of probiotics, including pH, hydrogen peroxide, oxygen, storage temperature, among others (Shah, Lankaputhra, Britz, & Kyle, 1995). Different approaches that increase the resistance of these sensitive microorganisms against adverse conditions have been proposed, including appropriate selection of acid and bile resistant strains, use of oxygen-impermeable containers, two-step fermentation, stress adaptation, incorporation of micronutrients such as peptides and amino acids, and microencapsulation (Sarkar, 2010).

The last option, microencapsulation, is one of the most efficient methods, and has been under especial consideration and investigation. Microencapsulation can be defined as the process in which cells are retained within an encapsulating membrane to reduce cell injury or cell lost, in a way that result in appropriate microorganism release in the gut (Sultana et al., 2000). Some benefits of microencapsulation of cells include: protection from bacteriophages and detrimental factors increasing survival during freeze drying, freezing and storage and converting them into a powder form easier to use, since it enhances their homogeneous distribution throughout the product (Mortazavian, Razavi, Ehsani, & Sohrabvandi, 2007).

Given the importance of microencapsulation, the aim of this article is to review the techniques for the microencapsulation of probiotics, as well as the components used during encapsulation, and its advantages. In addition, we analyze the effect of encapsulated probiotic in food and in some diseases.

2. Techniques for microencapsulation of probiotics

Currently, there are a lot of encapsulation technologies. Before selecting one of them, industry should have taken into account, the following point (Zuidam & Shimoni, 2010): (i) Which conditions affect probiotics viability? (ii) Which processing conditions are used during food production or processing? (iii) What will be the storage conditions of the food product containing the encapsulated prior to consumer use? (iv) Which particle size and density are needed to incorporate it properly in the food product? (v) What are the triggers and mechanisms of release? (vi) What are the cost constraints? We described below the most important technologies used to encapsulate probiotic cells. In this sense, as previously mentioned, it is known that probiotics are affected by different conditions such as moisture content, high temperatures, and agitation. In this respect food matrices should be produced in mild conditions, low temperature, controlled agitation, small presence of oxygen and moderate pH. Authors should test before introducing particles into food matrices the best storage conditions, most of the studies are carried out at 4 °C and room temperature. Particle size should be enough to protect the probiotic but not to cause gritty mouthfeel. It has been reported that soft, rounded particles are not perceptually gritty up to about 80 μm (Lawless & Heymann, 2010). The amount of particles that should be incorporated will depend on the dose of the probiotic required. The mechanism of release depends on the technology used and the material. In most of the cases, particles release their content because of pH changes (acid or basic), quelating agents and enzymatic action. Finally, the balance between cost and benefit should be taken into account, since some of the technologies described below required specific devices or materials that can increase production cost.

2.1. Extrusion technique

Extrusion technique is the most popular method because of its, simplicity, low cost and gentle formulation conditions that ensure high cell viability (Krasaekoopt, Bhandari, & Deeth, 2003). It involves preparing a hydrocolloid solution, adding microorganisms, and extruding the cell suspension through a syringe needle. The droplets are dripped into a hardening solution (Heidebach, Först, & Kulozik, 2012)

If the droplet formation occurs in a controlled manner (contrary to spraying) the technique is known as prilling. This is done by pulsation of the jet or vibration of the nozzle. The use of coaxial flow or an electrostatic field is the other common technique to form small droplets. When an electrostatic field is applied, the electrostatic forces disrupt the liquid surface at the needle tip, forming a charged stream of small droplets (Fig. 1). The method does not need organic solvents and it is easy to control the size of beads by varying the applied potential. Mass production of beads can either be achieved by multi-nozzle system or using a rotating disc (Fig. 1). Another process is the centrifugal extrusion which consists a coextrusion process. It utilizes a nozzle with concentric orifices located on the outer circumference of a rotating cylinder. The core material is pumped through the inner orifice and a liquid shell material through the outer orifice. When the system rotates, the extruded rod breaks up into droplets that form capsules (Kailasapathy, 2002).

2.1.1. Supporting material

2.1.1.1. Alginate. Alginate is a linear heteropolysaccharide extracted from different types of algae, with two structural units consisting of D-mannuronic (M) and L-guluronic acids (G). Depending on the source, the composition and the sequence in D-mannuronic and L-guluronic acids vary widely. In the same way, the functional properties of alginate as supporting material correlate strongly with the composition and

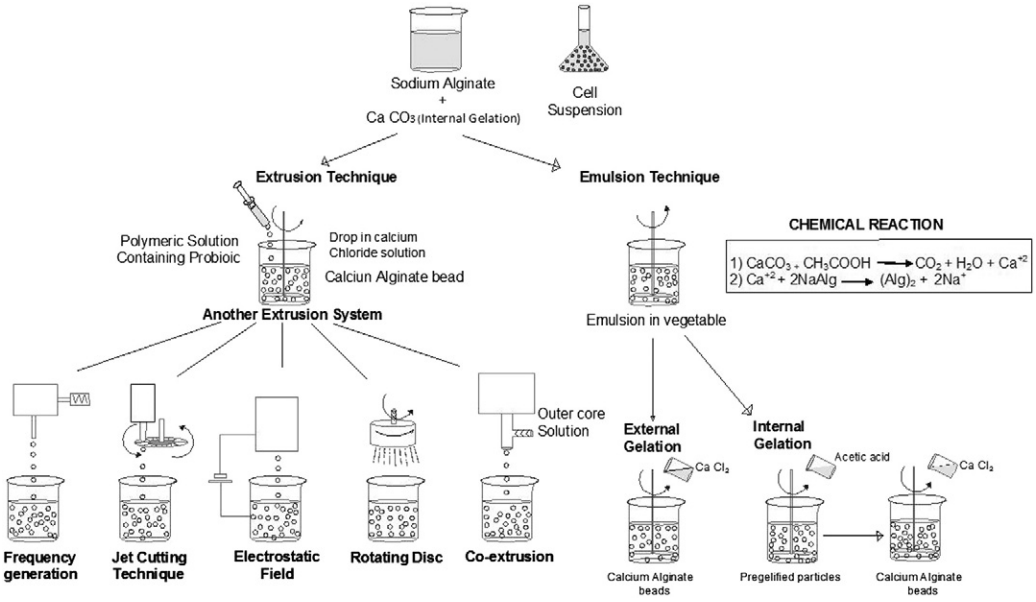

Fig. 1. Extrusion and emulsion technologies.

sequence of M-units and G-units. G-units have a bucked shape while the M-units tend to be as an extended band. In this sense, two G-units aligned side by side, result in the formation of a hole with a specific dimension which is able to bind selectively divalent cations.

To form beads, a cell suspension is mixed with a sodium alginate solution and the mixture is dripping into a solution containing multivalent cations (usually Ca^{+2} in the form of $CaCl_2$). The droplets form gel spheres instantaneously, entrapping the cells in a three dimensional structure. This is because a polymer cross-linking occurs following the exchange of sodium ions from the guluronic acids with divalent cations (Ca^{2+}, Sr^{2+}, or Ba^{2+}). This results in a chain–chain association that constitutes the so-called "egg-box model".

The success of this technique is because of the gentle environment that it provides for the entrapped material, and its biocompatibility. The size and spheric shape of the bead depend mainly on the viscosity of the sodium alginate solution and the distance between the syringe and the calcium bath. In this way, high concentration increases the viscosity of the gel but decreases the size of the beads. The extruder orifice diameter is another important factor, which regulates droplet size. The composition of the alginate also influences bead size; small bead results from low guluronic alginates (Krasaekoopt et al., 2003).

2.1.1.2. Whey protein. Alternative to polymeric hydrogels, food proteins can also be used because of their high nutritional value and excellent functional properties (Gunasekaran, Ko, & Xiao, 2007). Whey proteins are a mixture of globular protein isolated from whey, the liquid material created as a result of the production of cheese. These proteins possess the ability to interact with a wide range of active molecules, which offer a wide spectrum of opportunities for protection and reverse binding of active molecules prior to their targeted release in the host. Another potential benefit associated with protein encapsulation matrices involves hydrolysis of food proteins by digestive enzymes. It may generate bioactive peptides that may exert a number of physiological effects in vivo. In this sense, Doherty et al. (2011) use whey protein as an encapsulation material. The particles were able to protect the probiotic during 3 h in vitro stomach incubation.

2.1.1.3. Pectin. Pectin is a heteropolysaccharide mainly extracted from fruits. It is used as a gelling agent in food, in medicines and as a source of dietary fiber. It remains intact in the stomach and the small intestine. Gebare et al. (2013) produced pectin microparticles coated with whey protein. This microencapsulation system conferred greater protective effect to *Lactobacillus acidophilus* as compared to the free cells. However, the coating of pectin microparticles with whey protein did not confer additional protection to probiotics when exposed to simulated gastrointestinal conditions. In contrast, Gerez, Font De Valdez, Gigante, and Grosso (2012) found an improvement in the survival of probiotic when they are microencapsulated into pectin particles coated with whey protein after exposure to gastric conditions.

2.1.1.4. Milk. Pure milk as an encapsulation carrier has been studied too. Shi, Li, Li et al., 2013 and Shi, Li, Zhang et al., 2013 developed milk microparticles coated with carrageenan and locust bean. These milk microspheres showed good protection for *Lactobacillus bulgaricus*. However, these milk microspheres had irregular shapes and poor mechanical characteristics. To improve it, a mix of alginate with milk was used by these authors (Shi et al., 2013). The studies demonstrated that the encapsulation of *L. bulgaricus* in these new microspheres, is an effective way to protect probiotics against extreme simulated gastrointestinal environment.

2.1.1.5. Human like collagen. Human like collagen (HLC) is produced by recombinant *E. coli* BL21 containing human like collagen cDNA. This collagen is used as a hemostatic material, a scaffolding biomaterial for organs or tissue regeneration and functional foods. Su et al. (2011), prepared microspheres using alginate and HCL by electrostatic droplet generation. The human like collagen incorporated into the solution of alginate forming intermolecular hydrogen bonding or other interactions improving bead stability. The results shown by these authors showed that the tolerance of probiotics in a simulated gastric juice was improved.

2.2. Emulsion technique

In this technique, the discontinuous phase (cell polymer suspension) is added to a large volume of oil (continuous phase). The mixture is homogenized to form water-in-oil emulsion. Once the water-in-oil emulsion is formed, the water soluble polymer is insolubilized (cross-

linked) to form the particles within the oil phase (Heidebach et al., 2012). The beads are harvested later by filtration (Fig. 1). The size of the beads is controlled by the speed of agitation, and can vary between 25 μm and 2 mm.

For food applications, vegetable oils are used as the continuous phase. Some studies have used white light paraffin oil and mineral oil. Emulsifiers are also added to form a better emulsion, because the emulsifiers lower the surface tension, resulting in smaller particles (Krasaekoopt et al., 2003).

2.2.1. Supporting material and technological conditions

There are many supporting materials used with the emulsion technique. We described below the most used ones.

2.2.1.1. Carrageenan and its mixtures. κ-Carrageenan is a neutral polysaccharide extracted from marine macroalgae, commonly used as a food additive. Carrageenan requires temperatures comprising between 60 and 90 °C for dissolution especially when applied at high concentrations such as 2–5%. Its gelation is induced by temperature changes. Probiotics are added to the polymer solution at 40–45 °C and gelation occurs by cooling to room temperature. After the beads are formed, K^+ ions (in the form of KCl) are used to stabilize the gel and to prevent swelling, or to induce gelation. However, KCl has been reported to have an inhibitory effect on some lactic acid bacteria. As an alternative to KCl, Rb^+, Cs^+ and $NH4^+$ ions have been recommended. These ions, in addition to solve the abovementioned problem, produce stronger gel beads compared with potassium ions (Krasaekoopt et al., 2003).

It has been reported that a proportion of 1:2 for carrageenan and locust gum gives a strong gel for microencapsulation (Miles, Morris, & Carroll, 1984). This mixture has also good efficiency in lactic fermented products (such as yogurt) due to its lower susceptibility to the organic acids. For this reason, it has been widely used for the microencapsulation of probiotics in fermented products (Arnauld, Laroix, & Choplin, 1992; Audet, Paquin, & Lacroix, 1988). However, the gel formation of κ-carrageenan and locust bean is dependent on calcium ions, which have adverse effects in the viability of *Bifidobacterium* spp. and in the human body because of undesirable effect on the electrolyte equilibrium of liquids in the body (Sun & Griffiths, 2000).

2.2.1.2. Sodium carboxymethyl cellulose. Sodium carboxymethyl cellulose (NaCMC) is a water soluble-cellulose ether derivative. It consists of linked glucopyranose residues with varying levels of carboxymethyl substitution. The gastric acid resistance and intestinal solubility properties of NaCMC enable its utilization in drugs and probiotic delivery (Kamel, Ali, Jahangir, Shah, & El-Gendy, 2008). Chitprasert, Sudsai, and Rodklongtan (2012) developed microcapsules using a mix of sodium carboxymethyl cellulose and rice bran (RB) as filler. Rice bran is an obtained by product of rice milling processes. It is considered to be a good filler. Furthermore, its low cost can help reduce the production cost of the microcapsules. Microcapsules were prepared using a cell suspension in NaCMC with and without RB emulsified with palm oil and then crosslinking with aluminum ions. The results obtained show that microencapsulation using NaCMC and RB improved the viability of *Lactobacillus reuteri* after heat exposure. For these reasons, these particles could be applied to the development of probiotic products as functional feeds that require heat treatment.

2.2.1.3. Cellulose acetate phtalate (CAP). This polymer is used for controlling drug release in the intestine because of its safety nature (Mortazavian et al., 2008). The advantage of CAP is that it is insoluble in acid media ($pH \leq 5$) but it is soluble when the pH is ≥ 6 as a result of the presence of phthalate groups. In this sense, the microencapsulation of bacteria with CAP might offer an effective way of delivering large numbers of viable bacterial cells to the colon (Burgain et al., 2011). Rao, Shiwnavain, and Maharaj (1989) found that preparing an emulsion with starch and oil and adding CAP improved the viability of probiotics in a simulated gastric environment. Other authors found similar results using the spray drying process (Fávaro-Tindale & Grosso, 2002).

2.2.1.4. Alginate and its combinations. Calcium alginate has been widely used for the encapsulation of probiotic bacteria, mainly in the concentration range of 0.5–5% (Jankowski, Zielinska, & Wysakowska, 1997; Kebary, Hussein, & Badawi, 1998; Kim, Baek, & Yoon, 1996; Krasaekoopt, Bhandari, & Deeth, 2004; Lee et al., 2000; Martin, Lara-Villoslada, Ruiz, & Morales, 2013; Shah & Rarula, 2000; Sheu & Marshall, 1991; Sheu, Marshall, & Heymann, 1993; Sultana et al., 2000; Truelstrup-Hansen, Allan-wojtas, Jin, & Paulson, 2002).

Alginate microparticles can be obtained by external or internal gelation (Fig. 1). In the first case, the microparticles are produced by the formation of a water-in-oil emulsion, usually stabilized by surfactants, such as Tween® 80. The alginate is then gelled by the addition of calcium chloride solution to the emulsion, as it is explained in Section 2.2. Although less common, the microcapsules may also be formed by internal gelation, in which the alginate in solution contains calcium carbonate. A water-in-oil emulsion is formed and after that an organic acid (acetic acid) is added. As it penetrates into the water phase it reacts with the calcium carbonate releasing calcium ions and carbonic acid. Calcium ions react with the alginate forming the egg-box structure (Cook, Tzortzis, Charalampopoulos, & Khutoryanskiy, 2012).

Some drawbacks are attributed to alginate microparticles. For example they are susceptible to acidic environments. They crack and lose their mechanical stability in these environments. Moreover, alginate gel is formed in the presence of calcium ions, thus its integrity is deteriorated when subjected to monovalent ions or chelating agents (phosphates, lactates and citrates). Other disadvantages include difficulties in industrial scale applications. These particles are also very porous, which causes a fast diffusion of moisture and other fluids through the beads. This fact reduces the barrier properties against unfavorable environmental factors (Gouin, 2004). The mentioned defects can be solved by blending alginate with other polymer compounds, coating the alginate with different substances or doing some structural modification of the alginate (Krasaekoopt et al., 2003).

Blending alginate with corn starch has improved the effectiveness of the encapsulation technology using different bacterial cells (Martin et al., 2013; Zou et al., 2011). Starch is a polysaccharide composed by α-D-glucose units linked by glycosidic bonds, produced by all green plants. Resistant starch (RS) is the starch which is not digested by pancreatic enzymes (amylases) in the small intestine. For this reason it can reach the colon where it will be fermented. This specificity provides good enteric delivery characteristic. Moreover, resistant starch is an ideal surface for the adherence of the probiotic cells to the starch granules (Anal & Singh, 2007) and this can enhance probiotic delivery in a viable and a metabolically active state to the intestine (Krasaekoopt et al., 2003; Sultana et al., 2000; Sun & Griffiths, 2000; Truelstrup-Hansen et al., 2002; Vivek, 2013) produced particles with high cell viability bleeding the alginate with a resistant starch.

In addition, to improve the survivability of the frozen cells at −20 °C alginate can be blended with glycerol, for its cryogenic effect (Sultana et al., 2000).

Another strategy to improve physical and chemical stability of alginate particles is to form semipermeable layers of chitosan around the capsules. This structure is tolerant against the deteriorative effects of calcium chelating and antigelling agents. Structurally, the beads are also denser and much stronger, thus avoiding breaking and cell release (Krasaekoopt et al., 2003). Low molecular weight is preferred rather than high molecular weight chitosan (Krasaekoopt, Bhandari, & Deeth, 2006), since it diffuses faster into the alginate matrix, resulting in the formation of spheres with higher density and strength.

Another way of coating is using calcium chloride (Chandramouli, Kailasapathy, Peiris, & Jones, 2004). This coating causes the generation of more stable beads with a higher protective effect on the probiotic cells, and as a result, higher viability.

Polyamino acids can be also used as a coating material. In this sense, poly-L-lysine (PLL) makes strong complexes with alginate matrix and gives it the advantages previously mentioned for chitosan. Generation of multilayer shells of PLL on the alginate capsules has also been investigated. The first layer of PLL on the particle surface produces positive charge, then the second alginate coat gives a negative charge to the bead surface. This technique can be repeated several times. Other alternatives of polycationic polymers are polyetylenamine and glutaraldehyde (Mortazavian et al., 2007).

In addition, modifying alginate itself by fatty acids can be used as an encapsulating material. Amine et al. (2014) developed palmitolated alginate microparticles using the emulsion technique. Furthermore, Le-Tien, Millette, Mateescu, and Lacroix (2004) elaborated microparticles using the technique (Fig. 1). Both kinds of particles were able to improve the stability of probiotic.

2.2.1.5. Chitosan. Chitosan is a linear polysaccharide with a positive charge which is obtained by deacetylation of chitin extracted from crustacean shells. It is water soluble at pH < 6 and like alginate, forms a gel by ionotropic gelation. Chitosan exhibited inhibitory effects on different types of lactic acid bacteria and for this reason is preferred as a coating material as it was explained before (Groboillot, Champagne, Darling, & Poncelet, 1993).

2.2.1.6. Gelatin. Gelatin is a protein derived by partial hydrolysis of collagen. It has a special structure and versatile functional properties, and forms a solution of high viscosity in water, which sets to a gel on cooling. Its amphoteric nature gives the ability of having synergistic effects with anionic polysaccharides such as gellan gum. The two mentioned polymers are miscible at a pH higher than 6, since they both carry net negative charges and repel one another. However, when the pH is adjusted below gelatin's isoelectric point, the net charge on the gelatin becomes positive, causing an interaction with the negatively charged gellan gum. The mixture of gelatin-toluene diisocyanate makes strong capsules which are tolerant against crackling and breaking, especially at higher concentrations. This can be attributed to the cross-link formation between these polymers. The mentioned mixture has been used for the encapsulation of *Lactobacillus lactis* ssp. cremoris (Hyndman, Groboillot, Poncelet, Champagne, & Neufeld, 1993). Gelatin has also crosslinked with genipin and coated with alginate to prevent the pepsin-induced degradation of the gelatin microspheres in the simulated gastric juice (Annan, Borza, & Hansen, 2008).

2.2.1.7. Chickpea protein. Chickpea protein was used as an encapsulating material because of its excellent functional attributes and nutritional importance. Chickpea is also attractive as a result of fewer allergen concerns. This protein is dominated by two salt-soluble globulin-type storage proteins: legumin and vicilin attributes. Wang, Korber, Low, and Nickerson (2014) developed a chickpea protein–alginate microcapsules using the emulsion technology. The particles offered good protection to *Bifidobacterium adolescentis* within the synthetic gastric juice. The beads produced using this design were < 100 μm in size. Thus, there were no perceived adverse effects on the sensory attributes of this ingredient into foods by consumers. The study suggests that chickpea protein–alginate capsule designs could serve as a suitable probiotic carrier intended for food applications. Klemmer, Korber, Low, and Nickerson (2011), used a mixture of pea protein and alginate to produce microcapsules by extrusion. The particles were able to protect *B. adolescentis* within the simulated gastric juice and simulated intestinal fluids. However, capsule sizes were too large for food applications.

2.3. Fluid bed

In this process, cell suspension is sprayed and dried on inert carriers using a Wurster based fluidized bed system. The advantages of this process are total control over the temperature and lower comparable cost. The disadvantages are that this technology is difficult to master and is of relatively longer duration. Before drying, it is needed that the probiotic culture is encapsulated in a supporting material such as skimmed milk calcium alginate or fats. Shellac, a purified product of the resinous secretion of the insect *Kerria lacca* (Coccoidea), has also been used. The physicochemical properties of shellac are variable depending on the strain of insect, host trees and refining methods (Buch, Penning, Wächtersbach, Maskos, & Langguth, 2009). Because of its natural origin, shellac is an acceptable coating material for food supplement products. In general, shellac possesses good resistance to gastric fluid, suggesting its use for enteric coating purposes. However, the low solubility of shellac in the intestinal fluid, especially in the case of enteric coating of hydrophobic substances limits its use as an enteric coating polymer. To improve the enteric coating properties of shellac, Stummer et al. (2010) used sodium alginate, hydroxypropyl methylcellulose and polyvinylpyrrolidone as additional water-soluble polymers, and glycerol and glyceryl triacetate as plasticizers. Fluid bead is easy to scale up. For this reason it is one of the most encapsulation technologies applied commercially to probiotics. Some companies have developed products using Probiocap® and Duaolac® (Burgain et al., 2011). It can be adapted to give multilayer coatings too. In this respect, Champagne, Raymond, and Tompkins (2010) used this method by applying a coating with two different fats.

2.4. Rennet-gelled protein encapsulation

Microcapsules can be produced using a food approved enzyme (rennet) and an aqueous milk protein solution. Rennet is a proteolytic enzyme complex, which is capable of cleaving the k-casein molecule, which produces the aggregation of the casein micelles (Heidebach, Först, & Kulozik, 2009). Non-covalent cross-links are then progressively formed between chains of flocculating micelles to form a final gel above 18 °C (Bansal, Fox, & McSweeney, 2007). These microcapsules are able to encapsulate probiotics, without significant loss of cells during the encapsulation process. The survival of encapsulated cells can probably be explained by a higher local pH value within the protein matrix of the capsules caused by the protein buffering capacity. It can protect the cells during incubation under simulated gastric conditions at low pH. Furthermore, these proteins alleviate the feasibility to control the capsule size of microcapsules, which is of high importance with regard to the sensory impact of the particles in the final products. For all those reasons, this technique seems to be a suitable approach for a more effective application of probiotic in food.

2.5. Freeze drying

Freeze drying has been used to manufacture probiotic powders for decades but the combination of freeze drying and encapsulation is a relatively new concept. The process is based upon sublimation, occurring in three phases; freezing, primary, and secondary drying. Typically, cells are first frozen and then dried by sublimation under a high vacuum (Santivarangkna, Kulozik, & Foerst, 2007). As the processing conditions associated with freeze drying are milder than spray drying, higher probiotic survival rates are typically achieved (Wang, Yu, & Chou, 2004). In this technique, the solvent is frozen and removed via sublimation (Solanki et al., 2013). However, freezing causes damage to the cell membrane because of crystal formation and also imparts stress condition by high osmolarity. A variety of protectants have been added to the drying media before freeze drying to protect the viability of probiotics during dehydration, such as skim milk powder, whey protein,

glucose, maltodextrine, and trehalose among others. Cryoprotectants may also be added to media prior to fermentation to assist in the adaptation of probiotics to the environment (Basholli-Salihu, Mueller, Salar-Behzadi, Unger, & Viernstein, 2014; Capela, Hay, & Shah, 2006). The mechanism of cryoprotectants is that they are able to accumulate within the cells, reducing the osmotic difference between the internal and external environments (Kets, Teunissen, & De Bont, 1996).

2.6. Spray drying

Spray drying is the most commonly used microencapsulation method in the food industry, since it is economical and flexible. The energy consumption of spray drying is 6 to10 times lower compared to freeze drying and it produces a good quality product. The process involves the dispersion of the core material, forming an emulsion or dispersion, followed by homogenization of the liquid, and then the atomization of the mixture into the drying chamber (Fig. 2). This leads to evaporation of the solvent. It is important to underline that in this technique, the product feed, gas flow and temperature should be controlled.

The advantage of the process is that it can be operated on a continuous basis. The disadvantage is that the high temperature used in the process may not be suitable for encapsulating probiotic bacterial cultures. On this point, outlet temperatures greater than 85–90 °C are lethal for probiotics. It is seen that under same inlet temperature conditions a higher inlet feed rate had a lower outlet temperature and an increased survival rate. This indicates that the cell survival is mostly dependent on outlet temperatures. Cellular membrane heat damage is one of the most susceptible target damage during spray drying. These high temperatures during spray drying cause the cellular pores to leak the intracellular substances (Anekella & Orsat, 2013). However, proper adjustment and control of the processing conditions such as the inlet and the outlet temperatures can achieve viable encapsulated cultures with a desired particle size distribution (Table 2). Other factors that affect spray dried probiotic viability are the types of strain and their tolerance to stress conditions, the carrier, drying temperature and time of exposure to heat (before spray drying process) and the water activity and storage conditions (after spray drying process). (See Table 1.)

2.6.1. Two-step drying

Normally, probiotics are spray dried at high inlet and outlet temperatures (Table 2), in order to obtain a dry powder with a moisture content below 4%, required for safe storage. As it was mentioned before, such drying temperatures are the most probable causes of unsatisfactory survival. Optimization of drying conditions in order to enhance their survival during storage is needed. In this sense, Chavez and Ledeboer (2007) developed a system based on the use of spray drying (Ti = 80 °C, to = 48 °C) and vacuum drying at mild temperatures (45 °C). The result shows that a two-step drying process is a realistic alternative to freeze drying in order to produce food powders containing viable probiotics. Furthermore, such a two-step process is estimated to be 3 times cheaper than freeze drying.

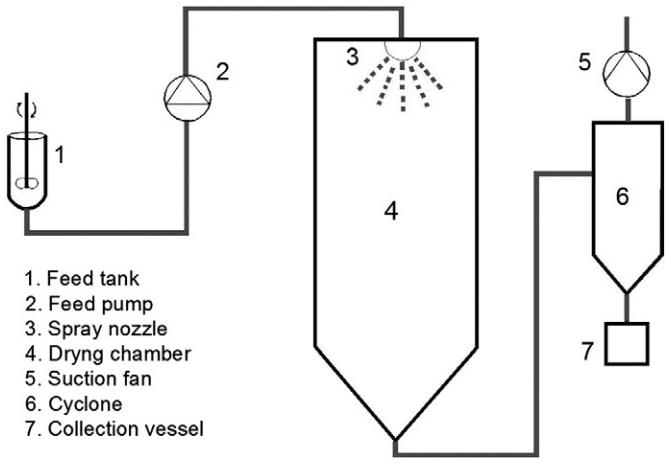

Fig. 2. Spray-drying technology.

2.6.2. Spray freeze drying

The spray freeze drying method combines processing steps that are common to freeze drying and to spray drying. Probiotic cells are in a solution which is atomized into a cold vapor phase of a cryogenic liquid such as liquid nitrogen. This step generates a dispersion of frozen droplets. Frozen droplets are then dried in a freeze dryer (Amin, Thakur, & Jain, 2013). This technique presents various advantages, like providing controlled size and larger specific surface area than spray-dried capsules. Moreover the capsules can be coated by an additional shell using the fluid bead method to give protection against adverse environmental conditions (Semyonov et al., 2010). However, this process has also some disadvantages including the use of high energy, the long processing time and the cost which is 30–50 times expensive than spray drying (Zuidam & Shimoni, 2010).

Semyonov et al. (2010) use a wall matrix maltodextrin, a polysaccharide that contributes in reducing the mobility of the cells in the glassy state. Another matrix component was a disaccharide and trehalose, that act as a protective excipient, which is able to improve the cell viability during freezing (cryoprotectant), freeze drying, as well as during the storage of the dried bacteria. Trehalose is known to create hydrogen bonds with proteins and the polar head groups of the lipid membrane of the cells prevent structural damage during dehydration. The authors demonstrated that spray freeze drying is an appropriate process to generate dried microcapsules with *Lactobacillus paracasei*. These particles are able to retain high viability during the spraying, freezing, and drying stages.

2.6.3. Spray chilling

Spray chilling is also called spray cooling and spray congealing. This process is similar to spray drying with respect to the production of small droplets. However, spray chilling is based on the injection of cold air, which enables the solidification of the particle. A molten matrix that contains the bioactive compound is atomized so that it forms drops that quickly solidify when they are in contact with the cold air (Champagne & Fustier, 2007).

The spray chilling mainly uses fat matrices as carrier. The microparticles that are produced can present some disadvantages, which include a low encapsulation capacity and the expulsion of core material during storage, as a result of the crystalline structure and polymorphic arrangement characteristic of many lipid materials during the solidification and crystallization processes (Sato & Ueno, 2005). However, spray chilling is considered to be the cheapest encapsulation technology that has the possibility of industrial scale manufacture (Gouin, 2004) Moreover, this technology could be used to generate smaller beads, which may be desirable in food processing. Pedroso, Thomazini, Barrozo Heinemann, and Favaro-Trindade (2012) using the spray chilling technology to microencapsulate *Bifidobacterium lactis* and *L. acidophilus* used as wall materials, interesterified fat with palm and palm kernel. The solid lipid microparticles developed were efficient in protecting the probiotics against the passage through gastric and intestinal fluids, and they could also be stored at low temperatures. In addition, the morphologies and sizes of the microparticles may facilitate the flow of material, while causing no harmful effects towards the food texture.

2.6.4. Ultrasonic vacuum spray dryer

A technique based on spray drying which minimizes the thermal and oxidative stresses during the drying process has been developed. This system uses an ultrasonic nozzle, low temperatures and vacuum atmosphere in the drier chamber. Semyonov, Ramon, and Shimoni (2011)

Table 1
Comparison of different encapsulation technologies.

Technique	Particle size	Typical materials	Special treatment	Physical and chemical stability	Technical limitations	Material cost[a]	Food	Authors
Extrusion	1.89 mm	Alginate	Use of low molecular chitosan	Chitosan did not much improve the survival of encapsulated probiotics in the yogurts	Big particle size. Difficult to scale up. Particles are not dried.	**	Yogurt	Krasaekoopt et al. (2006)
External gelation	0.5–1 mm	Alginate + Hi-maize starch (resistant starch)	Use of Hi-maize starch and glycerol	Bacteria were not protected from low pH conditions	Not uniform bead size. Perceptible gritty mouthfeel. Particles are not dried.	**	Yogurt	Sultana et al. (2000), Kailasapathy (2006)
Fluidized bed	15–40 μm	Whey protein	Use of palm oil and cellets®	Encapsulation did not improve probiotic viability in food during storage	Difficult to master Longer duration	*	Infant formula	Weinbreck, Bodnár, and Marco (2010)
Freeze drying	–	Sucrose, lactose, cellobiose, or trehalose	–	Sucrose and lactose protect lees than cellobiose and trehalose in simulated gastric juice	Freezing damage	***	Pasteurized low fat milk Grape and red beet juices	Basholli-Salihu et al. (2014)
Spray drying	3–75 μm	Whey protein	Use of milk fat	Milk fat did not improve the viability of probiotic	High temperatures (heat damage of bacteria)	**	Yogurt	Picot and Lacroix (2004)
	11.23 μm	Whey protein		Microparticles did not improve the survival of probiotic in bile salt		*	Dairy dessert (white chocolate flavor vigor Delicatessen®)	Picinin De Castro-Cislaghi, Dos Reis E Silva, Fritzen-Freire, Goulart Lorenz, and Sant'Anna (2012)
	–	Raspberry juice pulp	Preheating of bacteria	Microorganism were killed during pre-heating in raspberry juice		*	Possible inclusion in non-dairy probiotic food	Anekella and Orsat (2013)
Impinging	10–40 μm	Alginate		Encapsulation was not able to enhance the survival of probiotic in orange juice	Need of a specific equipment Particles are not dried	**	Orange juice and pear and peach snack	Sohail et al. (2012)

[a] Material cost is classified such as: the cheapest one (*) to the most expensive (***).

selected as wall material a mix of maltodextrin and trehalose, since as it was indicated before, these components can increase the survival by maintaining the probiotic cells' membrane integrity during the drying and storage as well as promote the stabilizing effect of the bacteria's proteins. The results showed that the combination of a protein and a carbohydrate contributed to the retention of a high viability after spray drying and the extension of survival rates during storage.

2.7. Hybridization system

The hybridization system is a dry encapsulation technique. It consists of a high speed rotating rotor with six blades, a stator and a powder recirculation circuit. The powder mixture (host and guest particles) placed in the vessel is subjected to high impaction in air stream generated by the blade rotating at high speed. During the process, the particles form an ordered mixture by embedding or filming the guest particles onto the surface of the host particles. The hybridization system results in high yields of microcapsules and minimizes heat induced bacterial damage using a cooling system that maintains temperatures below 30 °C (Takafumi, Honda, & Koishi, 1993). Some prebiotic substances have been tested with this technique such as: sorbitol, mannitol, lactulose, xylitol, inulin, fructooligosaccharide and raffinose. The results indicate that double microencapsulation by hybridization is useful to effectively provide beneficial effects of probiotics for the host (Ann et al., 2007).

2.8. Impinging aerosol technology

Impinging aerosol technology uses two separate aerosols. One with the microbial suspension in alginate solution and the other one with calcium chloride. The mixture of alginate is injected from the top of a cylinder meanwhile the calcium chloride is injected from the base. This technology produces alginate microbeads with an average diameter of less than 40 μm (Sohail, Turner, Coombes, Bostrom, & Bhandari, 2011). As no heat or solvent is used, impinging aerosol technology is suitable for encapsulating heat labile and solvent sensitive materials. Moreover, it has a large volume production capacity and microbeads could be sprayed or freeze-dried. Sohail et al. (2011) demonstrated that the microbeads obtained by impinging aerosol technology and extruded macrobeads (approximately 2 mm diameter) offered similar protection to *Lactobacillus rhamnosus* GG in the acid and bile tolerance study. Moreover, Sohail, Turner, Prabawati, Coombes, and Bhandari (2012) investigated the effect of the microencapsulation on the survival of *L. rhamnosus* GG and *L. acidophilus* NCFM and their acidification in orange juice at 25 °C for nine days and at 4 °C over thirty five days of storage. Unencapsulated *L. rhamnosus* GG was found to have excellent survivability in orange juice at both temperatures. However unencapsulated *L. acidophilus* NCFM showed a significant reduction in viability. The encapsulation of these two bacteria did not significantly enhance survivability but did reduce acidification at 25 °C and 4 °C. In conclusion, *L. rhamnosus* GG showed excellent survival in orange juice and the microencapsulation has potential in reducing acidification and possible negative sensory effects of probiotics in orange juice and other fruit-based products.

2.9. Electrospinning

The combined use of two techniques namely electrospray and spinning is made use of in a highly versatile technique called electrospinning (electrop + spinning). In this technique, a high electric field is applied to a fluid which may be a melt or solution coming out from the tip of a die, which acts as one of the electrodes. This leads to the droplet deformation and finally to the ejection of a charged jet from the tip towards the counter electrode leading to the formation of continuous fibers (Fig. 3).

Table 2
Microparticles produced by different spray drying process.

Probiotic	Encapsulation matrix	Spray drying conditions	Reference
Bifidobacterium PL1	Modified waxy maize starch	Ti = 100 °C To = 45 °C	O'Riordan, Andrews, Buckle, and Conway (2001)
L. acidophilus La-05 *B. lactis* Bb-12	Cellulose acetate phthalate	Ti = 130 °C To = 75 °C	Fávaro-Tindale and Grosso (2002)
B. breve R070 *B. longum* R023	Whey protein isolate (10% w/w) Milk fat	Ti = 160 °C To = 80 °C	Picot and Lacroix (2003)
Lactobacillus rhamnosus GG *L. rhamnosus* E800 *L. salivaris* UCC 500	Skim milk (20% w/v) Polydextrose (20% w/v) Inulin (20% w/v) Skim milk (10%) + polydextrose (10% w/v) Skim milk (10% w/v) + inulin (10% w/v) Raftilose® P9 and Synergy1 Raftiline® GR and HP	Ti = 140 °C To = 85–90 °C	Corcoran, Ross, Fitzgerald, and Stanton (2004)
B. longum B6 *B. infantis* CCRC 14633	Gelatin (10%) Soluble starch (10%) Skim milk (10%) Arabic gum (10%)	Ti = 100 °C To = 50 °C	Hsiao, Lian, and Chou (2004)
L. rhamnosus GG	Skim milk (20% w/v) Polydextrose (20% w/v) Inulin (20% w/v) Raftilose® P95 (20 w/v %)	Ti = 140 °C To = 70–100 °C	Ananta, Volkert, and Knorr (2005)
Lactobacillus kéfir CIDCA 8321 and 8348	Skim milk (11% w/v) Skim milk + sucrose (2% w/v) Skim milk + monosodium glutamate (1.25% w/v) Skim milk + frutooligosaccharides (2% w/v)	Ti = 160 °C To = 70 °C	Golowczyc et al. (2010)
L. rhamnosus GG	Whey protein isolate (32.7% w/w) + maltodextrine (65.3% w/w) Whey protein isolate (32.7% w/w) + maltodextrine (32.7% w/w) + glucose (32.7% w/w) Whey protein isolate (32.7% w/w) + inulin (65.3% w/w) Whey protein isolate (32.7% w/w) + inulin (32.7% w/w) + glucose(32.7% w/w)	Ti = 160 °C To = 65 °C	Ying et al. (2010)
Bifidobacterium BB-12	Skim milk (20% w/v) Skim milk (10% w/v) + inulin (10% w/v) Skim milk (10% w/v) + Orafti® Synergy1 (10% w/v) Skim milk (10% w/v) + oligofructose	Ti = 150 °C To = 55 °C	Fritzen-Freire et al. (2012)
L. casei *L. paracasei* *L. acidophilus* *L. plantarum*	Skim milk (20% w/v)	Ti = 170 °C To = 85 °C	Paéz et al. (2012)
Bifidobacterium BB-12	Whey	Ti = 150 °C To = 50–60 °C	Picinin De Castro-Cislaghi et al. (2012)
L. acidophilus NRRL B-4495 *L. rhamnosus* NRRL B-442	Raspberry juice + maltodextrine	Ti = 100 °C To = 50 °C	Anekella and Orsat (2013)
Lactobacillus acidophilus *Lactobacillus lactis* ssp.	Vegetable oil (10% w/v) Sodium caseinate (6% w/v) Fructooligasaccharides (2% w/v) D-Glucose (3% w/v) Mannitol (3% w/v)	Ti = 99 °C To = 50 °C	Dianawati, Mishra and Shah (2013)
Lactobacillus reuteri DSM 17938	Alginate (1% w/v) Calcium chloride (1% w/v)	Ti = 99 °C To = 50 °C	Malmo, La Storia, and Mauriello (2013)

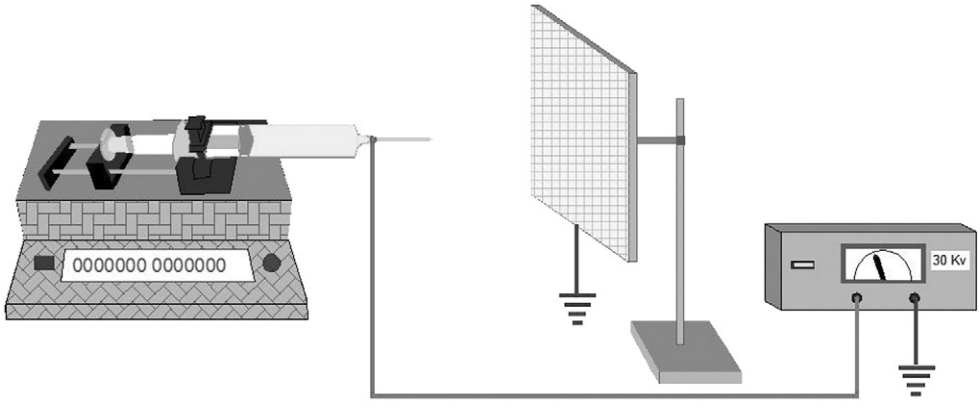

Fig. 3. Electrospinning.

The advantages of the electrospinning technique are the production of very thin fibers or capsules to the order of few nanometers with large surface areas. Moreover, the possibility of large scale productions combined with the simplicity of the process makes this technique very attractive for many different applications (Agarwal, Wendorff, & Greiner, 2008). In that regard, probiotic encapsulation has been carried through electrospinning using a protein based matrix (whey protein concentrate) and a carbohydrate based matrix (pullulan). Whey protein concentrate microcapsules have proved a greater improvement in cell viability when compared to the pullulan structures (López-Rubio, Sanchez, Wilkanowicz, Sanz, & Lagaron, 2012).

3. Encapsulated probiotic in food matrices

Although probiotics are normally considered as pharmaceutical products, the current trend is moving towards the health food sector, making true the Hippocrates' statement "let food be your medicine". Most probiotic foods in the current market are refrigerated dairy products. However, the analysis of these products in several different countries has confirmed that probiotic strains exhibit poor survival in food such as fermented dairy products (Shah, 2000). In this respect, probiotic microorganisms present in food should survive in a significant number (10^6–10^8 CFU/g), although the number varies from strain to strain. Growth, survival and death of these microorganisms in food are largely governed by properties of the food (water availability, pH, buffering capacity, among others) in addition to the storage conditions (temperature, relative humidity and atmosphere). It has to be pointed out that food matrices should help probiotics to survive through the gastrointestinal tract and regulate the colonization of the gastrointestinal tract. Therefore, the selection of suitable food systems to deliver probiotics is a vital factor that should be considered in developing functional probiotic foods. Microencapsulation can also improve the viability of probiotics in some food matrices. In fact during the past few years, food products containing encapsulated probiotic cells have been introduced on the market (Burgain et al., 2011).

4. Studies of the effects of microencapsulated bacteria on some pathologies

As we mentioned in Section 1, probiotics can produce beneficial effect on some pathologies. However, to get this beneficial effect, they have to reach the gut in adequate amounts. As a result of the harsh condition associated with the gastrointestinal tract, using encapsulated probiotic could be an interesting option. However, only a few in vivo studies have been carried out to test the beneficial effect of encapsulated probiotics in various pathologies.

In this respect, probiotics have been used to regulate the glucose concentration. These microorganisms are known to have health effects in reducing cholesterol levels (Bhatia, Rana, Sharma, Singla, & Randhawa, 2012) and immunomodulation (Kumar, Arora, & Bhatia, 2011). In fact, there is a direct correlation between diabetes and immunomodulation. The result obtained by Bhatia, Sharma, Sood, and Singla (2013) using encapsulated *Lactobacillus* (*Lactobacillus*) (LB10) isolated from healthy buffalo milk and commercial probiotic from LeeBiotic Capsule (LCap) show that encapsulated probiotics have better efficacy as antidiabetic agent than the same probiotic in unencapsulated form. Microparticles were prepared using the extrusion technology. In the groups treated with unencapsulated bacteria (LB10) and (LCap) the decreases in glucose levels observed were 37.85% and 36.50% respectively whereas in the group receiving encapsulated bacteria LB10 and the encapsulated commercial probiotic, decreases of 41.84% and 40.97% were observed respectively. Moreover the bacteria reduced the glucose level to normal within 14 days. Glibenclamide reduced the glucose level within 7 days. However this drug created hypoglycemic conditions. This result suggests that encapsulation improves the survival of bacteria under gastrointestinal conditions and produces a significant reduction of total blood glucose level. Hence, for a sustained beneficial health effect of probiotics, the encapsulation of bacteria could be an alternative to decreased blood glucose levels.

Another factor which may be responsible for health benefits of probiotics is the induction of the conversion of linoleic acid (LA) to conjugated linoleic acid (CLA). This fatty acid has shown to have anticholesterolemic action (Schlegel, Ringseis, Windisch, Schwarz, & Eder, 2012). The results obtained by Bhatia et al. (2012), show that encapsulated and unencapsulated *Lactobacillus* (isolated from healthy buffalo milk) as well as drug (Atorvastatin) reduced the cholesterol level. Microparticles were developed using the extrusion process. The percentage of the decrease in cholesterol level in encapsulated an unencapsulated bacteria is almost parallel to that obtained in drug treated mice. The study also indicated that the effect of probiotics is independent from the encapsulation. According to these authors, this result could be because the encapsulated bacteria could need a longer period of time to exert the effect since they are released in a slower but maintained way than the unencapsulated bacteria.

Furthermore, it is known that the micromilieu of solid tumors provides an ideal environment for the growth of facultative and strictly anaerobic bacteria (Cheng et al., 2008). It has been shown that certain species including *Lactobacillus* and *Clostridium* can colonize those environments leading to the regression of tumor growth (Cheng et al., 2008; Kim, Oh, Yun, Oh, & Kim, 2010; Matsuzaki, 1998; Tuo et al., 2010; Zabala et al., 2001). Such observations have given rise to the concept of bacteriolytic therapy where live microorganisms might be used to colonize the tumor and exert a tumorolytic effect. However, these lytic properties of some bacteria could also be detrimental for non-tumor cells. For this reason, it would be advantageous to explore a relatively non-pathogenic strain and provide some form of containment that would enable site specific injection and minimize the dispersion of the microorganism throughout the host. In testing the feasibility of such an approach, Dwivedi, Nomikou, Nigam, and McHale (2012) prepared microencapsulated formulations of *L. casei* NCDO 161 by external gelation. They demonstrated that these formulations were toxic for tumor cells in vitro. The authors also investigated the effects of the microencapsulated formulations on tumor growth in vivo following direct intratumoral injection. The study demonstrated a significant inhibition of tumor growth and suggested the potential therapeutic benefit of this approach in the treatment of solid tumors.

In addition, Ruan et al. (2007) developed gelatin microparticles to be tested in a hemorrhagic shock model, using *Bifidobacterium longun*, *Bifidobacterium bifidum*, and *B. adolescentis*. The authors demonstrated that rats pretreated with encapsulated and unencapsulated *Bifidobacteria* showed a decrease of total aerobes in cecum, magnitude of total aerobes to bacterial translocation levels of plasma endotoxin, and percentage of ileal villous damage when compared with rats treated with phosphate buffered saline. Encapsulated *Bifidobacteria* induced greater decreases than intact *Bifidobacteria* in this model, with the exception of a similar effect on ileal villous damage. Moreover, the incidence of bacterial translocation was decreased in hemorrhagic rats pretreated with *Bifidobacteria* compared with control. However, the magnitude of total anaerobes and *Bifidobacteria* were similar among hemorrhagic shocked rats receiving the different supplements.

5. Conclusion

Microencapsulation has been proven to be one of the most efficient methods for maintaining viability and stability of probiotics, as it protects probiotics during food processing and storage, as well as in gastric conditions. Besides the polysaccharides traditionally used as a matrix in microencapsulation, new materials are being tested and new technologies are developed such as electrospinning. However there is a need to develop new technologies or equipments that produce uniform particles for industrial applications. Further researches also have to be carried out to find appropriate carrier matrices, and bacterial strains. The

extra costs incurred by microencapsulation have to be estimated so that they can be minimized. Cost savings can be derived from easier technologies, lower waste of bacterial material and better health impact of the product. Nevertheless, the research is actually focused on expanding the use of encapsulated probiotic in different food matrices.

In addition, only a few in vivo studies have been carried out to test the beneficial effect of encapsulated probiotics. Although these studies show promising results, they have only been carried out in animals. Clinical trials, involving large numbers of patients, will be mandatory to achieve definite evidence of the preventive and curative role of encapsulated probiotics in medical practice. Information about correct formulations in terms of the amount of bacteria and viability and also the capability of these microorganisms to colonize their niche will be required. It is needed to standardize the administration schedule and to achieve homogeneous and comparable results.

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I.3. Effect of unmodified starch on viability of alginate-encapsulated *Lactobacillus fermentum* CECT 5716

Efecto del almidón no-resistente en la viabilidad de *Lactobacillus fermentum CECT5716* vehiculizado en micropartículas de alginato.

En los últimos años, las bacterias probióticas se han convertido en uno de los ingredientes más prometedores para la producción de alimentos funcionales y nutracéuticos, existiendo evidencia de su eficacia en ciertas patologías. A la hora de utilizar probióticos, el principal problema que se presenta es la escasa resistencia de estos a diferentes condiciones ambientales y tecnológicas. En este sentido, las técnicas de microencapsulación son un buen método para proteger a estos microorganismos, sin embargo no todas las técnicas son apropiadas. En concreto, el objetivo fundamental de nuestro trabajo ha sido utilizar la gelificación iónica interna como método para la microencapsulación de *Lactobacillus fermentum* CECT5716. Hemos evaluado la eficiencia de encapsulación de dos formulaciones diferentes en las que hemos utilizado como material de recubrimiento alginato (5 g/100 mL) y alginato (5 g/100 mL) más almidón no-resistente (1 g/100 mL).

Los resultados obtenidos muestran que la viabilidad del probiótico microencapsulado decrece 3 logaritmos de unidades formadoras de colonias en el caso de la fórmula con alginato y únicamente 0,3 logaritmos de unidades formadoras de colonias para las micropartículas elaboradas con alginato y almidón no-resistente. Además, la viabilidad se mantiene durante 45 días a 4ºC, siendo el tamaño de partícula adecuado (<30 μm).

Por tanto, podemos concluir que la Gelificación Interna usando alginato y almidón no-resistente nos permite obtener micropartículas capaces de conferir a las bacterias probióticas cierto grado de protección.

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Effect of unmodified starch on viability of alginate-encapsulated *Lactobacillus fermentum* CECT5716

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ABSTRACT

Probiotics are one of the most promising ingredients for the production of functional foods or nutraceutics. However, when incorporating probiotics into a food matrix, the main problem is their low resistance to different environmental and technological conditions.

Microencapsulation is a good method in order to protect probiotics. The aim of this study was to apply the Ionic Internal Gelation Technology to protect *Lactobacillus fermentum* CECT5716.

We evaluate the encapsulation efficiency of two different formulations, the first one with alginate (5 g/100 mL) as a supporting material and the second one with a mix of alginate (5 g/100 mL) and unmodified starch (1 g/100 mL).

Results show that the viability of probiotic decreases in 3 log cell numbers in the case of the formulae with alginate and in 0.3 log in the formulae with alginate and starch. Moreover, the last one allows us to obtain a suitable particle size (<30 μm) and the viability of probiotic was not modified after 45 days at 4 °C.

In conclusion, Internal Gelation Technology using alginate and starch seems to be a suitable procedure for protecting this probiotic strain and the formulae with alginate and starch is more efficient than the one with just alginate.

1. Introduction

Probiotics are defined by the World Health Organization (WHO) as live microorganisms, which when administered in adequate amounts confer health benefits (FAO/WHO, 2001). Considering that probiotics products are mainly for oral consumption, it would be reasonable to think that their beneficial effects would be mainly apparent in intestinal pathologies (Arribas, Rodriguez, Camuesco, Zarauelo, & Galvez, 2008). The manipulation of intestinal microbiota by probiotics has gained and increasing interest, since it is known that the microbiota has an impact in different functions of the human body, such as the functioning of the intestinal barrier and, especially the immune response, where the interaction between bacteria and the immune cells is key for the development of the immune system (Rijkers et al., 2010). This is a very interesting area for the food industry, as is demonstrated by the increasing number of probiotics foods as well as dietary supplements that are being commercialized in the last years (Kailasapathy, 2002).

However the main problem of probiotics is their low resistance to various environmental factors such as pH, post-acidification (during storage) in fermented products, hydrogen peroxide production, oxygen toxicity (oxygen permeation through packaging), and storage temperatures (Kailasapathy, 2002). A plethora of obstacles are further encountered during gastrointestinal (GI) transit (pH, enzymes, bile salts), all of which limit probiotic survival and functionality for the conveyance of health benefits (Doherty et al., 2011).

Thus, when incorporating a probiotic strain into a food matrix there are two main problems to be addressed, the resistance of the probiotic to the technological conditions of the food production (Saarela, Mogensen, Fondén, Mätö, & Mattila-Sandholm, 2000) and the maintenance of viability up to the expiring date of the food, since the manufacturer has to ensure an optimal administration of the probiotics throughout the life of the product. That could limit the use of probiotics in long-life products specially if they are not refrigerated during storage. For example, some studies have shown low viability of probiotics in products such as yoghurt and fermented milk (Schillinger, 1999; Vinderola, Bailo, & Reinheimer, 2000).

One of the most common methods used to preserve probiotics is freeze-drying. However, this method is not considered optimal, at

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least as the only strategy, since it just protects probiotics from the humidity but does not confer protection from certain environmental and technological conditions such as varying temperatures, oxygen toxicity or passage through the intestinal tract (Krasaekoopt, Bhandari, & Deeth, 2003). It is also frequent that probiotics experience decreased viability during or after freeze-drying.

In this sense, microencapsulation, which is defined as a process in which the cells are retained within an encapsulating matrix or membrane, may provide an approach for protecting probiotics. This technique would allow isolate the probiotic from the environment thus increasing its resistance to the conditions of production and would also improve its viability throughout the storage. The protective effect of microencapsulation is generally explained by limited diffusion of inhibitory substances such as metabolic product from starter cultures, H_2O_2, lactic acid, and bacteriocin into the capsules (Heidebach, Först, & Kulozik, 2012). Also, sodium alginate, used by internal gelation, is able to resist pH between 3 and 10 and is rather resistant to bacterial and enzymatic degradation. Moreover, alginate formed a hydrogel barrier in solution that retards the permeation of acid fluid (Klayraung, Viernstein, & Okonogi, 2009).

Some authors have indicated that the encapsulation of various probiotic stains in hydrocolloid gels enhance their survival during storage in yogurt at about 0.5 to 3 log cycles cfu (Heidebach et al., 2012). Many studies have reported the use of encapsulated probiotic cells and particularly in Cheddar cheese, frozen dairy dessert and chocolate (Burgain, Gaiani, Linder, & Scher, 2011).

However not all techniques of microencapsulation are suitable for probiotics. Some techniques such as spray-drying and extrusion have been used with food ingredients but have limited application with respect to probiotics due to their thermosensitivity and also because microparticles' size may be too large, as is the case for the extrusion technique. An emulsification internal gelation technique has been described by Poncelet et al. (1995) for immobilizing labile materials within alginate microparticles. Alginate is a linear heteropolysaccharide of D-mannuronic and L-guluronic acid extracted of various species of algae (Smidsrød, Haug, & Lian, 1972) which interesting characteristics, such as its relative inexpensiveness, structural simplicity, and biocompatibility (Martinsen, Skjak-Braek, & Smidsrod, 1989; Tanaka, Matose, & Veleky, 1984). This technique could also be suitable for probiotics, since the gel matrix consisting of alginate microparticles is able to retain bacteria (1–3 μm size) because its estimated pore size is less than 17 nm (Klein, Stock, & Vorlop, 1983). Furthermore, at low pH alginate is degraded and the materials encapsulated are released which would allow liberation of probiotics in the gastrointestinal tract (Gombotz & Wee, 1998).

Although this technique is suitable for probiotics, the use of alginate is limited due to its low physical stability in the presence of Ca^{+2}-chelating agents, monovalent ions and harsh environmental conditions (Smidsrød, & Skjak-Braek, 1990). There have been numerous efforts on the improvement on the chemical and mechanical stability of the alginate beads consequently improving the effectiveness of encapsulation (Krasaekoopt et al., 2003). Blending with other polymers such as starch (Sultana et al., 2000) provides to some extent alginate gels with additional features and it has been reported as an effective method for the encapsulation of probiotics. However, to date, the effects of the reinforcement based on alginate and unmodified corn starch on protection of the encapsulated probiotics were not reported by ionic internal gelation technology.

The probiotic strain used in this work was *Lactobacillus fermentum* CECT5716, a bacteria isolated from human milk of healthy mothers which have demonstrated, *in vitro*, *in animal models* and in human trials, its probiotic properties (Lara-Villoslada, Olivares, Sierra, Rodríguez, & Xaus, 2007; Martin et al., 2005). Thus, *L. fermentum* CECT5716 is a good candidate for food products aimed at improving or maintaining human health. By the moment, *L. fermentum* CECT5716 is present just in infant formula in powder and the microencapsulation technique could allow further food applications.

Nowadays, there is no report on the microencapsulation of this strain and thus, the objective of this work was to analyze the application of the emulsification/internal gelation technique to probiotic cells encapsulation, evaluating the encapsulation efficiency of two different formulations (alginate and a mix of alginate and unmodified starch). The best material, alginate and unmodified starch were selected, and the survivability was studied throughout time at different temperatures.

2. Materials and methods

2.1. Materials

Freeze-dried *L. fermentum* CECT5716, was kindly provided by Biosearch Life S. A. (Granada, Spain). Sodium alginate (*Natrii alginas*) was purchased from Fagron Iberica S. A. (Barcelona, Spain). Calcium carbonate and acetic acid were from Panreac Quimica S.A.U. (Barcelona, Spain). Soya oil, corn starch and Span 80 were purchased from Guinama S.L.U. (Valencia, Spain). Distilled water was prepared by a Millipore (Billerica, MA, USA) system.

2.2. Methods

2.2.1. Preparation of alginate microparticles

The emulsification/internal gelation technique to form alginate microparticles has been described previously by Poncelet et al. (1995). The procedure was modified as follows to encapsulate *L. fermentum*. Glassware and reagents used in the experiments were sterilized before used. A (5 g/100 mL) sodium alginate mixture was prepared by mixing with 1 g of lyophilized culture and 0.04 mol/l $CaCO_3$ and after homogenization, was added to 2 parts of soya oil (v/v) (continuous phase) containing 2.5% (w/v) Span 80®. The mixture was stirred at 700 rpm for 10 min with a mechanical stirrer to form a uniform water-in-oil-emulsion. With continuous stirring, 0.450 mL glacial acetic were added to the emulsion to initiate internal gelation. After 10 min under stirring, microparticles were separated from the oil dispersion by mixing with 0.45 mol/l calcium chloride solution. The phases were allowed to separate overnight. The supernatant was discarded and the microparticles were centrifuged, collected and washed using 100 mL sterile 20 g/l peptone water by vacuum filtration and stored at 4 °C.

2.2.2. Preparation of alginate and unmodified starch microparticles

The mixture was prepared by adding 0.4 g of corn starch to alginate solution and microparticles were prepared as previously described.

For lyophilization of these microparticles the following conditions were used: 1.5 h at −35 °C for freezing, then 18 h at 5 °C followed by 4 h at 20 °C for drying.

2.3. Microscopic examination of alginate beads

The morphology of the microparticles produced from each process with and without probiotics was characterized by optical microscopy using an Olympus BX40 microscope equipped with a camera Olympus SC35 (Tokyo, Japan). The average size of microparticles was determined by measuring the diameters of 100 microbeads at ×100 magnification using a calibrated micrometer scale and presented with standard deviation ($n - 1$).

The internal and external appearance of the alginate beads were also examined by scanning electron microscopy (SEM) using a ZEISS DSM 950 scanning electron microscope operated at 5 Kv.

Samples were chemically stabilized by immersion in 2.5% glutaraldehyde in 0.05 sodium cacodylate buffer, pH 7.4 for 2 h at 4 °C and were postfixed with 1.0% osmium tetroxide for 1 h at room temperature. Samples were then dehydrated with series of 50%–70% ethanol concentrations, and critical point dried with carbon dioxide, using a Polaron CPD 7501. Dried samples were frozen in liquid nitrogen and fractured. Samples were mounted on a carbon adhesive to the sample holders and were coated with gold. This method allowed the visualization of whole and fracture alginate microparticles, as well as details about the entrapped bacteria.

2.4. Enumeration of bacteria entrapped within microbeads

Entrapped bacteria were released from the beads according to the methods of Sheu and Marshall (1993). Briefly, one gram of beads was re-suspended in 10 mL of phosphate buffer (0.1 M, pH 7.0) followed by homogenization with an orbital shaker at 300 rpm. To ensure that bacteria were released, it was verified that microparticles were degraded by optical microscopy (data not shown). The number of colony forming units per gram of beads (CFU/g) was determined by plating on MRS agar and incubating at 37 °C for 24 h in anaerobic conditions. All samples were analyzed in triplicate. For the lyophilized microparticles the enumeration method was the same but the probiotic was previously homogenized by the addition of 0.5 g of the lyophilized microparticles in 50 mL of buffer and stirred for 30 min.

The encapsulation yield (EY), which is a combined measurement of the efficacy of entrapment and survival of viable cells during the encapsulation procedure, was calculated as follows:

$$EY = (N/N_0) \times 100$$

where N is the number of viable entrapped cells released from microspheres and N_0 is the number of free cells added to the biopolymer mix during production of the microparticles.

2.5. Stability assays

To examine the viability of the microencapsulated probiotics, the microparticles were stored at three different temperatures: 25 °C, 4 °C and −20 °C for 45 days. The number of viable cell counts was determined at 0, 1, 3, 7, 14, 28, 35 and 45 days using the method described in Section 2.4. All the samples were analyzed in triplicate.

Dried microparticles were also stored at 25 °C, 4 °C and −20 °C for 30 days, and the number of viable cell counts was determined at 0, 14 and 30 days using the same method.

2.6. Statistical analysis

Data were subjected to normal distribution tests (both *Kolmogorov–Smirnov and Shapiro Wilk*). Particle size was analyzed by a U Mann–Whitney test (Fig. 2). Results in Tables 1 and 2 were presented as mean ± Standard Deviation (SD) of replicated determinations (in logarithmic scale). Statistical significant differences were measured by Student's *t*-test. Data showed in Tables 1 and 2 were subjected to an independent *t*-Test and paired *t*-Test, respectively. Data from Fig. 4 were analyzed by means of two-way repeated measures ANOVA (time and temperature). Statistical significance was set at $p < 0.05$. All analysis were performed using SPSS version 19.0 for Windows (SPSS, Chicago, Illinois, USA).

3. Results and discussion

3.1. Microscopic examination of alginate beads

Optical microphotographs of microparticles showed a spherical shape, with a defined limit. Particles are isolated without adherence to each other. Fig. 1B shows particles more opaque than microparticles without probiotics (Fig. 1A) due to the presence of bacteria the presence of cell.

In contrast to other authors (Zou et al., 2011), we did not report rejected material such as filamentous masses of polymer, which could negatively affect the textural and organoleptic properties of food products in which the microparticles could be added (Anal & Singh, 2007). This result shows that the time and the shaking speed used in the elaboration of microparticles seem to be suitable for the diffusion of calcium ions through alginate gel which is a key step in the formation of the microparticles. White arrows in Fig. 1B show small and dark droplet into the particles which is due to the presence of residual oil used in the synthesis of microparticles.

Particle size distribution (Fig. 2) showed polydispersity and unimodal size distribution. The polydispersity may be explained because of encapsulation technology used and different amount of probiotic inside of beads (Zou et al., 2011). In the alginate formulation most of the microparticles had a diameter between 30 and 60 μm, whereas in the same formula with probiotics the mean size, was significantly lower 32, 15 ± 15 *vs.* 49 ± 17 ($p < 0.05$) with a higher percentage of microparticles in the range 0–30 μm. In contrast, the addition of starch to the alginate formulation increases the size of microparticles 70 ± 17 *vs.* 49 ± 17 ($p < 0.05$), with a significantly higher percentage in the range 60–90 μm. Probiotics also decrease significantly the size of microparticles in the starch formulation 26 ± 12 *vs.* 70 ± 17 ($p < 0.05$). To our knowledge, no other report has shown that probiotics decrease microparticle size. Further studies are needed to elucidate if this effect could be due to changes in the zeta-potential of microparticles. In contrast, the increasing effect described for the starch has been previously reported by Zou et al. (2011), who obtained a larger bead size of microparticles (178 μm), using the same emulsion-internal gelation technology. This may be attributed to the fact that the starch used by these authors, as well as the probiotic strain, are different from those used in our work. Interestingly, the particle size obtained is suitable for the application in food, since it has been reported that soft, rounded particles are not perceptually gritty up to about 80 μm (Lawless & Heymann, 2010).

Rosenberg and Sheu (1996) and Thu et al. (1996) reported that the internal organization of the microparticles, which includes the spatial distribution of the components, has implications for

Table 1
Number of cells entrapped compared to initial number of cells and encapsulation yield.[a]

Microparticles type	Initial number of cells (log CFU)	Microparticles weight (g)	Number of cell entrapped		Encapsulation yield (EY)
			Log CFU total	Log CFU/g	
Alginate	11.00 ± 0.10	31.0 ± 1.00	8.18 ± 0.26	6.69 ± 0.24	74.41 ± 1.76
Alginate blended with starch	11.30 ± 0.06	35.0 ± 3.61	10.99 ± 0.09*	9.44 ± 0.09*	97.26 ± 0.33*

*$P < 0.05$ *vs.* alginate.
[a] Values are mean ($n = 3$) ± standard deviation. Log CFU: colony forming units.

Table 2
Viability at different times and temperatures of microencapsulated *L. fermentum* in alginate blended with starch beads after freeze-drying process.[a]

Alginate blended with starch microparticles	Log CFU total post-freeze drying		
	−20 °C	4 °C	25 °C
0 days	11.17 ± 0.16	11.17 ± 0.16	11.17 ± 0.16
14 days	_	10.30 ± 0.00*	11.21 ± 0.11
30 days	8.66 ± 0.06*	8.51 ± 0.22*	0

*$P < 0.05$ *vs.* 0 days.
[a] Values are mean ($n = 3$) ± standard deviation. Log CFU: colony forming units.

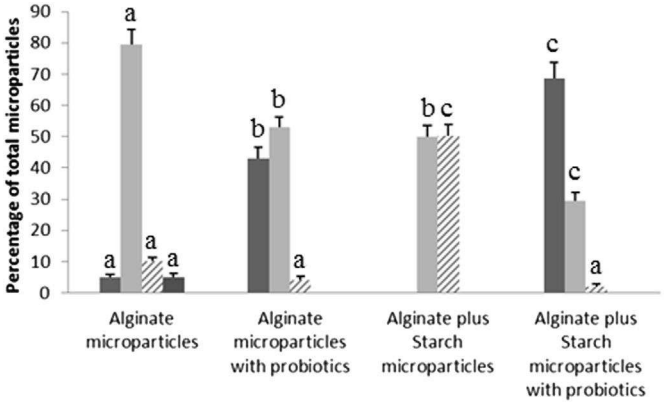

Fig. 2. Particle size distribution expressed as percentage of total particles. (■) 0–30 μm, (□) 30–60 μm, (▩) 60–90 μm, (■) >90 μm. Bars represent the average of percentages of particles with standard deviation. Bars with different letters are significantly different ($P < 0.05$ between different formulas).

microparticles properties and retention of microstructure. In order to study the surface, the integrity and porosity of the wall material, scanning electron microphotographs (Fig. 3) of fractured frozen microparticles were used.

According to the data obtained by optical microscopy (Fig. 1), SEM shows probiotic microparticles with spherical shape and smooth surface without pores (Fig. 3). Porosity of alginate is important in maintaining the viability of the bacteria as they move through the digestive tract (Allan-Wojtas, Truelstrup Hansen & Paulson, 2008). As a result a less porous matrix is more protective for the bacteria (Prakash & Jones, 2005; Thu et al., 1996). Microparticles with probiotics seem to be rougher than microparticles without bacterias. Moreover, beads without probiotics tend to adherence to each other which can explain the big size of these particles.

In addition, it can be seen that some of the bacteria presented on the surface of the particle are covered by a thin layer of polymer material, and some of them are also attached to the surface (Fig. 3A, B).

The inside of the microparticles was composed of a mesh-like network of alginate, through which bacteria were distributed, sequestered in voids (Fig. 3C, D). These pictures show that the presence of the bacteria during gelation seems to cause local changes in the gelation process with the occurrence of the 'void space' phenomenon also observed in fermented dairy products. This phenomenon consists of the formation of cavities in the matrix because of the presence of bacteria in these spaces (Allan-Wojtas et al., 2008).

3.2. Number of cells entrapped

Viability of probiotics was evaluated for every formulae, to identify the most effective encapsulating method. Table 1 shows the number of cells entrapped in the formulae with alginate and in the formulae with the mix of alginate and starch.

The results indicate that microencapsulation in alginate beads with starch was more appropriate for microencapsulation of *L. fermentum* CECT5716 than the same technique without starch, since the reduction of viability of probiotics during the microencapsulation process was significantly lower. Thus, the EY for viable cells was significantly higher ($p < 0.05$) for microparticles blending with starch 97.26 ± 0.33% as compared to microparticles without starch 74.41 ± 1.76%. A previous report showed less survival with the same technique and a resistant starch (Zou et al., 2011), in which the EY for *Bifidobacterium bifidum* was 50.3 ± 2.3%. On the other hand other techniques such as extrusion (Chávarri et al., 2010) and coacervation (Annan, Borza, & Truelstrup, 2008) found lower EY compared to our results. However, it should be taken into account that not all probiotics have the same properties and thus, the results of comparison of different reports with different bacteria is limited, even in the case of using the same microencapsulation technique.

Although more studies would be needed to better characterize the mechanism of microencapsulation, the better protection of probiotics during the microencapsulation process with the mix of alginate and starch, could be attributed to the effect that corn starch had in the stabilization of the alginate microparticles (Tal, Van Rijn, & Nussinovitch, 2000). During particle formation, starch favors the formation of polymeric networks where the cells are entrapped and partially isolated from the environmental conditions. Although more studies will be needed to elucidate this mechanism, it is similar to that previously proposed (Heidebach et al., 2012; Klayraung et al., 2009). Kailasapathy (2006) indicate that the addition of starch to alginate protect probiotics from bacteriophages, thus increasing probiotic survival during acid challenge. The mixture of starch and alginate is used as a technological additive in the production of yogurt, ice cream, frozen dairy desserts,

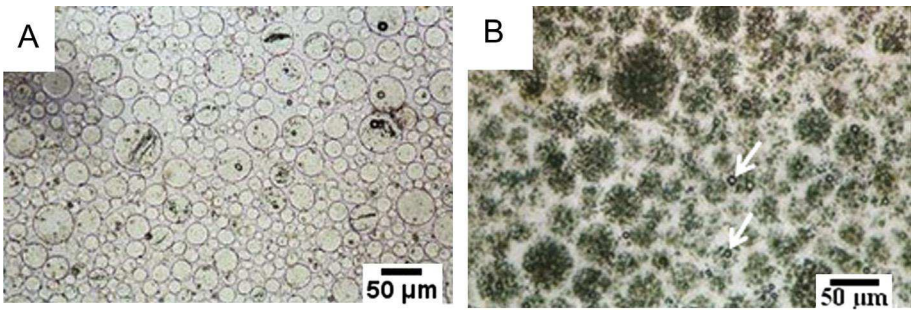

Fig. 1. Optical microphotographs of calcium alginate blended with starch microparticles. (A) Microparticles without *L. fermentum*. (B) Microparticles with *L. fermentum*. Particles in figure B are more opaque than in figure A. White arrows in figure B show residual oil drops.

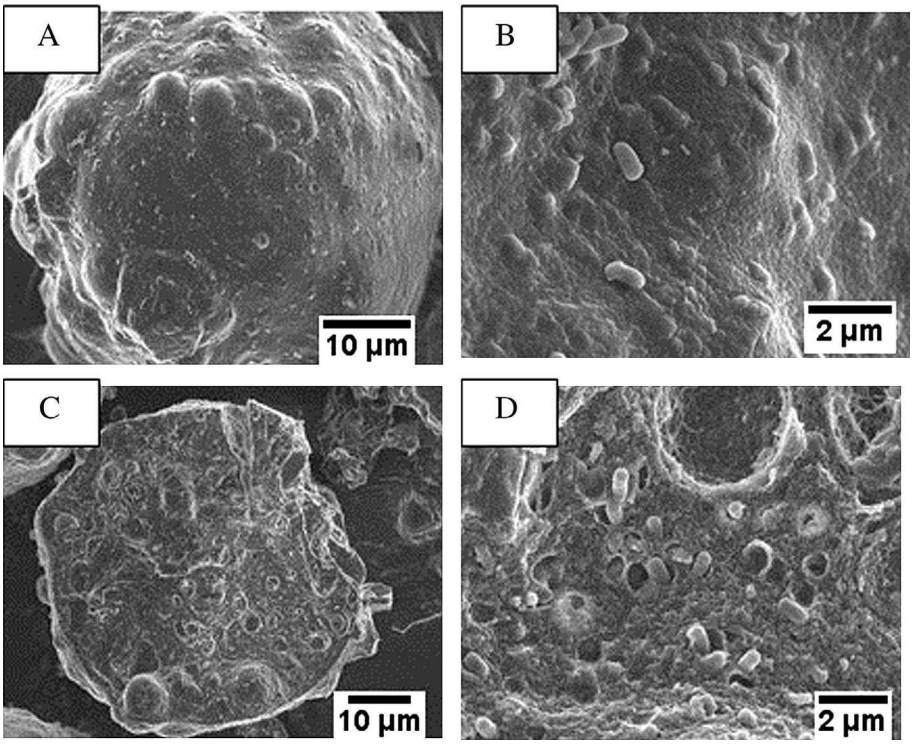

Fig. 3. SEM images of alginate and starch beads with *L. fermentum*. (A) Surface of a microparticle. (B) Surface of a microparticle at high magnification. (C) Fractured alginate and starch microparticle loaded with *L. fermentum*. (D) Fractured alginate and starch microparticle loaded with *L. fermentum* at high magnification.

and mayonnaise. Starch and alginate tend to be synergistic in gelling and allow sufficient diffusion of nutrients and metabolites to maintain growth of encapsulated cells. In addition, starch is a commonly used food stabilizer in the manufacture of yoghurt to prevent syneresis, so its biocompatibility with the bacteria and its toxicity have been widely tested.

The release of probiotics from alginate capsules is outside of the scope of this study. Although some authors have reported that alginate capsules are dissolved in vitro or ex vivo (Iyer, Kailasapathy,& Peiris, 2004; Iyer, Phillips, & Kailasapathy, 2005; Mandal, Puniya, & Singh, 2005), this has not be confirmed in vivo. In fact, Van Venrroy (personal communication, 2004) reported that alginate-microcapsules remained intact during the gastrointestinal passage and did not release the encapsulated probiotic bacteria being excreted unchanged in feces. More studies are needed to elucidate if the alginate microparticles obtained in the present study are able to release the probiotics in vivo.

3.3. Stability assays

Due to the better results obtained with the mix of alginate and starch, this formula was chosen for further stability assays. As shown in Fig. 4, the viability of the bacteria at room temperature (RT) has decreased by 1.7 log after 24 h and the loss of viability reached 100% after 2 weeks at RT. These results are probably due to the humidity which has been widely described as a deleterious factor in the survival of anaerobic bacteria.

In contrast, the viability of the probiotics was better at 4 °C and at −20 °C. The number of microencapsulated cells remained relatively constant after 45 days of storage at 4 °C. Using the extrusion technology with a mix of *Lactobacillus gasseri* and *B. bifidum* Chávarri et al. (2010) found a loss higher than 2 log after one month. Whereas Lee, Cha, and Park (2004) found a loss of 0.8 log after 28 days using *Lactobacillus bulgaricus*. Kailasapathy (2006) using a mix of *Lactobacillus acidophilus* and *Bifidobacterium lactis* encapsulated by external gelation found a loss of 1.15 log after 5 weeks. As mentioned above, variability in the bacteria used for microencapsulation could also be involved in the differences observed in the stability.

The viability of probiotic decreased in 0.8 log after 45 days of storage at −20 °C. It could be expected a better survival at −20 °C than at 4 °C, but our results could be explained by the detrimental effect of crystallized water, formed during the freezing process, on the bacteria wall. However, survival of probiotics was still 91% after 5 weeks of storage at −20 °C. In contrast, Sheu, Marshall, and Heymann (1993) observed that the viability after 14 days remains only around 50% using external gelation and *L. bulgaricus*.

From these results it can be suggested that the application of a freeze drying process previous to the storage seems to be a good alternative in order to eliminate the residual water, which could be beneficial for maintaining the viability both at RT and at −20 °C.

With this purpose beads were freeze-dried and the viability of bacteria was evaluated. No significant differences ($p > 0.05$) were

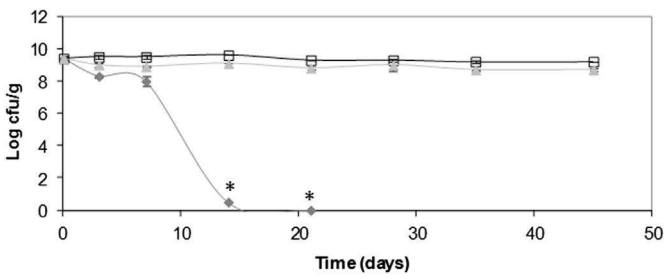

Fig. 4. Survival of microencapsulated *L. fermentum* in alginate blended with starch beads at different temperatures and times (◆) 25 °C, (□) 4 °C, (▲) −20 °C. *$P < 0.05$ *vs.* 4 °C and −20 °C.

found between dried particles and wet particles, thus suggesting that the freeze-drying process does not affect the viability of microencapsulated *L. fermentum* CECT5716. In contrast, the results obtained by Sultana et al. (2000), and Zárate and Nader-Macias (2006) showed that the viability immediately after freeze-drying declined between 0.05 and 2 log, and 3.4 log respectively. However, the viability during storage of lyophilized microparticles decreased in 2.5 log after 30 days at both 4 °C and −20 °C and there is no survival at 25 °C (Table 2). It is surprising that the stability during storage at 4 °C was worse in lyophilized microparticles compared to those non-lyophilized, since it is suppose that elimination of residual water during freeze-drying could improve it. Burgain et al. (2011) indicated that starch improved culture viability during drying and storage but the starch used in our work may not have been able to protect cells from injury. Probiotic organisms are sensitive to freeze-drying due to deterioration of the physiological state of the cells, so the process of drying without protective agents resulted in an almost complete inactivation of bacteria. Further studies are needed to elucidate if the addition of a cryoprotectant could improve the survival of lyophilized microparticles through time.

4. Conclusion

These results show that *L. fermentum* CECT5716 resists the process of microencapsulation in alginate beads with starch, and the resultant microencapsulated probiotic preparation is stable at 4 °C and, in a lesser extent, also at −20 °C.

We have also shown a significant effect of starch and probiotics in the microparticle size. The addition of the probiotic strain decreases the microparticle size in both formulations (with and without starch), thus leading to a product suitable for application in food.

In conclusion, ionic internal gelation technology with alginate and starch seems to be a suitable procedure for protecting *L. fermentum* and could allow the addition of this probiotic to food and pharmaceutical preparations.

More studies are needed to elucidate whether the addition of technological coadjuvants, such as, cryoprotectants, would be suitable to obtain a good survival of the microencapsulated probiotic through time. Future works will also be aimed at studying the functionality of the microencapsulating probiotic in a final matrix and whether it improves survival through gastrointestinal tract.

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I.4. Survival of *Lactobacillus plantarum* WCFS1 to encapsulation by spray-drying with different carriers and the effect of infant formula as food matrice

Estudio de la viabilidad de *Lactobacillus plantarum* WCFS1 encapsulado mediante *spray-drying* utilizando diferentes materiales, así como el efecto de su incorporación a una fórmula infantil como matriz.

Cuando incorporamos probióticos a una matriz alimentaria, el principal problema que se presenta es la escasa resistencia de estos microorganismos, a diferentes condiciones ambientales y tecnológicas. En este sentido, diferentes estudios demuestran que las técnicas de microencapsulación son un buen método para protegerlos, sin embargo no todas las técnicas son apropiadas. Por ello, el objetivo fundamental de este estudio ha sido evaluar la adecuación del *spray drying* como método de microencapsulación de *Lactobacillus plantarum,* usando tres tipos diferentes de material de recubrimiento: leche en polvo y dos medios prebióticos (inulina+ FOF y maltodextrina). El tamaño de partícula obtenido, osciló entre 10.7 y 33.3 μm. La estabilidad de las bacterias microencapsuladas fue estudiada a temperatura ambiente. Se observó que las micropartículas elaboradas con leche en polvo, mostraron los mejores resultados de viabilidad a dichas condiciones de conservación (8.01±0.11 logaritmos de unidades formadoras de colonias). Por ello, estas micropartículas fueron incorporadas a una matriz alimentaria, en concreto, una fórmula infantil. En este caso, la viabilidad del probiótico descendió 1 logaritmo de ufc. En definitiva, no se observan diferencias estadísticamente significativas entre las partículas y las micropartículas incorporadas a la fórmula infantil durante su almacenamiento a temperatura ambiente durante 60 días.

Este trabajo se encentra actualmente bajo revisión en Journal of Functional Food

Survival of *Lactobacillus plantarum* WCFS1 after spray-drying using different carriers and their effect after incorporation into an infant formula

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Abstract:

When incorporating probiotics into a food matrix, the main problem is their low resistance to environmental and technological conditions. Different studies have probed that microencapsulation is a good way to protect probiotic. However, not all techniques are adequate for probiotics. The aim of this study was to evaluate the feasibility of spray drying to produce dry preparations containing *Lactobacillus plantarum* using three different carriers: skimmed milk, and two prebiotic media (inulin+FOS and maltodextrin). Particle size ranged from 10.7 to 33.3 μm. Moreover, particles show a spherical shape. The stability of encapsulated bacteria was tested at room temperature. The best viability was reported for the particles elaborated with skim milk (8.01±0.11 log CFU). These particles were introduced into an infant formula. The viability of probiotic in infant formula decreased around 1 log. No significant differences were reported between particles and the mixture of particles and infant formula stored during 60 days.

Key words: spray drying, probiotics, maltodextrin, skim milk, inulin, oligofructose.

1. Introduction:

Probiotics are live organisms which, administered in adequate amount, are associated with beneficial health effects, and may be selected for prevention and treatment of diseases (FAO/WHO 2001). This fact has stimulated interest in the inclusion of probiotic in food products to improve health for the general population.

The majority of probiotic species applied in functional food industry are lactobacilli like *L. casei* and *L. rhmanosus* and bifidobacteria (Holzafpel and Schillinger 2002). These organisms are very susceptible to food processing conditions such as spay drying, which is a very common operating unit for foods suitable for probiotic application. Within this context, microencapsulation of probiotic is currently drawing more and more attention as a possible alternative to improve the stability of probiotic organisms in food products, during both processing and storage until the end of the shelf life. However, not all techniques of microencapsulation are suitable.

Freezing and freeze drying are the most common formulation technologies for probiotics, nevertheless the use of spray drying would have several industrial advantages such as scale, costs and process continuity. The survival under standard spray drying settings is however very poor. Its application to generate probiotic preparations has received considerable interest. The key stress factor for survival during freeze drying is the high processing temperature encountered (~80°C). These high temperatures are needed to evaporate the water during the passage of bacteria through the drying chamber. Cell membrane damage is often evident following spray-drying, and this has been attributed primarily to the effects of heat and dehydration. Others parameters that affect the survival of bacteria during spray-drying and subsequent during storage include the drying conditions, species, strain, and the carrier agent used (Desmond, Ross, O´Callaghan, Fitzgerald, & Stanton, 2002).

In this sense, reconstituted skimmed milk is an encapsulating agent that has shown a favorable effect on the improvement of cell survival during the spray drying process REF). Another approach to increase the viability of probiotic is the use of prebiotic. Prebiotics reduced the moisture content and the thermal analysis suggests a higher stability of these kinds of particles. Fritzen- Freire, Prudêncio, Amboni, Pinto, Negrão-Murakami, & Murakami, (2012) evaluated the viability of *Bifidobacterium* BB12 microencapsulated by spray drying using a mixture of skim milk plus inulin and oligofructose. These microcapsules showed high initial count and good protection of the

probiotic during storage. Moreover, prebiotic are nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and or activity of bacteria in the colon. It will increase the host´s natural resistance to invading pathogens (Cummings and Macfarlane 2002)

Furthermore, maltodextrine can also potentially acts as a prebiotic as well as a microencapsulating agent during spray drying. In addition, maltodextrine can reduce the caking and stickiness to the walls and increase the free flowing nature of the spray dried powder (Anekella, & Orsat, 2013).

As it mentioned before, a growing interest has arisen in the inclusion of probiotic in foods. Another important point is to improve the viability of probiotics during long term shelf life (months) at ambient temperatures (Golowczyc, Gerez, Silva, Abraham, De Antoni, & Teixeira, 2010). The food matrix encapsulation must act as a buffer during storage. The probiotic microorganism present in food should survive in significant numbers, this number vary from strain to strain (Anekella, et al., 2013). Cell survival during shelf-life is particularly affected by storage temperature and the presence of atmospheric oxygen (Weinbreck, Bodnár, & Marco, 2010).

This is of special relevance in infant formulas. Breastfed infants develop a probiotic-rich gut microbiota with less pathogenic bacteria, compared with formula-fed individuals. Probiotic bacteria present in human milk (Lara-Villoslada et al. 2009) could play a role in the beneficial effects of breastfeeding and for this reason infant formulas are actually supplemented with probiotics.

The objective of the current study was (i) to evaluate the feasibility of spray drying to produce dry preparations containing *Lactobacillus plantarum* using three different drying media: skim milk, and two prebiotic media(inulin+FOS and maltodextrine), (ii) to investigate the stability of bacteria encapsulated in these different carried at room temperature, (iii) characterize the microcapsules in relation to their physical properties, (iv) finally, the best carriers were selected and the survival of probiotic introduced into a food matrix (infant formula) was evaluate.

2. Materials and Methods

2.1. Materials

Lactobacillus plantarum WCFS1 was obtained from NIZO Collection. Cells were grown in MRS broth (de Man, Rogosa, and Sharpe (Sigma-Aldrich (Madrid, Spain).

Culture was maintained in glycerol (20%) stocks at -40°C. The carrier solutions used were skim milk powder, oligofructose-enriched inulin (Orafti® Synergy 1, Orafti, Tienen, Belgium), which is a 1:1 mixture of oligofructose (DP 2-8) and long-chain inulin fraction(DP 10-60) and maltodextrine 19 (MD19) (Syral, France).

2.2. Microencapsulation by spray drying

2.2.1. Preparation of bacterial suspension

WCFS 1 was inoculated directly from a frozen stock in MRS, and incubated overnight at 37°C to stationary phase. OD600 was measured. Cells were harvested by centrifugation and resuspended in carrier solutions normalized to OD600 of 1.0.

2.2.2. Preparation of drying media

Three feed solution were prepared following the procedures described by Fritzen-Freire,et al. (2012). All carriers: Skim milk, FOS and maltodextrine were prepared in a concentration of 200g/L. All the media were homogenized pasteurized at 80ºC for 30 min.

2.2.3. Spray drying

The spray drying process was performed in a laboratory scale spray dryer (Buchi B 290 mini spray dryer, Flawil, Switzerland). The spray dyer was allowed to reach uniform process temperature prior to the start the process. The outlet temperature was adjusted to 70 ± 2º C. Ice water was continuously used to cool the nozzle. The dried powder was collected in a cyclone separator and thoroughly mixed with a spatula. The samples were placed in sterile plastic bottles and store at RT. Three different microcapsules were obtained using the drying media mentioned in section 2.2.2. S1 (200g /l Skim milk), S2 (200 g /L of Maltodextrine 19), S3 (200 g/l of oligofructose- enriched inulin).

2.3. Differential scanning calorimetry analysis (DSC)

The DSC curves of the microcapsules were obtained using a Mettler FP 85 T A cell monitored by Mettler FP 80 central processor unit (Mettler Toledo, Barcelona, Spain). Samples of approximately 5 mg of powder were placed in aluminum sealed pans, and heated from 30 ºC to 300 ºC at a heating rate of 5 ºC/min^{-1}. The DSC equipment was preliminarily calibrate with a standard reference of indium.

2.4. Moisture content

The residual moisture contet of microcapsules was determined using the Sartorious MA 35 (Sartorious Mechatronic, Germany) gravimetric analyzer. Approximately 2 g of powder was spread on an aluminium pan and put in the moisture content analyzer, which heated the powder until the mass equilibrated to a final value. This involved determination of the difference in weight before and after drying, expressed as a percentage of the initial powder weight.

2.5. Enumeration of probiotic after spray drying

To determine the survival rate of probiotic bacteria, dried samples were rehydratated with steril phosphate buffer (pH 7.0). To release cells from the microcapsules, samples were placed in a shaker (RT, 300rpm /30min). Afther that, samples were serally diluted and drop plated in duplicate on MRS agar. Plated were placed in an anaerobic jar and incubated at 37º C for 24 hours.

The encapsulation yield (EY), which is a combined measurement of the efficacy of entrapment and survival of viable cells during the encapsulation procedure, was calculated as follows:

$EY = N/N_0 \times 100$

Where N is the number of viable entrapped cells released from microspheres and N_0 is the number of free cells added to the dried media before spray drying process.

2.6. Particle size and morphological analysis

Particle size distribution was examined by light diffraction using Mastersizer 2000 (Malvern Instruments Ltd., Malvern, UK) with a size range from 0.02 to 2000 μm yielding the volume distribution of the particles. Characterization parameters were the diameters D(0,1), D(0,5) and D(0,9), that are the particle diameters determined at the 10th, 50th and 90th percentile of the undersized particle distribution curve. Also the mean diameter over the volume distribution (D(4,3)) and polydispersity expressed as the Span factor were calculated (equation 1). A high value of SPAN indicates a wide distribution in size and a high polydispersity. Scanning electron microscope (Hitachi, S-510, Tokyo, Japan) was used to evaluate the morphology and corroborate the particle size. Spray-dried powder were attached to brass stubbs and gold coated using deposition

parameters of 0.03 sputter amps for 30 s. Caoted perparations were visulaized with a scanning electron microscope using an acceleration voltage of 25 kV.

Span= D(v, 0.9)-D(v,0.1)/D(v,0.5)

2.7. Storage test

Viability of the probiotic strains was determined on the day of powder manufacture and during powder storage for 60 days at room temperature, using the same methodology described in section 2.5.

The viable bacteria number was expressed in CFU/g of microcapsules. The storage inactivation data were expresed as logarithmic value. N refers to the bacterial count at a particular storage period, whereas N_0 represents the bacterial count at the beginning of the storage and t is the storage time in days. The viability loss during storage was assumed to follow first-order reaction kinetics (Ying, Sun, Sanguansri, Weerakkody & Augustin, 2012):

$$\text{Log } N_t = \log N_0 - R_m t$$

where, R_m is the absolute value of the regression coefficient of each regression line. The value R_m was defined as the rate of mortality (Chávez & Ledeboer, 2007)

2.8. Viability of microencapsulated probiotic incorporated into food

Microparticles with the best viability (skimmed milk and maltodextrine particles) were mixed with an infant formula (Nutrilon Standaard, Nutricia, The Netherlands). The initial concentration of microorganisms in the supplemented formula was around 10^7 colony-forming units (CFU) per g of formula powder. Viability of the probiotic strains was determined on the day of mixture was made and during powder storage for 60 days at room temperature, using the same methodology described in section 2.5.

2.9. Statistical analysis

The mean values and the standard deviations were calculated from the data obtained with duplicate trials. Statistical differences were analyzed by using an analysis of variance (ANOVA) followed by the Duncan´s multiple range method. Statistical significance was set at $p < 0.005$. All analyses were performed using Statgraphics version 5.1 for windows 7.

3. Results

3.1. Differential scanning calorimetry analysis (DSC)

It is desirable to keep the cell membrane in the liquid crystalline phase upon drying to reduce viability loss. In this sense, carriers can help to maintain this condition. Perdana, Fox, Siwei, Boom,& Shutyser (2014) indicated that the protective effect of a carrier is correlated with a high glass transition temperature. Vitrification can still occur at higher outlet temperatures providing stability to bacteria when enclosed in a glassy matrix. In this regard, the suitability of the different carriers was tested using a differential calorimetric analysis.

The DSC graphs of maltodextrine 19 (MD19), skimmed milk and Inuline:FOS are shown in figure 1. The MD19 curve exhibited a very broad endothermic effect, between 35 ºC and 130º C, which reached a minimum at around 90.3 ºC. Furthermore, Inulin: FOS also show a broad endothermic effect.In the case of skimmed milk a broad endothermic effect was reported between 35 ºC and 130 ºC, which reached a maximum around 87.1 ºC.

The results obtained show that all the carriers tested have higher glass temperatures (≈ 90 ºC) than the outlet temperature used in the experiment (80 ºC). In this sense, bacteria will be enclosed in a glassy matrix in every carrier, giving increased protection

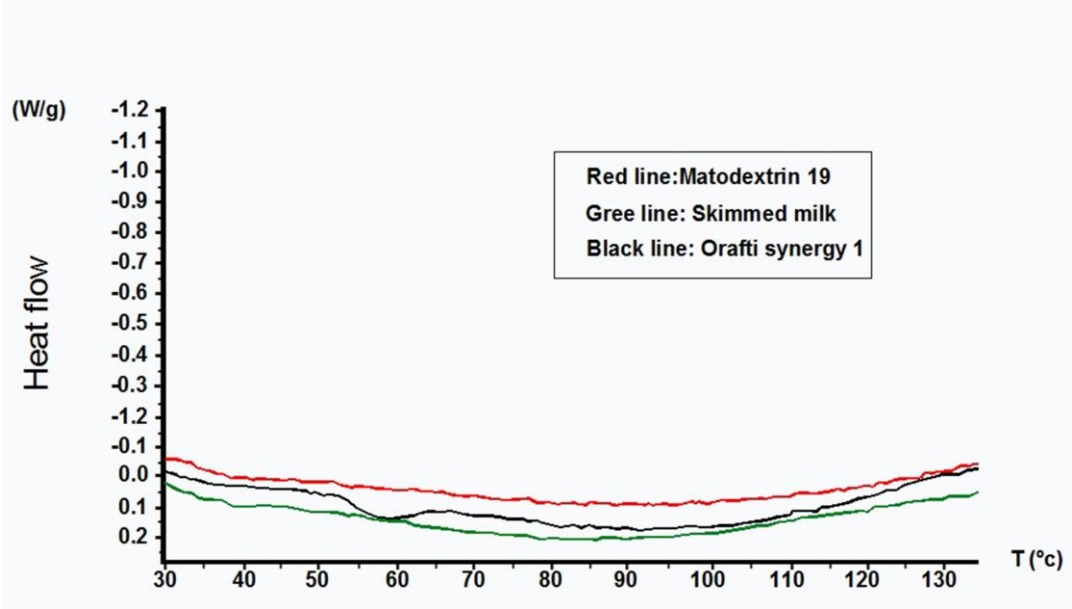

Figure 1. Differential scanning calorimetry analysis of skimmed milk, Maltodextrine 19 and Orafti synergi 1

3.2. Moisture content

Water content is an important parameter for the stability of dried cultures. In general, microorganisms survive better at low-water activity. Simpson (2005) indicates that the moisture content has to be less that 4% H_20/ g particle and Chavez an Ledeboer suggest that has to be below 5 % in order to be categorized as stable product. However, overdrying may diminish the viability and stability of microorganisms. Besides, it is reported that optimum residual moisture content varies with the composition of the fluid in which the microorganism are dried, with the storage atmosphere and with the species of organisms (Wang, Yu, Chou, 2004). Table 1 shows the moisture content of particles. It is ranged from 3.6 to 5.4 %. In concordance with Fritzen- Freire, et al. (2012) inulin: FOS microparticles have low moisture content. These authors suggest that these prebiotics are able to reduce the moisture content. Moreover, these particles are the biggest one. Tonon, Brabet, Pallet, Brat & Hubinger, (2009) indicate that big particles exposed low surface area and consequently adsorb less water from the ambient air.

Carrier	Initial number of cells (Log CFU)	Number Of cell entrapped log CFU/g	EY	Moisture content
Skimmed milk	9.28±0.12	8.01±0.11	5.94±0.90	5.4±0.1
Inulin: FOS	8.57±0.18	5.69±0.06	0.12±0.01	3.6±0.1
Maltodextrine 19	9.23±0.12	6.73±0.09	0.31±0.07	5.2±0.1

Table 1. Number of cell entrapped compared to initial number of cells, encapsulation yield and moisture content.

3.3. Enumeration of probiotic after spray drying

The suitability of different drying media was evaluated. The total solids content of the spray drying medium was held constant at 20 % (w/v). Substantial differences between the evaluated media could be observed regarding their protection capacity against thermal effects upon dehydration at an outlet temperature of 70 ºC. This outlet temperature was selected taking into account the results obtained by Perdana et al., 2013. These authors found a high viability of *Lactobacillus plantarum* WCFS1 using outlet temperatures around 70ºC.

Skimmed milk microparticles show the best viability after spray drying, when compared to the microcapsules produced with inulin: FOS or maltodextrine (Table 1). In contrast with the results obtained by Perdana et al., (2013), a large mortality occurred in all of

the cases. This fact is illustrated by the low EY (<1%) for all the carriers tested. This may be attributed to the different devices used in the experiments. Perdana et al. (2013) used a Buchi B-190 spray dryer equipped with a two-fluid nozzle meanwhile in this experiment a Buchi B 290 mini spray dryer was used. These authors also use different carriers and conditions (time and volume).

By comparing the results with Fritzen- Freire, et al. (2012), inulin:FOS was not able to protect probiotic. The carrier used by these authors is a mixture of skimmed milk and inulin:FOS and their result of probiotic survival is higher, thus suggesting a protective effect of skimmed milk. According to our results skimmed milk confers a better protection than inulin:FOS.

3.4.Storage test

Since the effectiveness of probiotic consumption is related to their viability, it is of utmost importance not only to minimize cell death during the spray drying process but also to ensure minimal loss in the viability of the dried bacteria during storage.

The three media were compared for the effect on the viability of *Lactobacillus plantarum* during prolonged storage at 25 ºC. Figure 2 shows the loss of viability during storage at 25ºC.

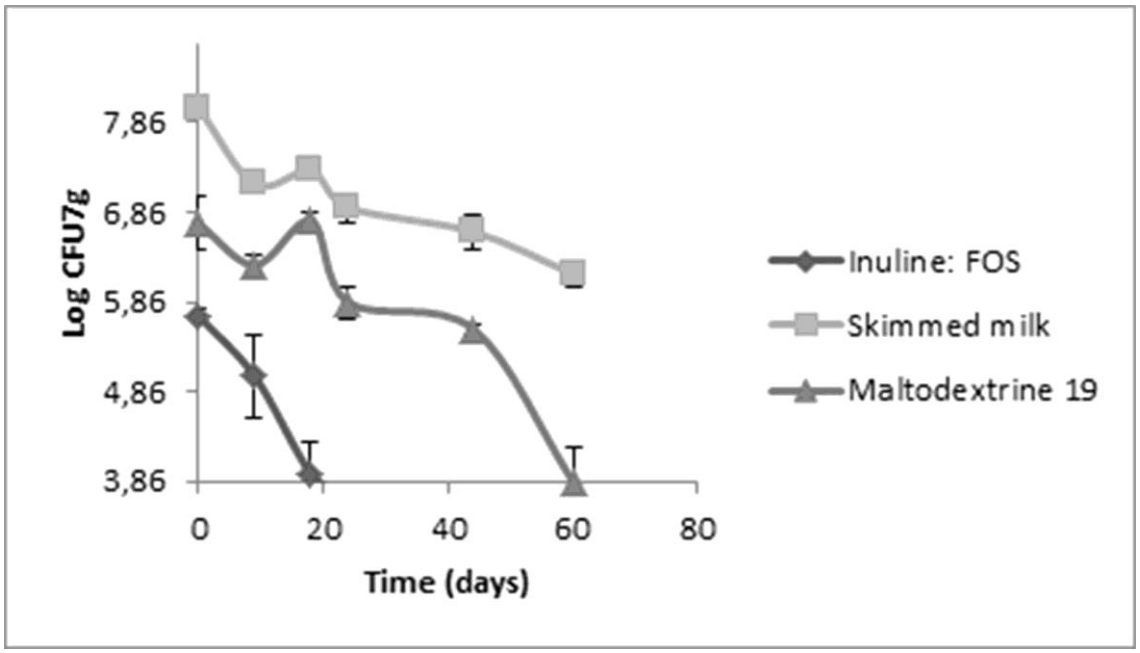

Figure 2. Viability of encapsulated probiotic at 25ºC during 2 months.

The decline of the bacterial load was represented by the logarithmic values of the survival fractions after different storage periods. This observation is quantitatively described in section 2. R_m was calculated over a period of 60 days of storage at room temperature and is shown for each dried media. This constant expresses the ability of carriers to preserve cellular architecture and to keep bacteria in a dormant state without metabolic activity, that is, to function as a barrier to diffusion of moisture and of present oxygen, as well as to from a glassy state material. R_m is therefore useful to predict the potential and limitations of a carrier for a long term protection, or to estimate the required initial load of aviable probiotic. To obtain the rate of Lactobacilli inactivation during storage, the regression line was determined from the plot of the common logarithm of the residual lactobacilli count (log10cfu/g) versus storage period (month) for each drying media temperature. The absolute value of the slope (regression coefficient) determined from the regression line was used as the inactivation rate constant (log10cfu/g/month) only when the correlation coefficient (R^2) of the regression line was greater than 0.75 ($R2 > 0.75$). Regression coefficient (R^2) value refers to the correlation of the points on the line of best fit, 1 being a strong positive correlation and 0 being no correlation between the points (figure 3).

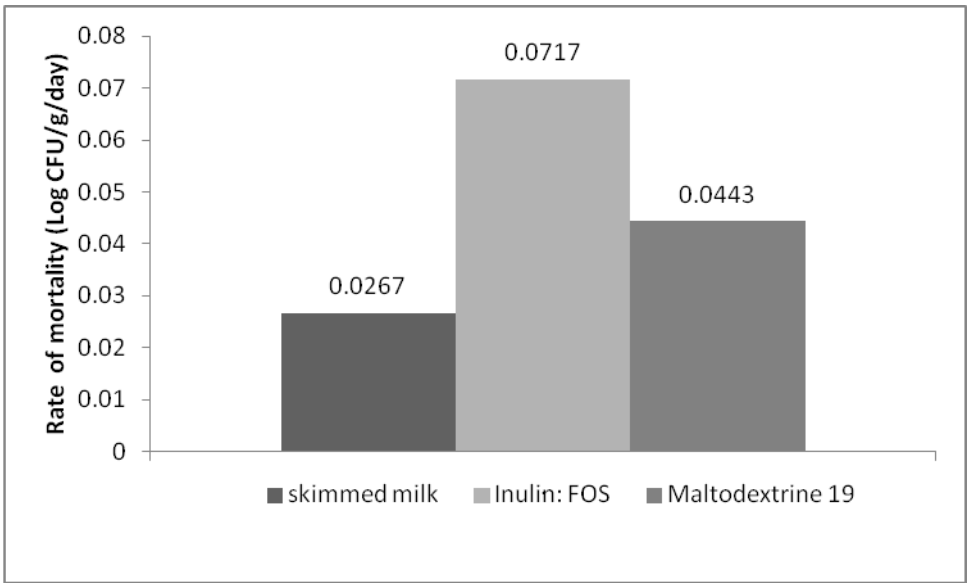

Figure 3. Rate of mortality of microencapsulated probiotic

The results of this study are in line with Chávez and Ledeboer, (2007) who found an exponential relationship between storage time and viable counts of lactobacilli. Skim milk microcapsules show the high survival for a period of 60 days (R_m= 0.0267). The count of viable probiotic cell was above the recommended levels for probiotic food

throughout the whole storage time (greater or equal that 6 log CFU /g) in this case. This fact suggests that skim milk had a positive effect on the protection of *Lactobacillus plantarum* during the encapsulation process. In the light of the water replacement theory, skim-milk constituents seemed to be superior in directly interacting with the polar headgroups of membrane phospholipids, and thereby reducing the damage on the cellular membranes during spray drying and prolonged storage. Principally, disaccharide was regarded as being effective in protecting both bacterial membranes and proteins during drying. It is thought that lactose might play a dominant role in bacterial protection by means of direct interaction with sensitive biomolecules. However, Perdana et al. (2014) found that lactose crystallization negatively affect viability during drying. The positive effect in the viability of probiotic can be related to another component of the skim milk such as the protein.

Several hypotheses have been proposed to explain the effect of these protectors on the process of cell dehydration. Among these, the presence of compatible solutes, such as sugars, quaternary amines and some amino acids, may equip the cells against hyperosmotic stress, which occurs during the drying process, bearing a direct relationship between the accumulation of these solutes and survival under adverse conditions (Kets et al., 1996).

According to Santivarankna et al. (2008), it is believed that the protective effect of these solutes occurs due to their preferential exclusion, which is the main mechanism of protection of macromolecules against moderate loss of water. Still according to the same authors, the preferential exclusion occurs where a macromolecule has greater affinity with water than with the solute, with the consequence of the latter becoming in excess on the surface of the macromolecule

Reconstituted skim milk (RSM) is another carrier matrix utilized in the production of dehydrated probiotics. In studies investigating the protective effect of different food systems in the survival of cells subjected to drying, the results obtained with RSM stood out in comparison to those yielded by other compound such as gelatin and starches (Lian et al., 2002). Fu & Chen (2011) suggest that their effective protection may be related to the presence of lactose in the composition of RSM, since this is the disaccharide that interacts with the cell membrane and helps to maintain its integrity in a similar way as non-reducing disaccharides do with trehalose and saccharose. However, this mechanism has not yet been totally elucidated as yet. In another way RSM might

create a porous structure in the lyophilized product, which would make its rehydration easier (Carvalho et al., 2004).

3.5. Particle size and morphological analysis

Microphotographs of microparticles showed a spherical shape, with a defined limit. It can be observed concavities typical of materials produced by spray drying. Some particles are also adhered to each other (Figure 4).

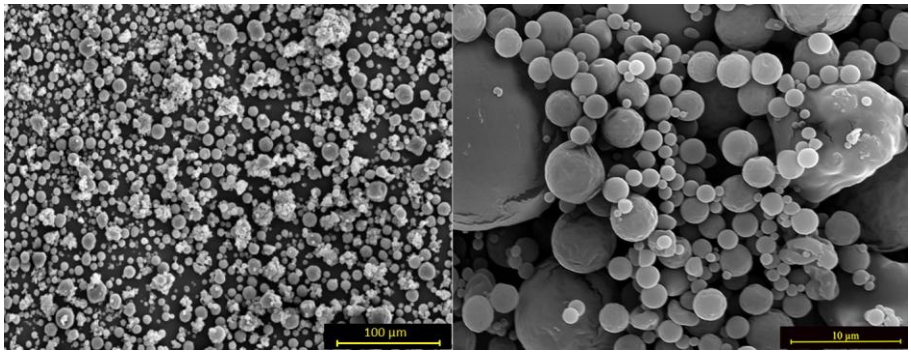

Figure 4. SEM microphotograph at different magnification

Particle size distribution (table 2) showed polydispersity, indicated by the high values of SPAN and bimodal size distribution. Spray-dried particle sizes ranged from 10.7 to 33.3µm. This particle size is suitable for the application in food, since it has been reported that soft, rounded particles are not perceptually gritty up to about 80 µm (Lawless & Heymann, 2010)

Carrier	D [4, 3]µm	SPAN
Skimmed milk	22.3	2.3
Inulin: FOS	33.3	2.0
Maltodextrine 19	10.7	2.0

Table 2. Particle size and SPAN factor

3.6. Incorporation into food matrices

An infant consuming approximately 800mL/day will ingest about $1x10^5$-$1x10^7$ commensal bacteria while suckling. In acordance to Weizman, Ghaled, & Alsheikh (2005) the concentration of microorganims in each supplemented formula was around $1x10^7$colony-formin units (CFU) per g of formula powder. Since the best results were obtained with skimmed milk these particles were chosen for infant formula enrichment at a concentration of $1x10^7$ cfu/g. The viability of probiotic bacteria in infant formula

decreased around 1 log during 30 days and 2 log in 60 days, which resulted in a final concentration of $7.34x10^5 \pm 7.57x10^4$ cfu/g, which is not acceptable, since the quantity to be consumed by the infant to achieve what is considered an optimal dose is 10^9cfu/day. In order to analyze the efect of the food matrice on encapsulated probiotic viability, we compared the results obtained with the particles with those of the infant formula. As shown in figure 5, there were no significant differences between both ($p>0.05$), thus suggesting that infant formula do not confere an additional protection to encapsulated probiotic in skimmed milk microparticles. This is probably due to the fact that infant formulas are mainly composed by milk protein and lactose, wich are also the main components of skimmed milk.

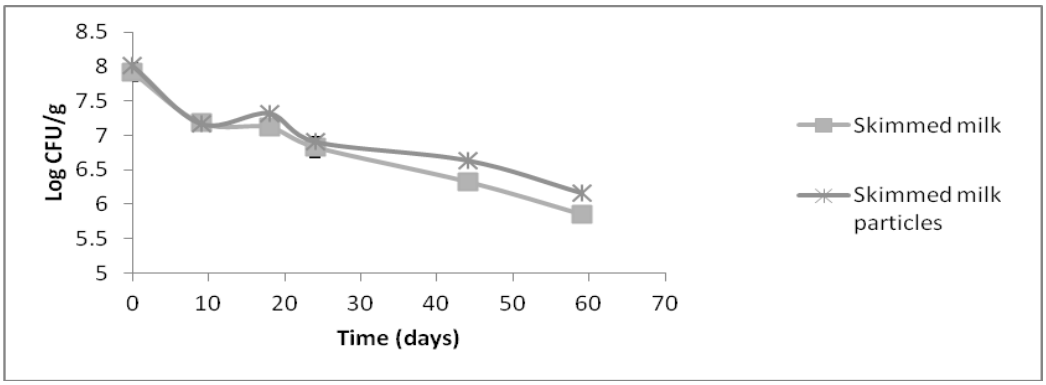

Figure 5. Viability of skimmed milk encapsulated probiotic mixed with the infant formula in comparison with skimmed milk particles at 25ºC during 2 months.

4. Conclusions

Comparing the three carrier system tested in this study showed that skimmed milk showed the best potential to protect probiotics. Overall, the low storage stability of probiotic bacteria in the different carrier tested at non-refrigerated temperatures showed that the bacteria were not sufficiently protected. This evidence justified the need to find suitable medium for the large-scale production of shelf-stable spray-dried probiotic bacteria. Further experiments are needed to further analyze the best carrier for spray-drying and to evaluate the retention of the functional characteristics of probiotic microencapsulated.

In addition, next studies are under way to validate the effectiveness for protecting sensitive probiotic bacteria in other food systems such as yogurt. In this respect, formulating and enriching food with probiotics would not only improve public health but also the diversity in food choices

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CAPÍTULO II.

Diseño y elaboración de comprimidos de liberación modificada con probióticos

II.1. Estudio teórico de formas farmacéuticas sólidas orales: comprimidos

III. 1. COMPRIMIDOS

Los comprimidos son formas farmacéuticas sólidas de dosificación unitaria, obtenidas por compresión mecánica de granulados o mezclas pulverulentas de uno o varios principios activos con adición, en la mayoría de los casos, de diversos excipientes.

Estas formas farmacéuticas reflejan una gran diversidad en lo relativo a su forma, tamaño y peso. A las formas tradicionales, se han incorporado otras (figura 1) comprimidos con secciones cuadra, ovoide, rómbica, etc. El tamaño suele oscilar entre 5 y 17mm y el peso entre 0.1 y 1g, dependiendo de la dosis de principio activo, de sus características y del uso a que esté destinado el comprimido.

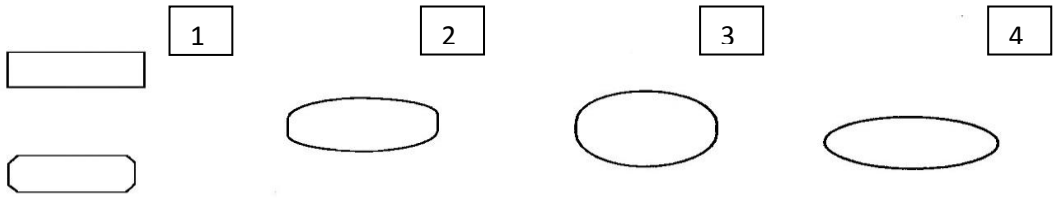

Figura 1. Formas más frecuente de los de los comprimidos del 1 al 4 (de caras planas, suevamente biconvexa, fuertemente biconvexa y lenticular). (García Sánchez & Santos Buelga, 1997).

III.1.1. VENTAJAS E INCONVENIENTES

Los comprimidos son las formas farmacéuticas más utilizadas, esto es consecuencia de las numerosas ventajas que presentan, entre las que pueden destacarse las siguientes:

- Constituyen la forma farmacéutica para administración oral con mayor precisión en la dosificación
- Permiten enmascarar con facilidad características organolépticas desagradables, bien utilizando técnicas de recubrimiento o incorporando correctivos a la formulación.
- Por su forma, estructura compacta y reducido tamaño son de fácil administración.
- Son las formas orales con mejores propiedades de estabilidad mecánica, química y microbiológica. Además si se toman las debidas precauciones en la formulación, no se plantean incompatibilidades entres sus componentes

- Es posible modular mediante un diseño adecuado la velocidad y el lugar de liberación del principio activo, en función de los objetivos terapéuticos.
- Los métodos de fabricación, facilitan una producción a gran escala, con elevados rendimientos.

Sin embargo, algunas limitaciones alejan a los comprimidos de la forma posológica ideal. Estas salvedades son debidas, en ocasiones, a las características del principio activo, como cuando se exige una dosificación elevada o se trata de sustancias difícilmente humectables o inestables a la compresión. Otros inconvenientes están relacionados más directamente con la forma farmacéutica:

- Algunos pacientes, en especial los lactantes, ancianos, adultos en grave estado o pacientes con sonda nasogástrica, no pueden ingerirlos.
- A pesar de los avances tecnológicos, la fabricación de comprimidos es compleja y exige numerosos controles a fin de garantizar una óptima dosificación y absorción de los principios activos.

III.1.2. COMPONENTES DE LA FORMULACIÓN

Los principios activos constituyen, desde el punto de vista terapéutico, los componentes esenciales de un comprimido. Por lo que un análisis detallado de las características del mismo, resulta fundamental para el posterior desarrollo de la formulación. Esto permitirá determinar la dosis que hay que incorporar en el comprimido, el tamaño final, la forma y el peso, las posibles incompatibilidades con otros componentes de la formulación, la estabilidad y la solubilidad. Otro aspecto fundamental, estrechamente relacionado con el principio activo, es el lugar y la extensión que se desea para su absorción en el tracto gastrointestinal.

La obtención de comprimidos requiere que el material que se va a comprimir posea ciertas características físicas y mecánicas: capacidad de fluir libremente, cohesividad y lubricación. La mayoría de los principios activos no poseen, por si mismos, todas estas propiedades, y es necesaria la adición de una serie de adyuvantes, materiales inertes, conocidos como **excipientes**, que se pueden clasificar de acuerdo con la función que cumplen en el comprimidos (Tabla 1). Así, un primer grupo lo constituyen los

materiales que tienen por objeto conferir a la formulación características adecuadas para su manipulación y compresión satisfactorias, tales como el flujo y la cohesividad. En este grupo, se incluyen los diluyentes, aglutinantes, disgregantes y agentes antifricción (deslizantes, antiadherentes y lubrificantes). En otro grupo se incluyen los excipientes usados con fines específicos, como absorbentes, correctores de la humectabilidad y solubilidad, así como todos los empleados para conseguir liberación modificada o bien para otros usos más específicos. Por otro lado encontramos los correctores de propiedades organolépticas, principalmente colorantes, aromatizantes y edulcorantes autorizados.

TIPO DE EXCIPIENTE	**EJEMPLOS**
Diluyentes	lactosa, sacarosa, glucosa, manitol, sorbitol, fosfato cálcico, hidroxipropilmetilcelulosa, etc.
Disgregantes	Almidón, polivinilpirrolidona reticulada, glicolato sódico de almidón, carboximetilcelulosa sódica, etc.
Aglutinantes	Gelatina, polivinilpirrolidona, polietilenglicol, almidón, metilcelulosa, etc.
Lubrificantes	Estearato de magnesio, acido esteárico, polietilenglicol, estearilfumarato sódico, parafina liquida, etc.
Deslizantes	Sílice, estearato de magnesio, talco, etc.
Antiadherente	Estearato de magnesio, acido esteárico, polietilenglicol, laurilsulfato sódico, estearilfumarato sódico, parafina liquida, etc.

Tabla 1. Excipientes usados frecuentemente en la formulación de comprimidos (Alderborn,2004)

Los comprimidos pueden obtenerse por las siguientes técnicas:

- Compresión directa
- Doble compresión
- Compresión de un granulado obtenido por vía húmeda

Para la elaboración de los comprimidos que se detallan en el apartado II.2., se utilizo la técnica de compresión directa, por eso no centraremos en ella.

III.1.3. COMPRIMIDOS OBTENIDOS POR COMPRESION DIRECTA

Por compresión directa, se entiende la compresión de fármacos pulveriformes o de mezclas de estos con coadyuvantes, sin tratamiento previo.

Las maquinas de comprimir constan de una serie de elementos fundamentales punzones, matriz y tolva. Los punzones son los elementos mediante los cuales se va a aplicar la fuerza axial. Son piezas metálicas, en general de acero inoxidable y habitualmente de forma cilíndrica. Su superficie puede ser plana, en mayor o menor grado, o cóncava, lo que da lugar a diferentes formas de comprimidos.

La matriz está constituida por una pieza metálica perforada con uno o varios orificios, según se utilicen punzones simples o múltiples, de sección generalmente circular, aunque, al igual que los punzones, puede adoptar diversas formas geométricas para adaptarse a aquellos.

El sistema de alimentación está constituido por una tolva en la que se introduce el granulado o polvo y ocasionalmente, por un dispositivo para facilitar el llenado homogéneo de la matriz.

El proceso de compresión, puede dividirse en tres etapas (Figura 2) García Sánchez & Santos Buelga, 1997):

- Primera fase (1-3): descenso del punzón inferior dentro de la matriz, lo que da lugar a una cavidad en la que el polvo o granulado fluirá por gravedad. La profundidad a la que se sitúa el punzón inferior en la matriz determinara el volumen de la cámara de compresión y, en consecuencia, el peso del comprimido.

- Segunda fase (4-5): aplicación de la fuerza por descenso del punzón superior únicamente o por acción simultánea de ambos punzones, ejerciendo sobre las partículas la presión necesaria para formar un comprimido consolidado.
- Tercera fase ascenso del punzón superior (6-7), al tiempo que sube el punzón inferior hasta alcanzar el tope de la matriz y eyección del comprimido.

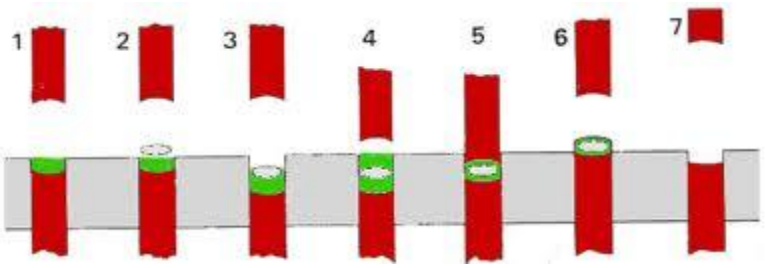

Figura 2. Proceso de compresión.

III.1.4. CONTROLES

Una vez que se obtienen los comprimidos, las variaciones entre ellos, dentro de un mismo lote y entre lotes, se reducen al mínimo, introduciendo controles apropiados durante el procesado y observando las prácticas de buena fabricación (GMP).

Los controles que habitualmente se realizan sobre muestras tomadas al azar de lotes de comprimidos terminados, son múltiples y de diferente naturaleza, incluyendo características físicas, químicas e indicadores de las propiedades biofarmacéuticas (tabla 2). Estos controles son también básicos, a nivel de la preformulación de los comprimidos.

La eficacia y seguridad del tratamiento constituyen el objetivo final de un medicamento; para asegurar ambas, es preciso que este se mantenga integro hasta ser consumido por el paciente, y que presente unas buenas características de disgregación y disolución, de ahí la importancia de estos ensayos.

CARACTERÍSTICAS	PARÁMETROS
Organolépticas	Aspectos Olor Textura Sabor
Geométricas	Forma y marcas Dimensiones
Mecánicas	Resistencia a la fractura Resistencia mecánica (friabilidad)
Estabilidad	Ingrediente activo Color Frente a la humedad, luz y calor
Posológicas	Uniformidad de peso Uniformidad de contenido
Indicadores biofarmacéuticos	Tiempo de disgregación Velocidad de disolución

Tabla 2. Controles realizados a los comprimidos (García Sánchez & Santos Buelga, 1997).

III.1.5. TIPOS DE COMPRIMIDOS Y APLICACIONES

En función de su presentación y de la forma de administración recomendada los comprimidos pueden clasificarse en comprimidos convencionales y especiales.

III.1.5.1.Comprimidos convencionales

Están destinados a ser ingeridos y liberar el principio activo en el tracto gastrointestinal para ejercer una acción local o una acción sistémica, previa absorción del principio activo. La introducción de nuevos materiales, especialmente polímeros, permite controlar el proceso de liberación, lo que hace posible espaciar la administración y mejorar el rendimiento terapéutico del medicamento.

III.1.5.2.Comprimidos especiales

Se incluyen dentro de esta categoría a aquellos comprimidos que presentan alguna característica farmacotécnica diferente de las descritas para los comprimidos convencionales. Son comprimidos en los que se modifican los componentes, la técnica de obtención o ambos parámetros. Entre ellos encontramos:

Comprimidos de Disgregación previa: Solubles; Dispersables y Efervescentes.

Comprimidos destinados a tenerlos en la cavidad oral hasta que se disuelvan, disgreguen o absorban: Comprimidos solubles y bucodispersables; comprimidos masticables; comprimidos sublinguales y comprimidos buco-adhesivos.

Comprimidos obtenidos por recubrimiento: grageas y recubrimiento pelicular.

Comprimidos de liberación modificada: Son comprimidos recubiertos o no recubiertos, que se preparan con excipientes especiales, o por procedimientos particulares o por ambos medios conjuntamente, con el fin de modificar la velocidad, el lugar o el momento de liberación del principio o principios activos.

Incluyen comprimidos de liberación prolongada, comprimidos de liberación retardada, comprimidos de liberación pulsátil y comprimidos de liberación acelerada. En este grupo se encuentran: comprimidos estratificados; comprimidos moteados; Comprimidos matriciales; comprimidos osmóticos y comprimidos flotantes

Otros: Comprimidos vaginales y comprimidos de implantación. En estos casos es la vía de administración la que es fundamentalmente diferente, y las características de estos comprimidos también lo son.

Por su parte la real farmacopea Española (RFE) clasifica los comprimidos de la siguiente manera:

- comprimidos no recubiertos
- comprimidos recubiertos
- comprimidos efervescentes
- comprimidos solubles
- comprimidos dispersables
- comprimidos gastrorresistentes
- comprimidos de liberación modificada
- comprimidos para utilizar en la cavidad bucal

III.1.6. COMPRIMIDOS DE LIBERACIÓN MODIFICADA

Por ser una de las formas farmacéuticas desarrollados en la presente tesis doctoral, nos centramos en los comprimidos de liberación modificada. La RFE (2010) los define como aquellos que contienen excipientes especiales o se preparan por procedimientos especiales, con el fin de modificar la velocidad, el lugar o el momento de liberación del principio activo. Dentro de ellos se incluyen García Sánchez & Santos Buelga, 1997):

- Comprimidos de liberación prolongada
- Comprimidos de liberación retardada
- Comprimidos de liberación pulsátil.

III.1.7. FORMAS FARMACÉUTICAS DE LIBERACIÓN RETARDADA

Liberan el principio activo transcurrido un tiempo de latencia, y esto no ocurre hasta que la forma farmacéutica se encuentra en la zona del tracto digestivo, donde se desea que se active el sistema. Ejemplo de ello son los comprimidos gastrorresistentes, este tipo de comprimidos están destinados a resistir la acción del jugo gástrico y a liberar su principio activo en el fluido intestinal.

Estos comprimidos pueden obtenerse por distingos métodos. Uno de los más simples es la incorporación del principio activo en una matriz de naturaleza polimérica que forme una barrera mucilaginosa que controle la difusión del principio activo o que se erosione lentamente permitiendo la liberación gradual del mismo. Otra forma de controlar la liberación consiste en el recubrimiento de los comprimidos con cubiertas especiales. Es importante destacar que en estos casos, se requiere la realización de ensayos de disolución adecuadamente adaptados, que permitan demostrar si la liberación del principio activo se realiza de acuerdo con la cinética prevista en su diseño.

III.1.7.1. SISTEMAS ENTÉRICOS

Como ya se ha indicado anteriormente, uno de los métodos para proteger al principio, de su posible degradación a nivel gastrointestinal, es la utilización de polímeros de solubilidad dependiente de pH.

Para este fin se han propuesto, la utilización de polímeros de distinta naturaleza, fundamentalmente derivados celulósicos y acrílicos.

MATRICES HIDRÓFILAS
Derivados celulósicos:Metilcelulosa,hidroxipropilcelulosa,Hidroxiporpilemetilcelulosa,etc.
Gomas
Alginatos
Derivados acrílicos: Eudragit L, Eudragit S, etc.
Derivados del oxido de polietileno
Gelatina
Carbopol

Tabla 3. Polímeros que dan lugar a la formación de matrices hidrófilas. (Alderborn, 2004)

El alginato y algunos derivados celulósico y acrílico, se engloban generalmente, dentro de lo que se conoce como **matrices hidrófilas** (Tabla 3). Los comprimidos elaborados

con este tipo de polímeros, al entrar en contacto con el agua forman rápidamente un gel en toda su superficie (Figura 3). Este gel establece una barrera de difusión para las moléculas del principio activo. A medida que el excipiente polimérico que constituye la matriz se hidrata, la gelificación avanza hacia el núcleo sólido, donde el polímero se encuentra en estado no hidratado. Puede presentarse asimismo un mecanismo de erosión de la capa gelificada externa que también contribuye al proceso de liberación del principio activo (Maderuelo et al., 2011). Estos mecanismos pueden actuar independientemente, a la vez, o consecutivamente.

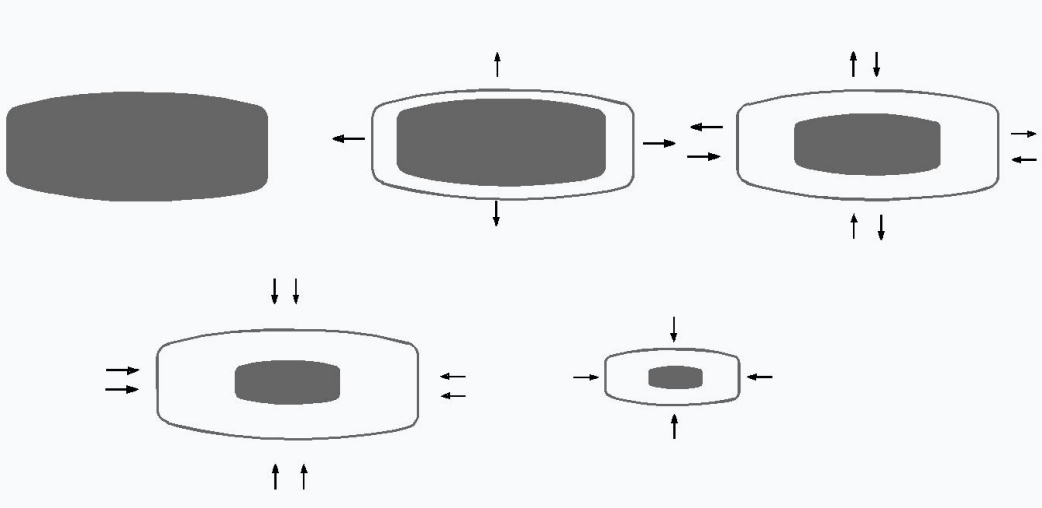

Figura 3. Simulacion de un corte de un comprimido de eleborado con una matriz hidrofila.

Conforme va penetrando el agua en el sistema, la capa de gel va experimentado un progresivo aumento de volumen. Poco a poco, las capas mas externas sufren paulatinamente un proceso de erosion. El proceso concluye con una total gelificacion del sistema y con la practica liberacon del principio activo bien por difusion, bien por erosion o por una mezcal de ambos fenómenos.

Estos comprimidos de matriz hidrófila se suelen elabora mediante compresión directa, o previa granulación por vía húmeda.

Ventajas de los sistemas de matriz hidrófila

- Su elaboración es relativamente sencilla.
- Los excipientes suelen ser baratos y considerados seguros.
- Pueden llevar grandes cargas de principio activo.
- Son biodegradables, lo que reduce la aparición de restos de matriz en las heces.

III.1.7.2. MATRICES HIDRÓFILAS EMPLEADAS OBEJTO DE ESTUDIO

Hidroxipropilmetilcelulosa

La Hidroxipropilmetilcelulosa (Figura 4) se conoce también con las denominaciones de hipromelosa o Methocel E, F, J y K (Rowe, Sheskey, Quinn, 2009).

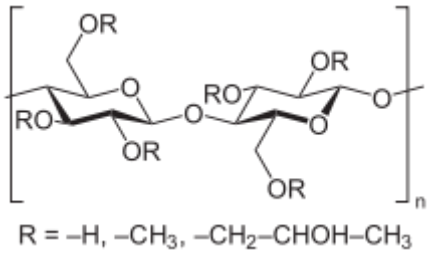

Figure 4. Hidroxipropilmetilcelulosa

Es un derivado de la celulosa que se clasifica atendiendo al grado de sustitución de la siguiente manera (tabla 4):

	Grupo Metoxilo %	Grupo hidroxipropil (%)
Methocel E	29	8.5
Methocel F	28	5.0
Methocel J	18	27
Methocel K	22	8.2

Tabla 4. Grado de sustitución del Methocel.

El grado de sustitución, a su vez, afecta a la solubilidad y a la temperatura a la que gelifican en soluciones acuosas estos polímeros.

El Methocel se utiliza como aditivo alimentario (E-464) con función emulsificante, espesante y estabilizante.

En la industria farmacéutica, la hipromelosa ha sido ampliamente utilizada en el desarrollo de formulaciones orales, oftálmicas, nasales, y tópicas. En las formas farmacéuticas orales, se emplea principalmente como aglutinante (2- 5% p/p), para formar película de recubrimiento, y como matriz para elaborar comprimidos de liberación modificada (10-80% p/p). También se utiliza en las formas de dosificación orales líquidas como las suspensiones en concentraciones del 0.25% al 5% (p/p).

Alginato

El alginato es un polisacárido que se obtiene de algunas "algas marrones", algas de gran tamaño, entre las que se encuentran fundamentalmente *Laminaria hyperborea*, *Laminaria digitata*, *Laminaria japonica*, *Macrocystis pyrifera*. Todas estas algas contienen entre el 20% y el 30% de alginato sobre su peso seco.

Los alginatos son las sales del ácido algínico, un polisacárido lineal constituido por dos unidades monoméricas, el ácido ß -D-manurónico (**M**) y el ácido α -L-glucurónico (**G**). Estas se agrupan en bloques de secuencias **MM**, **MG**, unidos por enlaces glucosídicos α (1-4); y bloques **GG**, **GM**, unidos por enlaces glucosídicos ß (1-4).

Se ha demostrado que la cadena polimérica que constituye el ácido algínico y sus sales se compone de tres tipos de regiones o bloques. Los bloques G, que contienen sólo unidades derivadas del ácido L-glucurónico, los M que se basan enteramente en ácido D-manurónico y las regiones MG, que consisten en unidades alternadas de ambos ácidos.

En las figuras 5 y 6 se muestran las configuraciones espaciales que adoptan los bloques M y G debido a los diferentes enlaces glucosídicos entre los carbonos C-1 y C-4 de las unidades monoméricas. Las regiones de bloques M corresponden a cadenas lineales, mientras que los bloques G presentan una estructura en forma de bucle.

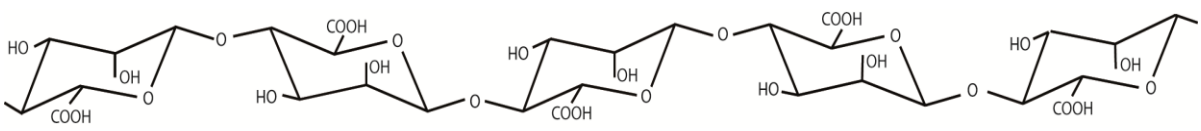

Figura 5. Bloques MM

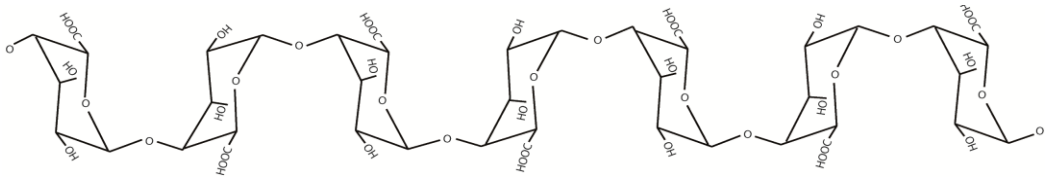

Figura 6. Bloques GG

Cuando dos cadenas de bloques G se alinean lado a lado resulta un hueco en forma de diamante, con una dimensión ideal para acomodar en su interior un ión calcio, formándose una estructura dimérica. Éste modelo fue propuesto por Grant y cols. en 1973 ("egg-box model") para explicar las propiedades gelificantes de los alginatos al reaccionar con sales cálcicas. Es importante destacar que, según los porcentajes de regiones de bloques G y M, que son diferentes en las distintas especies de algas, también variaran las características de los geles de alginato.

El alginato se degrada con facilidad en presencia de oxígeno, disminuyendo su viscosidad. La forma ácida es la menos estable, y la sal sódica la más estable.

Los alginatos en general son insolubles en solventes miscibles con el agua como son los alcoholes y cetonas. Las soluciones acuosas (1%) de la mayoría de los alginatos toleran la adición de 10-20% de tales solventes; pero proporciones mayores impiden una correcta hidratación de las moléculas.

La viscosidad de las soluciones de alginato de sodio es casi independiente del pH en el intervalo entre 5 y 10, presentando un valor ligeramente mayor cerca de la neutralidad (pH 6-8) debido a efectos repulsivos de los grupos carboxilos cargados negativamente (COO^-), estos efectos mantienen extendidas las cadenas del polímero e incrementan su capacidad de unión a las moléculas de agua. Por debajo de pH 4.5 la viscosidad tiende a incrementarse por la disminución de la solubilidad del ácido algínico libre, el cual precipita en forma de gel a un pH de 3-3.5.

Acetoftalato de Celulosa (CAP)

El acetoftalato de celulosa (Figura 7) es el producto de la reacción del acetato de celulosa y el anhídrido ftálico. Debe contener no menos de 21.5 por ciento ni más de 26.0 por ciento de grupos acetilo (C_2H_3O) y no menos de 30.0 por ciento ni más de 36.0

por ciento de grupos ftalil(o-carboxibenzoil) ($C_8H_5O_3$) calculados sobre la sustancia anhidra y libre de ácidos.

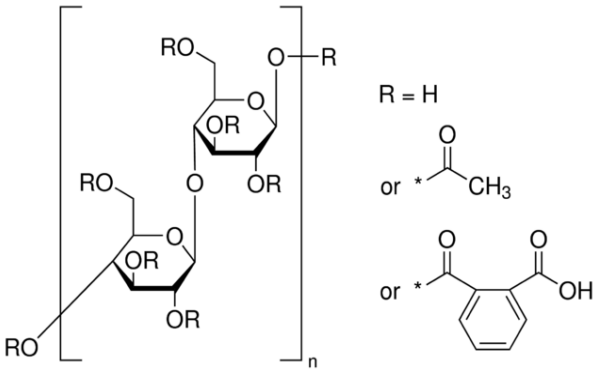

Figura 7. Acetoftalato de celulosa

Se trata de un polvo blanco o en forma granular que fluye fácilmente, es higroscópico y fácilmente soluble en acetona y en dietilenglicol. Prácticamente insoluble en agua, etanol y cloruro de metileno (RFE, 2010). Se disuelve en soluciones alcalinas diluidas (pH>6).

Eudragit®

Bajo la denominación de Eudragit® se engloban los copolímeros del ácido metacrílico y diferentes ésteres acrílicos o metacrílicos de interés farmacéutico. Estas resinas son particularmente atractivas en este campo, como consecuencia de su elevada estabilidad química, de su compatibilidad con diferentes materiales y del considerable número de variedades disponibles, que cubren un amplio espectro de características de solubilidad y permeabilidad y diferentes formas de presentación. Los Eudragit son biocompatibles y farmacológicamente inertes y, en el organismo, se excretan sin sufrir metabolismo sistémico. Además, cuentan con una elevada tolerancia por parte de la piel y de las mucosas. Tomando como base su esqueleto molecular, los Eudragit pueden dividirse en los diferentes grupos que se indican en la Figura 8 (Rowe, Sheskey, & Quinn, 2009).

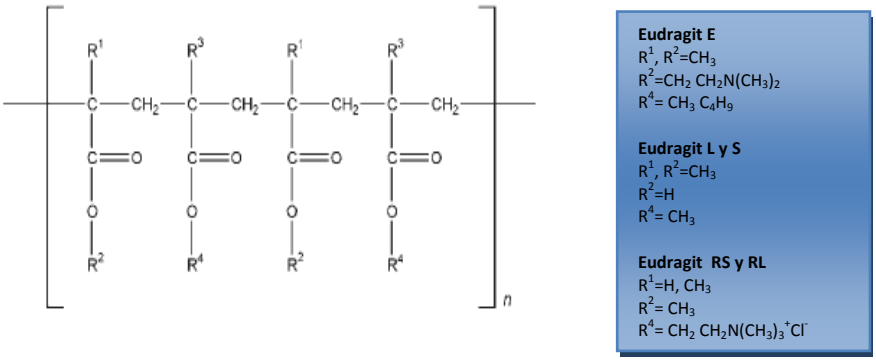

Figura 8. Polimetracrilatos.

En la tabla 5 se señalan las principales propiedades de los más importantes. Asi, dependiendo de su composición, estos polímeros son insolubles a valores de pH inferiores a 6 (Eudragit L) o 7 (Eudragit S), pero se disuelven rápidamente tras la desprotonización de los grupos carboxílicos a valores superiores de pH (Figura 9). La utilización de esta alternativa tecnológica trata de explotar el supuesto incremento del pH que se detecta al ir avanzando en el tracto intestinal, y que alcanzaría su valor máximo a nivel del colon.

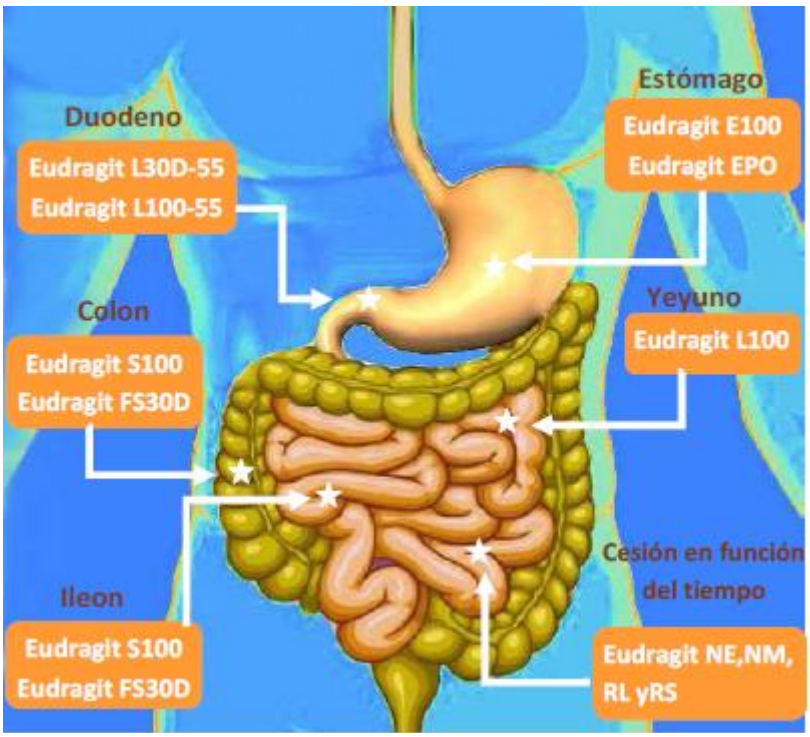

Figura 9. Liberación del principio activo atendiendo al tipo de Eudragit® utilizado.

Tipos	Variedades	Presentación	Solubilidad/Permeabilidad	Aplicaciones
Aniónicos	**L-100-55** **L30D-55**	Polvo Dispersión acuosa 30%	Soluble pH> 5.5	Liberación en el duodeno
	L 12,5	Disolución en alcohol isopropílico	Soluble pH> 6.0	Liberación en el yeyuno
	L 100	Polvo		
	S 100 **FS30D**	Polvo Dispersión acuosa 30%	Soluble pH> 7.0	Liberación en íleon y colon Liberación en colon
Catiónicos	**E100** **E12,5** **EPO**	Gránulos Disolución en alcohol isopropílico/acetona 12,5% Polvo	Soluble en fluido gástrico hasta pH=5,0	Enmascaramiento organoléptico Cubierta aislamiento
	RL 12,5 **RL100** **RLPO** **RL30D**	Disolución en alcohol isopropílico/acetona 12.5% Gránulos Polvo Dispersión acuosa 30%	Insoluble, alta permeabilidad	Formulaciones de liberación controlada
	RL 12,5 **RL100** **RLPO** **RL30D**	Disolución en alcohol isopropílico/acetona 12.5% Gránulos Polvo Dispersión acuosa 30%	Insoluble, baja permeabilidad	Adecuado para estructura matriciales
Neutros	**NE30D** **NE40D**	Dispersión acuosa 30% con 1.5% emulsificante Dispersión acuosa 30% con 0.7% emulsificante	Insoluble y permeable	Formulaciones de liberación controlada Adecuado para estructuras matriciales

Tabla 5. Características y propiedades de los diferentes tipos de Eudragit®(Rowe, Sheskey, & Quinn, 2009).

La mayor parte de los sistemas entéricos de liberación descritos hasta el momento son comprimidos recubiertos fundamentalmente con Eudragit S o L, derivados de la celulosa o microparticulas elaboradas con estos polímeros o a partir de mezclas con otras variedades poliméricas.

II.2. Development of gastro-resistant tablets for the protection and intestinal delivery of *Lactobacillus fermentum* CECT 5716

Desarrollo y caracterización tecnológica de comprimidos de liberación modificada con *L.fermentum* y evaluación de la viabilidad a lo largo del tiempo.

En la actualidad, diversos estudios han atribuido numerosos efectos beneficiosos sobre la salud del hospedador a *Lactobacillus fermentum* CECT 5716. Sin embargo, al igual que el resto de los probióticos se caracteriza por una baja resistencia a diversos factores ambientales y tecnológicos. Por ello, el objetivo fundamental de este estudio ha sido el diseño de comprimidos elaborados a partir de polímeros funcionales (Fórmula 1: Methocel K-15-alginato sódico; Fórmula 2: Eudragit® L-100- alginato sódico; Fórmula 3: Acetoftalato de celulosa) que contribuyan a la estabilidad y viabilidad del probiótico en el seno de la formulación y a su paso por el estómago. Asimismo, los comprimidos se han elaborado de acuerdo con un método de compresión directa con el que se consigue perder únicamente 1 logaritmo de unidades formadoras de colonias durante la operación. Todas las formulaciones estudiadas han demostrado mejorar la supervivencia de las bacterias cuando se exponen a medio ácido en comparación con las bacterias libres, siendo los comprimidos de Eudragit L-100 los que más protección conferían bajo dichas condiciones, manteniendo la viabilidad tras 2 horas de incubación en medio ácido. Finalmente, esta formulación resulto estable durante el período de tiempo estudiado, 6 meses a 4ºC, sin que existan diferencias estadísticamente significativas entre la viabilidad de los comprimidos a tiempo cero y transcurridos 6 meses a 4ºC ($p>0,05$). En conclusión, los comprimidos desarrollados son adecuados y confieren a las bacterias la protección deseada.

Este trabajo se encuentra actualmente bajo revisión en International Journal of Pharmacuetics.

Development of gastro-resistant tablets for the protection and intestinal delivery of *Lactobacillus fermentum* CECT 5716

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Abstract

Different studies have attributed health benefits to *Lactobacillus fermentum* CECT 5716. However, the main problem associated with probiotics, is their low resistance to environmental and technological factors. The aim of this study was to design tablets made out of functional polymers (Formula 1: Methocel K-15-sodium alginate; Formula 2: Eudragit® L-100- sodium alginate; Formula 3: Cellulose acetate phthalate) that improve the stability and survival of probiotics. Rigid tablets were produced through direct compression with a bacterial content as high as 10^9 CFU. Tablets were shown to improve the survival of cells when exposed to an acidic medium as compared to free cells. Eudragit® L-100-sodium alginate was found to be the most suitable excipient for the protection of probiotic within gastric conditions, resulting in the survival of 10^9 CFU after 2 h of incubation. Finally, these tablets were found to be very stable over a 6 month period when stored at 4ºC. No significant differences were reported between the number of cells at time cero and after 6 months of storage at 4ºC ($p>0.05$). In conclusion, these tablets seem to be a suitable procedure for protecting this probiotic.

Key words: probiotics, tablets, *lactobacillus fermentum*, protection

1. Introduction

Probiotics are live microorganisms which, when administered in appropriate quantities, offer health benefits to their host (FAO/WHO, 2006). Several studies have attributed health benefits to probiotics. These benefits may include a modulation of the immune

system, a reduction of symptoms of viral and antibiotic-associated diarrhea and an alleviation of lactose intolerance, among others (Singh, et al. 2013). Specifically, *Lactobacillus fermentum* CECT 5716 is a well-known probiotic of human origin. Its usefulness has been demonstrated in the treatment of mastitis (Arroyo, et al. 2010) and inflammatory bowel diseases (Mañé, et al. 2009). Moreover it has been shown that an infant formula with *L.fermentum* CECT5716 reduces the incidence of gastrointestinal and respiratory infections in children (Maldonado et al. 2012; Gil-Campos, et al. 2012).

In order to offer these health benefits, probiotic microorganisms must be capable of surviving the adverse conditions encountered during product production (Saarela, et al. 2000). The main problem associated with probiotics is their low resistance to factors such as pH, post-acidification (during storage) in fermented products, hydrogen peroxide production, oxygen toxicity (oxygen permeation through packaging), and storage temperatures (Kailasapathy, 2002). Indeed, several studies have revealed that the number of viable bacteria in some commercial products is below the desired level (Vinderola, et al.2000; Kailasapathy & Chin, 2000). In addition to production conditions, numerous additional obstacles are encountered during gastrointestinal (GI) transit (pH, enzymes, bile salts, etc.), limiting the survival and functionality of probiotics and their health benefits after intake.

By the moment, probiotics in general are added to food matrices, oral rehydration solutions, infant formulas and urogenital formulas. *L. fermentum* CECT5716 in particular is added to infant formulas and it is also present in the nutraceutical market in form of capsules. Whereas food is related to the maintenance of health and the reduction of disease risk, pharmaceutical forms are considered as primary therapeutic solutions. In this sense, most of the probiotic pharmaceutical products are present in the form of capsules or sachets, but tablets are less frequent (Sánchez, et al. 2015). Both pharmaceutical forms present some disadvantages such as lower production speed that tablets, greater weight variation, shape and size limited and supplier dependent. Moreover older patients perceived as being more difficult to swallow. Thus, the development of gastrorresitant tablets could represent further applications and advantages.

In fact, during the last year increased attention has been paid to the desing of probiotics tablets using functional polymers that improce the stability and survival. These tablets permit accurate dosage, ease administration, good patient acceptance, stability of

storage and large-scale production. In this study, direct tableting has been used for the production of probiotic tablets, as tableting method that can best adapt to the conditions of these microorganisms. Although this method has been used by other authors for the manufacture of probiotic tablets, we study to design a formulation which allows us to obtain gastro-resistant tablets of appropriate technological features using low compression pressures so as to achieve minimize the loss of viability during this technological operation and storage over time.

For this purpose, different formulations have been studied, mainly based on sodium alginate (Chan & Zhang, 2002; Chan & Zhang, 2005). Sodium alginate is preferred due to the fact that it is natural, safe and widely accepted in the food, drink and pharmaceutical industries. In addition, it confers physical integrity in gastric fluid and minimizes solvent penetration at acidic pH values. Moreover, cellulose acetate phthalate (CAP) has been also used as excipient for elaborating controlled release tablets (Sousa et al. (2013). CAP has been used for several decades as a pharmaceutical excipient, due to its solubility which is dependent upon the pH of the aqueous media.

Based on this knowledge, we investigate in the present study whether it is possible to design tablet formulations for probiotics that protect them from degradation at low pH and deliver them to the intestinal tract in viable form. Using CP and sodium alginate in combination with hydroxypropylmethylcellulose (Methocel) is also used to form the hydrophilic matrix system that is among the most widely-used means of providing controlled release of drug in solid oral dosage forms. Meanwhile, Eudragit®L-100 forms an effective and stable enteric coating offering fast dissolution in the upper bowel. Using a combination of these functional polymers in obtaining tablets probiotics allow to apply low pressures compression, obtaining suitable technological characteristics, and stability over time and release in the intestinal tract. All this is a challenge in developing such solid forms as possible to optimize production and improve the efficiency of administration.

The aim of this study was to test the efficacy of these different excipients on the protection of *Lactobacillus fermentum* CECT 5716 during gastrointestinal transit. To accomplish this, we prepare freeze-dried *Lactobacillus fermentum* matrix-type tablets consisting of sodium alginate-Eudragit®L-100, sodium alginate-Methocel®K-15 and cellulose acethopthalate. These tablets were characterized by ability to maintain bacterial viability during gastric incubation and stability over time. Cell release time

was also monitored in order to predict the potential site of cell release within the human gastrointestinal tract.

2. Materials and methods

2.1. Materials

Freeze-dried *Lactobacillus fermentum* CECT 5716 was kindly provided by Bioserch Life (Granada, Spain). Methocel®K-15 was purchased from Palex (Jaén, Spain), Eudragit®L-100 from Degussa (Darmstadt, Germany), and sodium alginate was obtained from Guinama (Valencia, Spain). Cellulose acetate phthalate (CAP) was supplied by Sigma-Aldrich (Missouri, USA).

2.2. Tablet preparation

Tablet with a constant weight of 303mg were prepared by direct compression of a homogeneous mixture of excipients and freeze-dried *Lactobacillus fermentum* CECT 5716 (LAB) using a Manual Hydraulic Press equipped with a 10 mm diameter flat-faced punch (Specac, Kent, UKA). All the punch pieces were disinfected with 70 % ethanol before each tablet was produced. A total number of twenty six formulations were developed using compression force range from 5kN to 30kN and period of time of 30 and 60s (Table 1). The powder contained also a suitable amount of magnesium stearate as lubrificant and talcum as antiadherant (0.1 % and 0.9 %, w/w, respectively).

Formulation nº	Compression force (kN)	Time (s)	Composition (303 mg)				
			LAB	Methocel k-15	Sodium Alginate	Eudragit® L-100	CAP
1	25	60	100	180	20		
2	25	60	100		100	100	
3	25	60	100				200

Table 1. Probiotic tablet formulation.

2.3. Tablet evaluation

The probiotic tablets obtained were evaluated for their disintegration, tensile strength and friability according to USP(United States Pharmacopeia). Taking into account the results obtained in the friability test only the formulations of table 1 were considered in further studies.

Disintegration of tablets was examined by means of a disintegration apparatus (Erweka, Heusenstamm, Germany). The tablets were placed separately in the test chamber, and then immersed in PBS pH 1 and subsequently in pH 6.8 as the disintegration medium at 37 ºC.

The tablet mechanical strength (n=10) was determined by using Erweka TBH 20 (Heusenstamm, Germany). The tensile strength was calculated by the following equation. Where *P* is the measured crushing force *D* is diameter and t is the thickness of the tablet. The tablet friability was measure by using a friabilator (Erweka, Heusenstamm, Germany).

$$\sigma = 2P/\pi Dt$$

Tablet specific volume (V_{sp}) was calculated as follows:

$$V_{sp} = \pi x R^2 x\, t/w$$

Were *W* is the tablet weight, *t* is the tablet tightness and *R* is the tablet radius. Each measurement was performed in triplicate.

Tablets were also examined by scanning electron microscopy (SEM) using a S510 electron microscope (Hitachi High−Technologies Europe GmbH, Krefeld, Germany).

2.4. Test of bacterial viability

2.4.1. Bacterial Viability after tableting

In order to determine the relationship between applied pressure and bacterial mortality, tablets formed at the pressure values indicated in tablet 1 were evaluated.

In accordance with a slightly modified Klayraung, et al. (2009) method, after compression, each table was immediately broken and dispersed in 100 mL of phosphate buffer (pH 6.8). A serial dilution of this suspension was made in 0.1 % sterile peptone water until a suitable cell density was obtained and subsequently plated in triplicate in MRS agar plates. These plates were incubated at 37ºC for 24 h in a 2.5 L AnaeroJar using an AnaeroGen sachet to create an anaerobic environment. The colony forming units (CFU) were finally enumerated and converted to log CFU.

The initial number of CFU in 1 gram lyophilized *L. fermentum* was determined by following the same procedure without compressing the powder mixture.

2.4.2. Viability after acid challenge

The survival of free and encapsulated cells in simulated gastric fluids (SGF) of pH 1 was studied.

All tests were conducted in accordance with the USP 2 paddle method at 100 rpm, 37ºC with 300 mL of SGF. The encapsulated and free cells were exposed to the respective SGF. To determine the survival of encapsulated cells, the tablets were first aseptically removed from the acidic medium and were subsequently homogenized with 100 mL of phosphate buffer solution pH 6.8. Cell enumeration was carried out according to the previously described method.

As a control, 100 mg of non-compressed, freeze-dried *lactobacillus fermentum* was treated following the same procedure.

2.4.3. In vitro determination of cell release time

In order to verify the results obtained in the disintegration test, the encapsulated cell was first exposed to the respective SGF for 2 hours and was subsequently transferred to a simulated intestinal fluid for dissolution. The simulated intestinal fluid was prepared from phosphate buffer solution (PBS) pH 6.8. The cells were allowed to thoroughly dissolve in the dissolution medium. The time required for total dissolution (including 2 h in SGF) was recorded. As previously mentioned, dissolution tests were carried out in accordance with the USP 2 paddle method at 100 rpm and 37ºC.

2.4.5. Stability of the probiotic tablet

For stability testing, the tablets used in section 2.4.3 were stored in light-resistant containers at 4ºC and at room temperature for a period of six months. The stability of the bacterial cells in terms of cells viability in the tablet across the storage period was examined. The plating procedure method that was described in Section 2.4.1 was used to determine cell viability.

2.5. Data analysis

All analyses were performed using SPSS version 19.0 for windows (SPSS, Chicago, Ilinois,USA). Values are **e**xpressed as mean ± standard deviation. Data were compared using the student t-test. Statitistical significance was set at $p < 0.05$.

3. Results and discussion

3.1. Tablet preparation

A total of twenty six formulations were developed with compression forces ranging from 5kN to 25kN and with time periods of 30s and 60s (Table 1). Of these, only three kinds of tablet were selected for the subsequent experiments, based on the results of technological assays (table 2). Working pressure was established at 25kN/60 min for formulations 1 and 2 and at 15kN/60min for formulation 3.

Formulation Nº	Tensile Strength (N/mm^2)	V_{sp}(cm^3/g)	Total release time (h) (±0.05)
1	2.24±0.13	0.7185±0.04	3.50
2	4.20±0.32	0.7489±0.04	3.10
3	6.68±0.43	0.759±0.006	3.51

Table 2. Effect of different formulation on tablet properties. The number in the brackets are standard errors.

3.2. Tablet evaluation

The selected conditions allowed for the creation of rigid (Friability < 1%) and easy-to-manipulate tablets. Moreover, tensile strengths ranging between 2.245 N/mm^2 and 6.68N/mm^2 are adequate, according to Muller et al. (2014), for pharmaceutical tablets (≈1.5N/mm^2) (Table 2).

3.2. Bacterial viability after tableting

The tablets contained 10^9 viable bacteria, what meets the general requirement of 10^9 living cells per serving for effective probiotic products. Higher compression force

(>25kN/60s) resulted in increased mortality levels (data not showed). Other authors have also reported these findings (Chan & Zhang, 2002; Poulin, et al. 2011).

Initial number of cells (log CFU)	**Formulation Nº**	**Number of cells after compression (CFU)**	**Number of cells after compression (log CFU)**
10.08±0.01	1	$1.04x10^9±5.03x10^7$	9.02±0.02
	2	$1.42x10^9±1.33x10^8$	9.15±0.04
	3	$2.40x10^9±6.06\ x10^8$	9.37±0.12

Table 3. Survival of *Lactobacillus fermentum* CECT 5716 after tabletting.

As shown in table 3, cells registered a loss of viability around one log for every formula. The highest viability was found in formulation 3. Plumpton, et al. (1986) reported that the lethal effect of compression may be attributable to shearing forces caused by interparticulate movement and pore size reduction within the matrix, leading to mechanical damage and death of the cells. The highest degree of mortality was found to correspond with the greatest decrease in specific volume. In fact, the size of the powder, particles and their physical behavior under pressure, namely brittle fracturing and/or plastic deformation, have a major influence on the extent of bacterial death. Furthermore, cell mortality is connected to the tablet's initial porosity so that when porosity is decreased, cell mortality is logically increased. The tablet's initial porosity is proportional to the ratio between tablet density (1/Vsp) and the polymer's true density (this data is unknown here), since when the powder bed has a greater density, it eliminates more of the air from the powder bed and voids in individual particles. The increased densification process (decrease in the tablet's specific volume) results in

lower tablet porosity. Thus, for each tablet, specific volume was measured as an approximation of the tablet's initial porosity (table 2). Moreover, SEM images of the surface and sections of the tablets were also taken. The specific volume was higher for formulation 3, followed by formulation 2 and then by formulation 1. This has been corroborated by the porosity revealed in the photographs (Figure 1). Table 3 shows the survival rates of the cells in the different formulas after tableting. These results are in line with the fact that the higher the tablet's specific volume, the lower the initial mortality

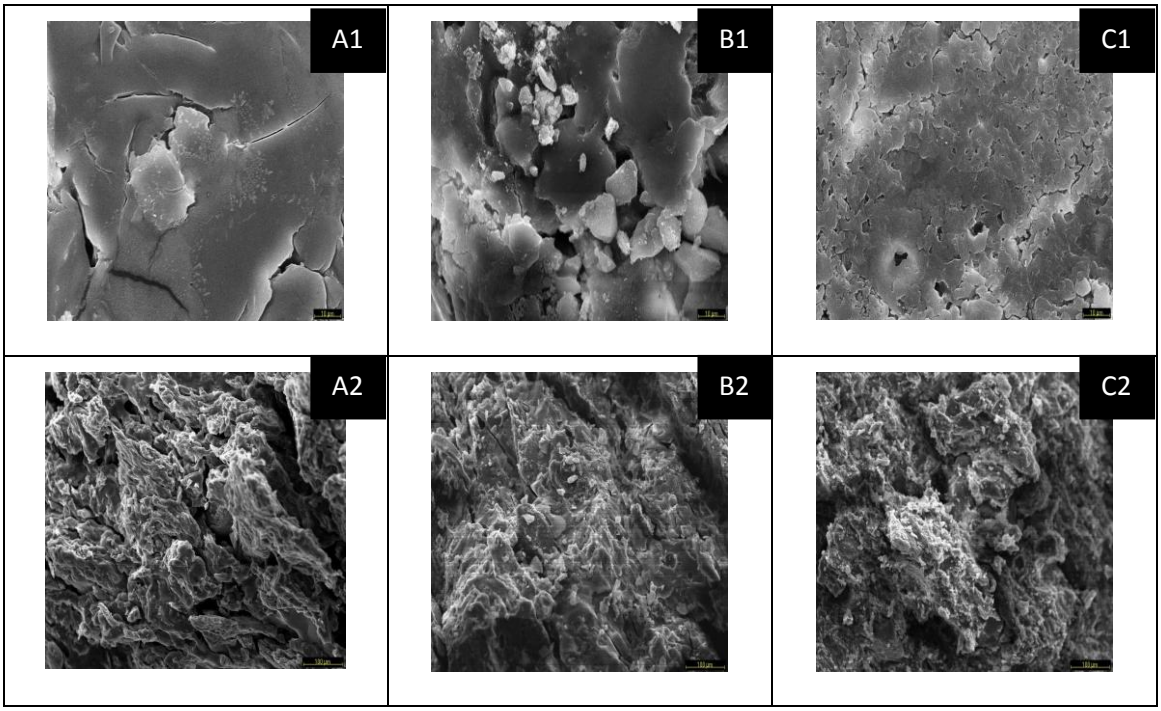


Fig. 1. Scanning electron microphotograph of tablets. A1) surface of Methocel- sodium alginate tablets. A2) section of Methocel- sodium alginate tablets. B1) surface of Eudragit L-100- sodium alginate tablets. B2) section of Eudragit L-100- sodium alginate tablets. C1) surface of CAP tablets. C2) section of CAP tablets.

3.4. Viability after acid challenge

Figure 2 shows the survival profile of cells when they were exposed to the SGF with pH 1 for 2 hours. The number of viable free cells decreases dramatically from 1.2 x 10^8CFU/mL to 6.5 x10^4CFU /mL after they were exposed to the SGF with pH 1 for 2 hours. Survival of the encapsulated cells was significantly higher than that of free cells

after 2 hours of exposure to SGF with pH 1. The effect of the type of coating material on the viability of encapsulated cells when exposed to SGF with pH 1 was also investigated. The viability of the probiotic was reduced by less than 1 logarithmic cycle for formulations 1 and 2 (0.9 log and 0.3 log, respectively) while for formulation 3 the decrease in viability was 1.27 log. Stadler and Vierstein (2003) considered that a decrease of 1 log unit after 2 hours of contact with an acidic medium was a good achievement. Formulation 2 was found to have a slightly higher viability and a proper time of release in the intestine (table 2).

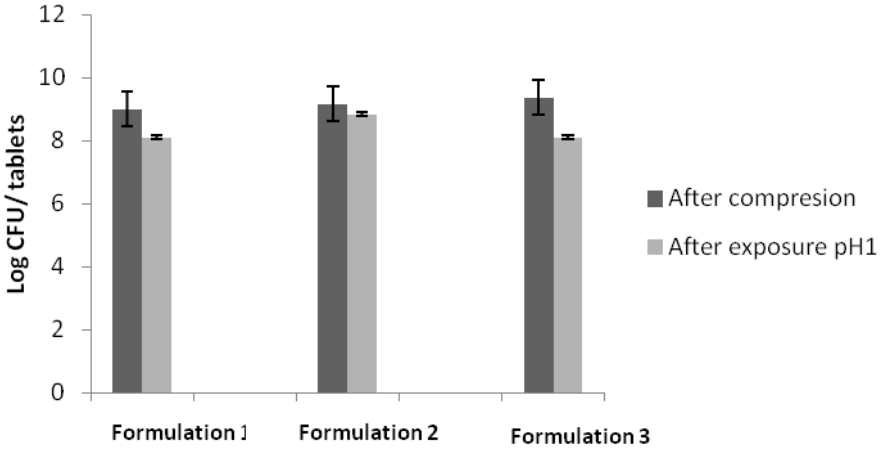
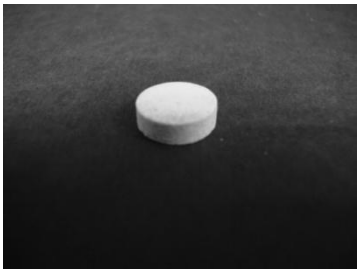
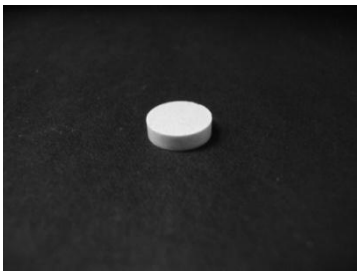


Figure 2. Survival of *Lactobacillus fermentum* CECT 5716 after tabletting and exposure at pH 1 for 2 hours.

3.5. *In vitro* determination of cell release

The release time found for the encapsulated cells may be used as an indicator of the site of cell release in the human gastro-intestinal tract. Since the mean residence time of a dosage form in the stomach is approximately 2 hours and the mean transit time of a dosage form in the small intestine of a healthy subject is roughly 3-4 h, it may be deduced that cells encapsulated by the previously described formulation and methodology would probably be released in the region between the near end of the small intestine and the beginning of the colon. This region would be an ideal site for probiotic bacteria delivery since it is the location of many indigenous microbiota in humans. This is in accordance with the results shown in the disintegration test (Table 2). All the tablets release their content in 3 hours.

Formulations 1 and 2 have higher rates of liquid permeation as compared to formulation 3 (Figure 3). In fact, the highest release time was found for formulation 3. This may be due to the fact that these tablets (formulations 1 and 2) contain sodium alginate. It has been seen that the pH of the medium may influence the rheology of alginate gel. It was observed that compacted sodium alginate powder was insoluble but relatively porous. The formation of a hydrogel is believed to be the basis for cell protection since the acidic fluid must permeate through the gel layer before reaching the cells (Poulin et al. 2012). Formulation 2 showed slightly higher survival rates after incubation in the acidic medium compared with the other formulations ($p<0.05$). Eudragit® L-100 and sodium alginate is the most protective mixture for *L.fermentum* CECT 5716, and a suitable disintegration as well as acceptable friability and tensile strength occurs at a pH of 6.8. This formula was subjected to stability testing.

Formulation	Before pH 1	After pH 1
1		
2		

3		

Figure 3. Tablets before and after exposure of pH 1 during two hours.

3.6. Stability of probiotic tablet

Figure 4 represents the evolution of the number of viable bacteria within Eudragit® L-100-sodium alginate tablets (formulation 2) stored for six months at storage temperature. When stored at 4ºC, the survival of *L. fermentum* within the tablets was significantly higher ($p<0.05$) than that obtained at room temperature. The overall reduction in the number of viable cells over 6 months was 0.2 log at 4ºC. No significant differences were reported between the number of cells at time cero and after 6 months of storage at 4ºC ($p>0.05$). Meanwhile, increasing the storage temperature to room temperature resulted in an increased mortality level. Specifically, after 6 months, the CFU level was below the therapeutic level ($7.57 \times 10^8 \pm 6.11 \times 10^7$ CFU/ tablets).

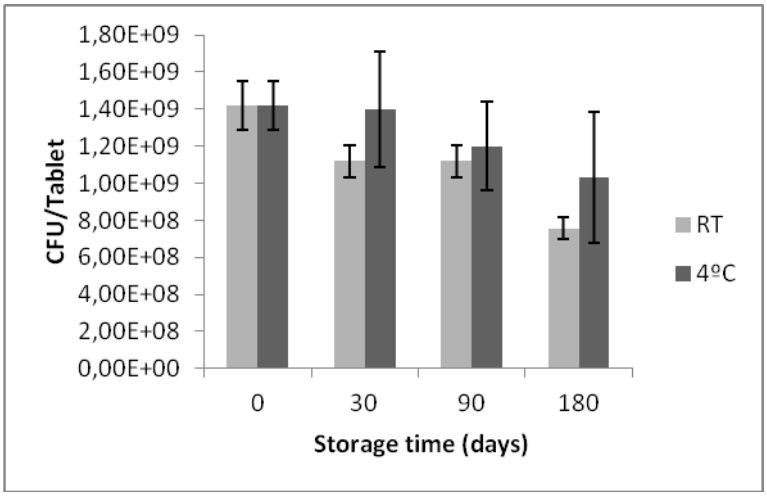


Figure 4. Storage stability of Eudragit® L-100 tablets with *L. fermentum* at room temperature and 4ªC during 6 months.

To explain the lost of viability during storage, Poulin, et al., (2011), hypothesized that it could be due to the impact of redox potential on probiotic storage. It is well known that the presence of oxygen (positive redox potential) in probiotic-containing products may

have a detrimental effect on the viability of probiotics, given that they are anaerobic. Like all chemical reactions, oxidation is temperature-dependent (Arrhenius law). Therefore, the higher rate of mortality occurring at room temperature as compared to that occurring at 4ºC could be the result of the increased oxidation phenomena at 25ºC. These results indicate that the formulated tablets are stable when refrigerated, a common practice for products containing probiotic microorganisms.

Our results were compared with those obtained by other authors who use the same technique, direct compression. However these authors use lower compression force, different strains of bacteria and excipients. Klayraung et al. (2009), reported that *L. fermentum* 2311 tablets made using hydroxypropyl methylcellulose phthalate 55 lost 1 log of viability after 6 months of storage at 30º C. No significant decrease of viability was found at 4º C. This result is in line with ours. However, numerous other authors have found greater levels of viability decrease during storage. For example, after 3 months, Poulin et al., (2011) found a reduction of 0.6 log and 5.2 log for *B. longum* when stored at 4 ºC and 25 ºC, respectively in tablets made with β- Lactoglobulin. Sousa et al. (2013) found a 2.67 log reduction in the viability of *L. paracasei L26* microencapsulated in whey protein particles after 60 days of storage at 23 ºC, using croscarmellose sodium and cellulose acetate phthalate. Chang and Zhang (2002) found a reduction of 2 log for *Lactobacillus acidophilus* after 30 days at 25ºC, using sodium alginate and hydroxypropyl cellulose. Survival of probiotic is going to depend of the intrinsic properties of the strain and the excipients used for the preparation of the tablets. Further studied comparing the same technique and excipients in different strains or comparing different techniques with the same strain would be needed to elucidate which technique is more suitable for each strain.

4. Conclusions

Eudragit® L-100-sodium alginate was found to be a suitable excipient for tablets containing probiotic bacteria and for the promotion of their survival within gastric conditions. The viability of the probiotic was reduced during tableting by less than 1 logarithmic cycle for formulations 1 and 2, while for formulation 3 the decrease in viability was 1.27 log. Tablets were shown to improve the survival of cells when exposed to an acidic medium as compared to free cells. Finally, these tablets were found

to be very stable over a 6 month period when stored at 4ºC. In addition, *in vitro* tests indicate that the probiotic cells shall most likely be released in an appropriate site within the human gastro-intestinal tract. It is believed that this method and formulation may also be applicable to the pharmaceutical, food and chemical industries.

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CONCLUSIONS

CONCLUSIONS

The main aim of this thesis was to develop techniques for probiotics in order to increase their protection to different environmental conditions, thus increasing the viability during the production process and allowing an adequate release of bacteria to the gut. Concerning this main objective, the following conclusions can be drawn from this research work.

M**icroencapsulation of *Lactobacillus fermentum* CECT 5716:**

1. Microencapsulation of probiotics has been poorly studied. Most of bibliography is very recent and the published reports have used a limited number of probiotics. Not all microencapsulation techniques commonly used in pharmaceutical technology are suitable for probiotics. Among all the techniques reviewed, internal gelation and spray dried were selected in this thesis, because a priori their technological characteristics (particle size, moisture, applicability in food and pharmacy, etc…) are suitable for probiotics viability.
2. Internal gelation technology was a suitable microencapsulation method for *Lactobacillus fermentum* CECT 5716.
    - 2.1. The mixture of sodium alginate and unmodified starch offers the best degree of protection for microorganisms and adequate release conditions.
    - 2.2. Technological characterization of microparticles shows an spherical shape, soft surface and particles size below 30µm. All these characteristics made microparticles suitable for oral administration.
    - 2.3. The viability of probiotic microparticles was not modified after 45 days at 4ºC, being those the recommended storage conditions.
    - 2.4. Freeze-drying does not affect the viability of microencapsulated *L. fermentum* CECT5716 but probiotic viability during storage of freeze-dryed particles is low. Further studies are needed to elucidate if the addition of a cryoprotectant could improve the survival of lyophilized microparticles through time.

Microencapsulation of *Lactobacillus plantarum* WCFS-1

3. Spray dried technology using the carriers tested (skim milk, inuline: FOS and maltodextrine) was not a suitable technology for the encapsulation of *L. plantarum* WCFS-1. The low storage stability of probiotic bacteria in the different carriers at non-refrigerated temperatures indicates that bacteria were not sufficiently protected.

3.1. Technological characterization of microparticles shows a spherical shape with concavities, and particles size ranged from 10.74 to 33.34 µm, what is suitable for oral administration.

3.2. Among all the matrices tested, skim milk showed the best protection.

Design and production of solid dosage forms for oral administration of *L. fermentum* CECT 5716.

4. Tablets suitable for oral administration of probiotics have been developed. Production process, storage conditions and probiotic release were adequate in all the formulations tested.

4.1. Methocel k 15- sodium alginate, cellulose acetate phthalate and Eudragit® L-100-sodium alginate tablets maintain the viability after technological operations. The percentage of viability was reduced by 90% in all type of tablets.

4.2. All the tablets elaborated meet the quality criteria required by the United States Pharmacopeia and Spanish Pharmacopeia referring to friability and disintegration test.

4.3. Protection of probiotics through passage to the stomach and delivery of probiotic in the intestine were verified in all cases. The best result was reported by Eudragit® L-100-sodium alginate tablets.

4.4. Tablets were very stable over a 6 month period when stored at 4ºC. No significant differences were reported between the number of cells at time zero and after 6 months of storage at 4ºC ($p>0.05$).

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